Pomegranate Rind Extract with Zn (II) Combination as a New Therapeutic Agent for Oral Care Products

A thesis submitted to Cardiff University in fulfilment of the requirements for the degree of **Doctor of Philosophy (PhD)**

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Abstract

Periodontal diseases, such as gingivitis and periodontitis, are amongst the most common diseases affecting most of the population and have been highly associated with dental plaque. The main strategy to prevent periodontal diseases is through control of dental plaque via physical and chemical methods. However, currently used anti-plaque agents have adverse effects such as tooth staining and taste alteration. Whilst increased antimicrobial resistance is another issue in the management of periodontal diseases. Therefore, the discovery of new alternative treatment agents is necessary. Pomegranate rind extract (PRE) is a well-established folkloric medicine and abundant with hydrolysable tannins, especially punicalagin, with beneficial health properties. More recently, addition of Zn (II) to PRE exerted a synergistic antimicrobial activity against microbes, including some bacteria and viruses. Thus, the aim of this study was to investigate potential use of PRE, Zn (II) and PRE/Zn (II) in relation to treating oral diseases. PRE (1-8 mg/mL), Zn (II) (0.39-6.25 mM) and PRE/Zn (II) demonstrated a broad-spectrum antimicrobial activity in planktonic and biofilm conditions against oral microbes. These agents also exhibited anti- adhesive properties by reducing attachment of *S. gordonii, S. mutans* and *C. albicans* to glass surfaces. Synergistic antimicrobial activity for PRE/Zn (II) was found against *S. gordonii* and *C. albicans*, it was associated with an increased reactive oxygen species (ROS) level which can be active against *C. albicans*. PRE and punicalagin were also investigated for wound healing properties with and without Zn (II). Results indicated that punicalagin had better antioxidant activity and addition of Zn (II) (0.1 mM) to punicalagin (0.1 μg/mL) increased the migration of human primary gingival fibroblast cells. Immunomodulatory activity of PRE and PRE/Zn (II) was evaluated using a *P. gingivalis* biofilm infected 3D *ex vivo* rat mandible model. This treatment caused a significant reduction in pro-inflammatory cytokines (IL-1b, IL-6 and TNF-α) in the culture medium of the 3D infected *ex vivo* rat mandible model. This work has provided a basis for using PRE and PRE/Zn (II) as a novel therapeutic system for periodontal diseases, such as gingivitis and periodontitis. Moreover, PRE and PRE/Zn (II) could be developed as cost-effective products with potential for improving oral health globally.

Presentations, Publications and Prizes

Presentations

1- Pomegranate rind extract and in combination with Zn (II) antimicrobial activity against to oral microbes. **Celiksoy, V.,** Moses, R. L., Sloan, A. J., Moseley, R., & Heard, C. M. *2nd International Conference of Traditional Medicine, Phytochemistry and Medicinal Plants,* Berlin, Germany (2019).

2- Pomegranate rind extract in combination with Zn (II) as an alternative oral topical care product. **Celiksoy, V.,** Moses, R. L., Sloan, A. J., Moseley, R., & Heard, C. M. *OBS Seminars*, Cardiff, UK (2019).

3- Pomegranate Rind Extract with Zn (II) as an Alternative Oral Care Product. **Celiksoy, V.,** Moses, R. L., Sloan, A. J., Moseley, R., & Heard, C. M. *Postgraduate day of Pharmacy School, Cardiff University,* Cardiff, UK (2019).

4- Assessment of antimicrobial activity and toxicity Pomegranate Rind Extract in Combination with Zn (II) on an *ex-vivo* rat mandible tissue model. **Celiksoy, V.,** Moses, R. L., Sloan, A. J., Moseley, R., & Heard, C. M. *CITER Conference*, Swansea, UK, (2019).

5- Wound Healing Potential of Pomegranate Rind Extract in Combination with Zn (II). **Celiksoy, V.,** Moses, R. L., Sloan, A. J., Moseley, R., & Heard, C. M. *CITER Conference*, Cardiff, UK, (2018).

Prizes

The best poster award 'The assessment of antimicrobial activity of pomegranate rind extract in combination with Zn (II) on some oral microbes in planktonic and biofilm conditions.' **Celiksoy, V.,** Moses, R. L., Sloan, A. J., Moseley, R., & Heard, C. M. *34th Annual School of Medicine and Dentistry Postgraduate Research Day,* Cardiff, UK (2020).

Publications

1- **Celiksoy, V.,** Moses, R. L., Sloan, A. J., Moseley, R., & Heard, C. M. (2021). Synergistic In Vitro Antimicrobial Activity of Pomegranate Rind Extract and Zinc (II) against Micrococcus luteus under Planktonic and Biofilm Conditions. *Pharmaceutics*, *13*(6), 851.

2- **Celiksoy, V.,** & Heard, C. M. (2021). Antimicrobial Potential of Pomegranate Extracts. In *Pomegranate*. IntechOpen.

3- **Celiksoy, V.,** Moses, R. L., Sloan, A. J., Moseley, R., & Heard, C. M. (2020). Evaluation of the in vitro oral wound healing effects of pomegranate (Punica granatum) rind extract and punicalagin, in combination with Zn (II). *Biomolecules*, *10*(9), 1234.

Chapter 1: General Introduction

1.1 Oral cavity

The oral cavity is a unique and dynamic environment, which comprises hard and soft tissues as well as the oral microbiome. It continuously faces mechanical stresses (*e.g.*, during mastication) and sudden temperature changes during food consumption. Furthermore, rapid changes that happen in the local pH, sensory changes, reflexes such as swallowing, and in salivary flow, further contribute to the complexity of the oral cavity which includes tooth, gingiva, oral mucosa, and tongue (Squier and Kremer 2001; Squier and Brodgen 2010).

1.1.1 Teeth

The main function of the human teeth is mastication that mechanically prepares food for digestion. Teeth also play a crucial role in speech. In addition to these primary roles, teeth are important for people's social life since the dentition is considered a factor of attractiveness and general hygiene of a person (Eli et al. 2001). Humans develop only two sets of teeth during their lifespan and thus the longevity of the second set of permanent teeth is critically important. During development, epithelial cells produce the hard, inert, acellular, hard enamel of teeth, that is supported by the formation of the less mineralised, more resilient, and vital hard connective tissue of dentine, which is made up of and supported by the dental pulp, a soft connective tissue (Figure 1.1). To accomplish its role, the tooth must be firm and well-rooted. Tooth-supporting connective tissues, which include cementum, periodontal ligament (PDL), and alveolar bone, bind teeth to the jaw and offer adequate flexibility to withstand the stresses of mastication (Nanci 2017).

1.1.1.1 Enamel

Enamel is a hard tissue that protects teeth from internal and/or external factors, that could give damage to the tooth surface. It has a chemically distinct nature with various non-collagenous matrix proteins expressed by ameloblasts, leading to the formation of

large hydroxyapatite mineral crystals. Ameloblasts are the cells responsible for enamel formation, but they lose their viability when the tooth appears in the oral cavity. Ameloblasts are lost via apoptosis and regression and the remaining cells are either shed during tooth eruption or incorporated into the tooth's epithelial connection to the oral gingiva (Lacruz et al. 2017). Enamel becomes a nonvital and insensitive matrix when ameloblasts are lost, and it cannot be replaced or regenerated naturally if it is removed by any means, such as wear or dental caries (Robinson et al. 2017).

Enamel has developed a high degree of mineralisation and a sophisticated organisation to compensate for this inherent constraint. These structural and chemical characteristics enable enamel to survive high masticatory stresses, as well as acid attack from food and microorganisms. Enamel is the body's most mineralised tissue, made up of more than 96% inorganic material in the form of hydroxyapatite crystals and a trace of organic material. Although enamel is a dead tissue in a biological sense, it is porous, allowing ionic exchange between the enamel and the oral cavity environment, particularly with saliva. This results in enamel degradation and the development of dental caries, one of the most common chronic disorders affecting individuals worldwide (Selwitz et al. 2007).

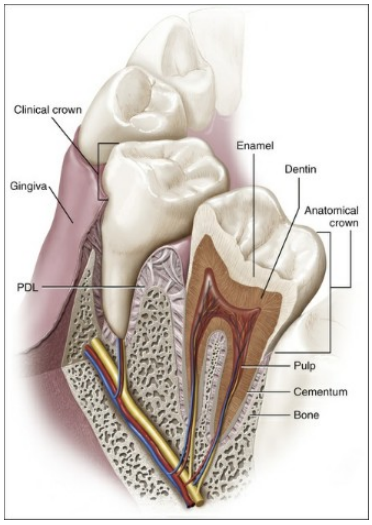


Figure 1.1: Anatomy of the tooth and surrounding tissue. Image adapted from Nanci (2017).

1.1.1.2 Dentine

Dentine, another mineralised layer beneath the enamel, forms the mass of the tooth and provides structural support. Dentine supports enamel as a resilient tissue since enamel can be fractured because of its high mineral composition. This tissue is formed by odontoblasts which, unlike ameloblasts, will continue to create dentine throughout the lifetime of the tooth (Couve 1986; Sloan et al. 1998; Sloan and Waddington 2009). Odontoblasts first secrete a predentine matrix, which is made up of collagenous (mainly collagen type I) and non-collagenous proteins. Dentine is composed of 45 % mineral hydroxyapatite, 33 % organic material (mostly type I collagen), and 22 % fluid identical to plasma, including albumin, fibrinogen, and immunoglobulin G (Knutsson et al. 1994; Marshall et al. 1997). Dentine and enamel are drastically distinct tissues, due to the presence of odontoblasts. Dentine is a delicate tissue that can be repaired because odontoblasts, or pulp cells, can be induced to deposit additional dentine during tertiary (reactionary or reparative) dentinogenesis, when it is required (Nanci 2017). Primarily, dentinogenesis happens during tooth development and involves the rapid creation of dentine. Secondary dentinogenesis occurs after tooth eruption and is marked by a slower rate of deposition and a different gene expression profile, which includes the up-regulation of matrix proteins, such as osteocalcin and dentine matrix acidic phosphoprotein-1 (DMP-1), and the downregulation of collagen type I and dentine matrix acidic phosphoprotein-1 (DSSP-1) (Simon et al. 2009).

After dentin formation by odontoblasts, the process continues with tubule formation within dentine. These long tubules ensure a hard, but porous, dentinal tissue. Fibrillar structures are seen within tubules, which were once thought to represent odontoblast processes' branches or ramifications (Szabo et al. 1984) but are now thought to anchor the odontoblast processes inside the tubules (Garces- Ortiz et al. 2015). Moreover, dentinal tubules can traverse the entire thickness of dentine and comprise cytoplasmic projections of odontoblast cells that make dentine a vital tissue, which can respond to external stimuli, such as pressure and temperature (Pashley et al. 1981; Camps and Pashley 2003). This tubular characteristic is also important in terms of infection, since it could be a means for bacteria and bacterial products to enter the dental pulp, where they can induce immuno-inflammatory responses to infection (Love and Jenkinson 2002). Moreover, the protein rich form (especially type I collagen) of the dentine and

pulp can further act as a favourable environment for the attachment of bacteria that have collagen-binding proteins, such certain streptococci (Ackermans et al. 1981; Avilés-Reyes et al. 2017).

1.1.1.3 Dental pulp

The pulp is a non-mineralised connective tissue that lies in the pulpal chamber, bordered by dentine at the centre of the tooth. In pulp tissue, most of the cells are fibroblasts, with dentine-forming odontoblasts bordering the perimeter. Blood vessels, neurons, and a variety of other cell types, including immune- inflammatory cells and mesenchymal progenitor cells, are also present. Pluripotent progenitor cells can develop into a variety of cell types, including odontoblasts that play a role in dentine formation during a tooth's lifespan (Gronthos et al. 2002; Mitsiadis et al. 2011; Nanci 2017).

The pupal extracellular matrix (ECM) is produced by pulp fibroblasts, and this largely consist of types I and III collagen, and non-collagenous components, such as proteoglycans, hyaluronan and glycoproteins (Goldberg and Smith 2004). While the ECM provides a mechanical support for fibroblasts and acts to maintain vascular and neural functionality within teeth, it can be also be a favourable environment for bacterial attachment during inflammatory pulpitis (Hannas et al. 2007).

1.1.2 Periodontium

The periodontium is a unique supporting system for the teeth, and its complex structure comprises both soft and hard tissues (Kinane et al. 2017). The primary actions of the periodontium are to attach the teeth to bones and to preserve the nerves, blood vessels and teeth from trauma. In addition, the periodontium acts like a barrier between underlying structures and the oral microbiome. The gingiva, mucosa-, and *PDL* are soft tissues, whereas the cementum and alveolar bone are hard tissues of the periodontium (Hughes 2015).

1.1.2.1 Periodontal ligament

The *PDL* is a dense, fibrous tissue, which aids tooth attachment to the alveolar bone. Attachment between the *PDL* and alveolar bone provides durability to teeth to overcome the forces of mastication. Collagen fibre bundles provide this support, as they transverse the extra cellular matrix (ECM) region between the bone and the tooth. The main fibres of the PDL are type I collagen, located in the alveolar bone, as well as the cementum of the tooth. As a result of their orientation, these fibres are called oblique fibres. The *PDL* also plays a role in sensory function (Nanci 2017). Furthermore, occlusal loads exerted on the teeth during mastication are absorbed and dissipated by the *PDL*. The *PDL*'s high elastin and hydrophilic proteoglycan serves to provide major elastic forces. The outcome of occlusal loading on a tooth is to cause hydraulic pressure that causes elastic rebound. Thus, they can move slightly and dissipate some of the load to the surrounding alveolar bone. Hence in anatomical places where the bone is particularly thin, such as the lower incisors, normal physiological movement of a tooth can result in little but noticeable tooth mobility (Hughes 2015).

1.1.2.2 Cementum

Cementum is produced by cementoblasts and is an avascular bond-like tissue that covers the root of teeth. It has been reported that cementum has a lower mineralisation rate, approximately 45 % inorganic material by weight; and thus, it is softer than other mineralised tissues due to its high content of cementum matrix. Cementum has two major functions: the first one is providing attachment to the tooth to the alveolar bone, via the participation of *PDL* fibres. The second function is to protect the root from resorption, during periodontium modelling (Hughes 2015). Moreover, it has been reported that *PDL* fibroblasts also play a role in cementum formation by secretion of extrinsic fibres (Bosshardt 2005).

Cementum is classified histologically into two types: acellular and cellular. Acellular cementum covers the upper (cervical) part of the root and is also called primary cementum. Cellular cementum covers the lower (apical) part of the root and is called secondary cementum (Bosshardt and Schroeder 1990; Nanci 2017).

1.1.2.3 Alveolar bone

The alveolar bone is an important part of the maxillofacial skeleton and forms the tooth socket surrounding teeth. The alveolar bone supports the teeth when mechanical stress is applied, and it undergoes ongoing bone remodelling (Huang et al. 2020). Like other bone tissues, osteoblasts and osteoclasts perform critical roles in the formation and resorption of alveolar bone with other bone extracellular matrix components. A complex cellular communication network, which includes osteocytes, macrophages, monocytes, neutrophils, and adaptive immune cells such T helper 17 cells (Th17 cells), also plays a significant role in maintaining tight bone coupling and alveolar bone homeostasis (Gruber 2019). Alveolar bone remodelling is not only a part of the skeletal system's bone turnover, but it also reflects skeletal bone state. It is reported that alveolar bone demonstrates considerably dynamic remodelling, as it has been shown that the rate of alveolar bone turnover in the mandible and maxilla is substantially greater than in the femur. The strong relation between teeth and periodontium is indicative of the highly dynamic remodelling of the alveolar bone (Huja et al. 2006; Marx et al. 2007). Since the maintenance and preservation of the alveolar bone is tooth-dependent, it is progressively resorbed down to the body of the jaw bones after tooth extraction. Complete tooth loss causes gradual bone resorption, which can lead to severe atrophy of the jaw bones, presenting major clinical challenges for implant insertion and dental prosthesis design (Hughes 2015; Li et al. 2021).

1.1.3 Oral mucosa

Oral mucosa is a mucosal membrane, and is the term used to describe the moist tissue of the oral cavity. Oral mucosa can be classified by three main structural features: oral epithelium, lamina propria and submucosa. The oral mucosa displays two distinct epithelial tissues, which are either keratinised or non-keratinised. Keratinised epithelium comprises tissues, such as the hard palate and gingiva, whilst the non-keratinised epithelium includes sublingual regions of the tongue and the buccal mucosa. Additionally, there are structural variations within the oral mucosa, which can be classified into three main types according to their main functions: masticatory mucosa, lining mucosa and specialised mucosa. Lining mucosa comprises the largest

part of the oral mucosa, approximately 60% of the total area, followed by masticatory mucosa and then specialised mucosa (Collins and Dawes 1987; Squier and Brodgen 2010).

Masticatory mucosa is comprised of hard palate and the gingiva, which is tightly attached to the underlying tissues and consists of a strong keratinised tissue, which gives an ability to resist damages associated with mastication. Lining mucosa includes the generally non-keratinised areas like the cheeks, inner aspects of the lips, floor of the mouth and ventral surface of the tongue. The dorsum of the tongue is specialised mucosa since it has taste buds and sensory nerve endings (Squier and Brodgen 2010).

As mentioned above, the oral cavity is a complex environment which is constantly exposed to potentially damaging factors. The fundamental role of the oral mucosa is to protect the underlying tissues. The oral mucosa provides protection from mechanical injuries and insults, resists the entry of microorganisms, and acts as a barrier to the permeability of toxic substances (Squier and Brodgen 2010).

1.1.3.1 Gingiva

Gingiva is an oral mucosa that immediately covers an erupted tooth. According to its functional aspect, gingiva can be classified into two parts: the region facing the oral cavity consisting of masticatory mucosa and the other region that faces the tooth, which is involved in attaching the gingiva to the tooth and forms part of the periodontium (Fujita et al. 2018). This gingival tissue covers the coronal side of the alveolar process and extends from the mucogingival line, which marks the boundary with the non-keratinised buccal mucosa. The gingiva terminates at each tooth's cervix, surrounds it, and connects to it by a specialised epithelial tissue, called the junctional epithelium. This epithelial attachment provides a constant epithelial lining between the oral cavity and the tooth surface. As the connection between the oral mucosa and the tooth is porous, antigens can readily pass through and cause inflammation in the gum tissue (marginal gingivitis) (Marsh et al. 2009; Clark and Clark 2018). Although the colour of healthy gingiva is described as ‘salmon’ or ‘coral pink’, it can show differences according to an individual’s ethnical origin (Solanki 2012). In addition, the width of the attached gingiva could also show substantial differences between

individuals from 1 mm to over 10 mm. The gingiva can be divided into the following sections: free marginal gingiva, the attached gingiva, the interdental gingiva, and the gingival crevice/sulcus that runs from the marginal gingiva to the junctional epithelium (Walmsley et al. 2007). A representation of dento-gingival area is presented in Figure 1.2.

The gingiva's junctional epithelium is the part of the gingiva that attaches the connective tissue to the tooth surface. This attachment is in a constant renewal process during its lifespan. The junctional epithelium has a faster turnover rate (4- 6 days), in comparison to oral epithelium (6-12 days) (Vriens et al. 2008). The junctional epithelium comprises non-keratinised cells and attaches to enamel via a basal lamina and intercellular hemidesmosomes. This attachment between soft gingival tissue and hard tooth tissue via the junctional epithelium has a critical importance for the maintenance of periodontal health. Since even without a clinical gingivitis condition, the junctional epithelium hosts many polymorphonuclear leucocytes (PMNs) moving through it towards the sulcus. However, as it is also permeable, the junctional epithelium could act as a pathway for diffusion of the metabolic products from plaque bacteria, such as toxins, chemotactic agents, and antigens (Kinane and Lappin 2002; Nakamura 2018).

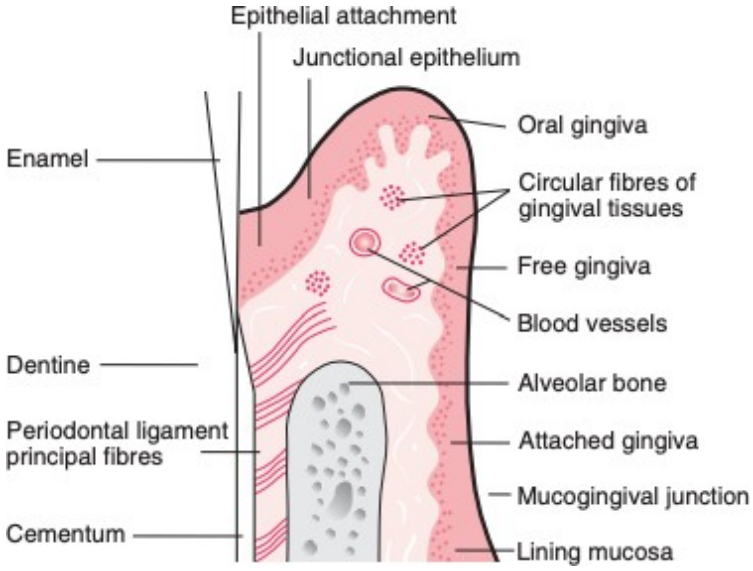


Figure 1.2: Dento-gingival region. Image adapted from Walmsley et al. (2007).

1.1.3.1.1 Gingival fibroblasts

Gingival fibroblasts help maintain the integrity of the dento-gingival tissues. They function attachment between the tooth root and alveolar bone. Gingival fibroblasts perform these functions by mediating ECM turnover and by preserving collagen fibre attachments to the root surface. The production and remodelling of gingival fibre attachment to the tooth root surfaces is mediated by gingival fibroblasts. The bulk of the connective gingival tissue includes type I collagen secreted by fibroblasts. Since type I collagen in periodontal tissue is rapidly turned over, deviations in the balance between collagen production and degradation may result in loss of loss of collagen and tissue architecture, leading to eventual tooth loss (Buckley et al. 2001; Nanci 2017). The primary cellular component of gingival connective tissues are gingival fibroblasts. Initial investigations of the innate immune responses of gingival fibroblasts have shown that stimuli such as lipopolysaccharide (LPS) induce these cells to generate different pro-inflammatory cytokines, such as interleukin (IL)-1, IL-6, and IL-8 (Takada et al. 1991; Tamura et al. 1992). Moreover, during wound re-epithelisation, fibroblasts produce numerous growth factors that orchestrate the creation of granulated tissue and re- epithelialisation, in addition to the production of a complex ECM, following re- epithelalisation. fibroblasts proliferation and migration capacity are directly related to all these processes (Haekkinen et al. 2000). It has been reported that gingival fibroblasts are phenotypically unique in adult tissues, can facilitate rapid healing of oral lesions with minimum gingival scarring, since gingival fibroblasts can rearrange and degrade the ECM quickly (Frantz et al. 2010).

1.1.3.2 Hard palate

The hard palate is a keratinised masticatory mucosa that forms the roof of the oral cavity. The palatine processes of the maxilla and the horizontal plates of the palatine bones form the underlying bony structure of the hard palate, which makes it an immovable hard bony segment. The hard palate is surrounded by mucoperiosteum, that is continuous with the connected gingiva and firmly linked to the underlying bone (Nanci 2017). One of the functions of the hard palate is it forms the floor of the nasal cavity and the roof of the oral cavity, officially separating the oral cavity from the nasal cavities. The ciliated pseudostratified columnar epithelium (respiratory mucosa) covers the hard palate superiorly and stratified squamous epithelium covers it

inferiorly (oral mucosa). The hard palate comprises rugaes on the mucous membrane of the anterior portion and facilitating the transfer of foods towards the pharynx. Moreover, three foramina/canals in the hard palate function as pathways for important neurovascular systems that feed the oral cavity. The hard palate provides a solid bottom to the nasal cavity, preventing nasal passages from closing by changes in pressure within the mouth. In addition, the hard palate also play role in phonation and rugaes in the hard palate facilitate the mastication and digestion of food (Hourfar et al. 2015; Olszewska and Woodson 2019; Helwany and Rathee 2021).

1.1.3.3 Soft palate

The soft palate is a continuation of the hard palate that makes up the posterior third part of the palate (Figure 1.3). Muscle fibres and connective tissue make up the soft palate, which is protected by a mucus barrier made up of a stratified squamous epithelium containing secretory salivary glands. In contrast to the hard palate, the soft palate has a flexible form without any bony structures. The soft palate elevates the nasopharynx, thereby blocking the oropharyngeal-nasopharyngeal connection. The soft palate comprises five muscles that help in breathing, phonation, and swallowing. The soft palate creates a vacuum in the oral cavity during swallowing, preventing food from entering the respiratory tract. Swallowing causes a temporary cessation in breathing due to the physical closure of the airway caused by elevation of the soft palate. The soft palate protects the nasal canal by directing some secretions to the oral cavity after a sneeze. In addition, the soft palate plays a part in the gag reflex; upon contact (Shigeta et al. 2010; Cho et al. 2013; Grimaldi et al. 2015; Helwany and Rathee 2021).

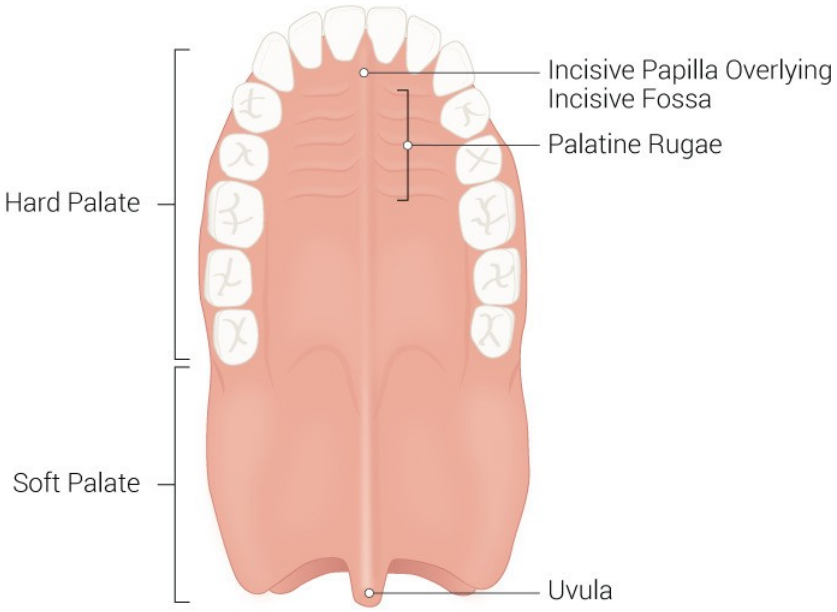


Figure 1.3: Representative illustration of the hard palate, soft palate, incisive papilla overlying incisive fossa, palatine rugae and uvula (Helwany and Rathee 2021).

1.1.4 Tongue

The tongue is a muscle organ that is involved in food processing, sensory reception in general and the sensory function of taste. The tongue's body is largely made up of voluntary muscle, with highly specialised, keratinised mucosa containing taste buds. Various papillae are distributed across the tongue's dorsal surface. On the dorsal surface, filiform papillae are most abundant and present clinically as short, hair-like projections. Large, pedunculated, mushroom-shaped fungiform papillae, which are concentrated on the anterior tip and lateral surfaces of the tongue, are among the filiform papillae. The circum vallate papillae are located near the intersection of the tongue's anterior two-thirds and posterior one- third. Each of the 12 to 20 papillae is bordered by a tiny depression and organised in an inverted V-shaped pattern. The circumvallate papillae have many taste buds (Nanci 2017). Many tongue lesions are unique to the tongue and are dependent on alterations in the tongue's specialised epithelial coating, particularly the filiform papillae (Tyldesley and Field 1995).

1.2 Oral microflora

1.2.1 Biofilm

Microorganisms are generally considered as simple life forms, when compared with higher organisms such as plants, and animals. However, it has been found that microorganisms have an ability for complex differentiation and behaviours (O'Toole et al. 2000). Biofilms can be simply and broadly described as a community of matrix enclosed microorganisms that are attached to a surface and/or each other (Geesey et al. 1977). Biofilms can comprise a single microbial species or multiple microbial species and can form on different biotic and abiotic surfaces. While multispecies microbial biofilms are the most found, single species biofilm exist in a variety of infections and on medical implant surfaces (Dickinson and Bisno 1993; Archibald and Gaynes 1997).

Biofilm formation includes stable biological cycle steps; initiation (attachment), maturation, maintenance, and dissolution (Figure 1.4). Adhesion is typically initiated in suitably nutritious environment. In nutrition deficient situations, microbes can detach from the surface and return to a planktonic form. This ability could allow microbes to search for new sources of nutrients (Kolter et al. 1993).

It was suggested that biofilm formation influences microbial development, which involves changes in metabolic activity that play a role in the life cycle of the organisms (Shimkets and Brun 1999). In this context, biofilm formation influences microbial development, although this effect could be subtle for some microorganisms (O'Toole et al. 2000). It is well known that there are distinct differences in microorganisms in their planktonic and biofilm states. Bacteria shows a transition from planktonic to biofilm level in which it must interact with other cells in proximity. Differences are observed in terms of cell functions, including physiology, cell surface composition and resistance to environmental differences such as pH, temperature, osmolarity, and oxygen (Henrici 1933).

Bacterial attachment to a biotic or abiotic surface is considered the first step of biofilm formation and commences when a microbe finds a suitable environment that induces

the microbe to start biofilm formation. Environmental factors involved include, nutrients, temperature, osmolarity, pH, iron, and oxygen (Stanley 1983; Pringle and Fletcher 1986; Palmer and White 1997; Wimpenny and Colasanti 1997; O'Toole and Kolter 1998a; O'Toole and Kolter 1998b; Pratt and Kolter 1999; Stoodley et al. 1999; O'Toole et al. 2000; Watnick and Kolter 2000). In addition to environmental factors, an organism's multiple genetic pathways play key roles in surface attachment (Fletcher 1996). Early attachment steps comprise cell-cell and cell-surface interactions, and then maturation of biofilm begins. Early/initial attachment mechanisms have not been fully elucidated, but some aspects have been investigated. In early studies, initial attachment was explained using simple chemical models (Marshall et al. 1971; McEldowney and Fletcher 1986; Fletcher 1996; Grasso et al. 1996). While these chemical steps influence cell-surface interactions, the complexity of initial attachment should not be ignored. Bacterial surface characteristics (such as pili) also contributes to initial attachment. These bacterial surface characteristics may regulate attachment of bacteria to an attachment surface. Moreover, surface characteristics can change according to environmental conditions (O'Toole et al. 2000).

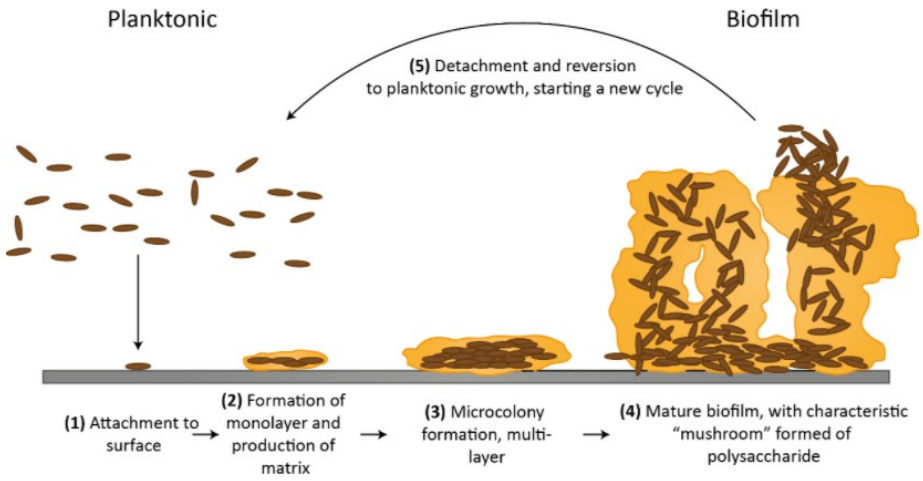


Figure 1.4: A schematic presentation of biofilm formation. (1) Biofilm formation starts with the reversible attachment of planktonic cells (brown ovals) to a surface (grey basement). (2) Microbial cells form a monolayer and produce an extracellular matrix

that provides irreversible attachment to the surface. (3) Monolayer becomes multilayer. (4) Biofilm changes to a mature biofilm and takes on a “mushroom” like shape due to polysaccharides. (5) Some cells detach to start a new biofilm cycle. Adapted from Vasudevan (2014).

After initial attachment, microbes adapt to live in a biofilm. When bacteria become part of a biofilm, the production of extracellular polysaccharides (EPS) and antimicrobial resistance increases. These features provide a protective environment for microorganisms by increasing their survival capacities and inducing virulence factors. In addition, bacteria in biofilms have other advantageous capabilities, such as increased resistance to ultraviolet (UV) light, increased rates of genetic exchange, enhanced biodegradative capabilities, and enhanced secondary metabolite production (Zobell 1937; Marshall 1992; Goodman et al. 1994; Wolfaardt et al. 1994; Ascon-Cabrera et al. 1995; Brazil et al. 1995; Gros and Logan 1995; Annachhatre 1996; Karamanev et al. 1998; Sarra et al. 1999). However, the molecular mechanisms in these changes have not yet been fully elucidated.

1.2.2 Dental plaque formation

The biofilm that forms on teeth is called dental plaque and comprises hundreds of microbial species. Dental biofilms are exposed to challenging environments, as the oral cavity can be a harsh place with regards nutrient availability (feast or famine), aerobic to anaerobic transitions and sudden pH or temperature alterations. Moreover, oral hygiene interventions may serve to regulate and manage biofilm communities (Carlsson 1997). Dental biofilm formation starts with initial attachment, with the surfaces in the mouth coated with glycoproteins, mucins and other proteins (commonly known as acquired pellicle). After conditioning of the surface, initial attachment of primary colonisers of dental plaque, occurs. Primary colonisers are mostly streptococci and then followed by *Actinomycetes* (Socransky et al. 1977; Syed and Loesche 1978; Nyvad and Kilian 1987; Bloomquist et al. 1994). The bacterial surface is one of the main factors mediating attachment to the tooth surface, for instance pili, with outer membrane proteins playing a key role in the interaction with the pellicle (Ganeshkumar et al. 1988; Clark et al. 1989; Kolenbrander and Andersen 1990a; Andersen et al. 1993). Streptococci, which are accepted as primary colonisers of dental

plaque, have a capacity to bind various components in the pellicle, such as proline-rich proteins and enzymes (Scannapieco et al. 1989; Gibbons et al. 1991).

Furthermore, streptococci play a role in cell-cell interactions or coaggregation, either with themselves or other types of microbes (Kolenbrander and Anderson 1986; Kolenbrander 1988; Kolenbrander and Anderson 1990a; Kolenbrander and Anderson 1990b). An example of this is with Type-I pili, playing a role in both the adherence to pellicle and cell-cell interactions in some organisms (Weiss et al. 1988). The multiple adhesion expression ability of early colonising bacteria provides an advantage over other bacteria, which have a reduced receptor recognition ability (Jenkinson and Lamont 2005). There are different mechanisms which play a role in the initial attachment, such as oligosaccharide receptors by protein adhesins, protein-protein interactions, ionic and hydrophobic interactions between microbial surface elements and the attached surface (Jenkinson and Lamont 1997; Li et al. 2001; Ruhl et al. 2004). Antigen I/II family polypeptides, which bind gp340, fibronectin and collagen, are considered some of the main streptococcal adhesins (Heddle et al. 2003; Jakubovics et al. 2005; Loimaranta et al. 2005).

Microbial interactions initiate rapidly after the initial attachment of primary colonisers (Bloomquist et al. 1994, 1996; Liljemark et al. 1997). In dental biofilm formation, after initial microbe-surface interactions, organisms show changes in gene expression. For instance, *S. mutans* exerted a 10- to 70-fold increase in glucosyltransferase BC (*gtfBC*) expression (which encodes a glucosyltransferase necessary to produce glucan polymers) in the biofilm state compared to its planktonic counterparts, but the expression of fructosyltransferases (*ftf*) (also involved in polymer synthesis) decreased approximately 100-fold (Burne et al. 1997). The microbial community starts to alter based on the physiology of individual organisms. *Fusobacterium* species act as a bridge between primary and later colonisers (Socransky et al. 1977; Kolenbrander and Anderson 1985; Nyvad and Kilian 1987; Kolenbrander et al. 1989; Kolenbrander and Anderson 1990a; Kolenbrander 1993).

The microbial composition of dental plaque is highly associated with the nature of the foundations. For example, *Streptococcus sanguinis* colonisation at an early age is correlated with the late colonisation of cariogenic *Streptococcus mutans* (Caufield et

al. 2000). Therefore, relationship between pioneering and subsequently colonising species could play an important role in dental plaque development (Palmer et al. 2003; Foster et al. 2004; Jakubovics et al. 2005; Jenkinson and Lamont 2005; Socransky and Haffajee 2005).

The microbial community varies between oral sites (Figure 1.5). After initial attachment, the accumulation of bacteria commences and the biofilm becomes more complex through recruitment of other community members and production of metabolic signals or attractants. In addition, availability of adherent substrates and deposited host salivary molecules is important in the formation of complex dental plaque. The disease related bacteria such as *Fusobacterium nucleatum* and *Porphyromonas gingivalis* are generally found in complex biofilm structures but are rarely found in early colonised communities where streptococci, *Actinomyces* and *Veillonella* are present. These pathogenic microorganisms, *P. gingivalis and F. nucleatum*, need a suitable environment and attachment with other microbes in the community for their colonisation. Studies showed that *P. gingivalis* colonisation could depends on other bacteria. For instance, the multivalent co-adhesive between *S. gordonii* and *P. gingivalis* has been established (Lamont et al. 1992; Love et al. 2000). The *P. gingivalis* long fimbriae (FimA) bind the glyceraldehyde-3-phosphate dehydrogenase which is found on the streptococcal surface, and the short fimbriae (Mfa) binds streptococcal SspA and SspB (antigen I/II) adhesins (Lamont et al. 2002). Therefore, it is crucially important to understand the specific binding mechanisms between oral bacteria and the shifting mechanism from microbiota in healthy states to disease states.

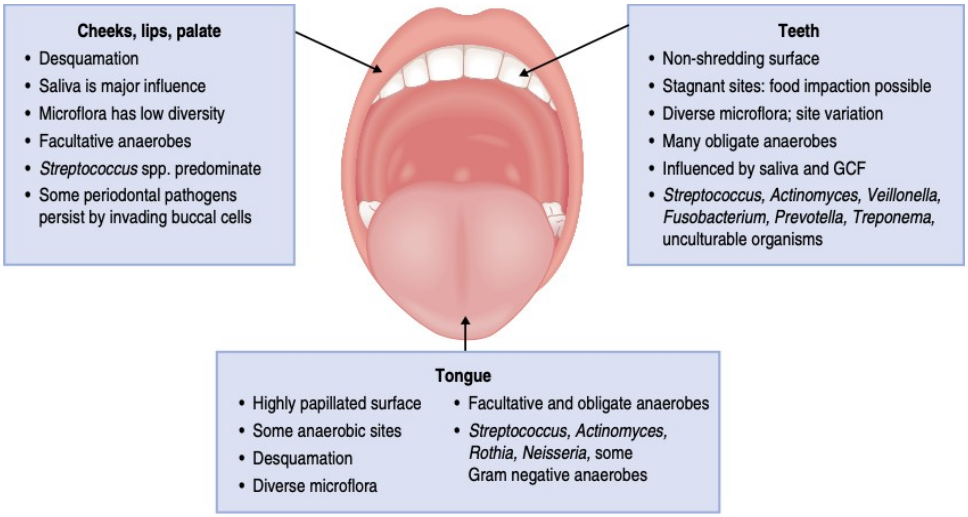


Figure 1.5: Microflora distribution at different sites in the oral cavity. Adapted from Marsh et al. (2009).

1.2.3 Development of oral diseases

There are 10-fold more bacterial cells in the human body than human cells, and 100-fold more bacterial genes than human genes (Turnbaugh et al. 2007; Yang et al. 2009). The oral cavity contains a rich microflora and poor oral health has been associated with systemic diseases, such as cardiovascular disease, diabetes, adverse pregnancy outcomes, oral cancer and gastrointestinal diseases (Watabe et al. 1998; Mercado et al. 2001; Seymour et al. 2007; Tezal et al. 2019). Thus, the microbiome and interactions between it and host are crucially important to health and disease (Wikoff et al. 2009; Archambaud et al. 2013). In addition to systemic effects, the most common oral diseases (dental caries and periodontitis) are most seen in populations of industrialised countries (Petersen and Lennon 2004).

Various hypotheses have been suggested to explain the shift in dental plaque between health and disease. The main hypotheses are the Traditional and Updated Non-Specific Plaque Hypothesis (NSPH), the Specific Plaque Hypothesis (SPH), the Ecological Plaque Hypothesis (EPH) and the Keystone Pathogen Hypothesis (KPH) (Loesche 1976; Miller 1984; Theilade 1986; Marsh 1994; Hajishengallis et al. 2012).

In traditional NSPH, the quantity of dental plaque is considered to play the primary role in pathogenicity. However, differences in virulence factors between different bacteria was not considered. It is suggested that the host has a limited capacity to eliminate bacterial virulence products, and with dental plaque accumulation the host cannot counter the increased level of bacterial products. This could result in infection in the oral cavity. Therefore, oral diseases could be prevented using non-specific mechanical removal of as much plaque as possible (Miller 1890; Black 1899; Theilade 1986; Loesche 1986). In SPH, disease- related microorganisms were investigated using culture- based techniques and microscopy, due to improved techniques in microbiology practices. This hypothesis suggested that use of antimicrobial agents against specific bacterial species could be used as a treatment and prevention method (Loesche and Nafe 1973; Loesche 1976,1977,1986). The updated NSPH suggested that in addition to the specific pathogens, sometimes common bacteria in health could cause the inflammation. Theilade (1986) suggested that any microbial colony could cause gingivitis when it reached a sufficient level in the gingival crevice and it was also stated that some subgingival bacteria can be more virulent than other bacteria by having a role in colonisation, a destructive effect on defence mechanisms and inducing inflammation and tissue destruction (Theilade 1986). In 1994, the EPH hypothesis was proposed (Marsh 1994). It was suggested that oral diseases could be the result of an altered homeostasis in the oral microflora caused by ecological stress. This ecological stress can result in an increased number of oral pathogens or disease-related microorganisms in the dental plaque. The new aspect of this hypothesis was that Marsh considered the ecological factors, such as pH, redox potential, nutrients, and essential factors, modulating in microbial composition (Marsh 1994, 2003). However, in this hypothesis, the genetic factors of host and host-dependent effects, that have a crucial role in dental plaque development and disease progression, were not considered (Mason et al. 2013). In 2012, KPH was proposed (Hajishengallis et al. 2012). KPH considers certain microbial pathogens, which can cause increased dental plaque accumulation and unfavourable alterations in the microbial composition of dental plaque. In the light of this hypothesis, *Porphyromonas gingivalis*, which has been considered one of the main pathogenic microbes in dental plaque, is able to manipulate both the host immune system and entire microbial community (Darveau 2010).

All these hypotheses provided steps to enhance our understanding of the mechanism of oral diseases, but they are not sufficient and need further investigation. It should also be recognised that in addition to bacteria, the oral cavity comprises other microbes such as viruses, protozoa, and fungi, which may have a significant role in the oral cavity as co-inhabitants. Furthermore, with improved microbiological techniques and methods, more data could be obtained related to pathogenic microbes and genetic actions of the host. However, an all-encompassing hypothesis is needed and is potentially only available when additional data is obtained from complex interactions between the oral microbiota and the host innate immune system (Rosier et al. 2014).

1.2.4 Antimicrobial resistance of biofilms

It is well-known that the treatment of biofilm infections is challenging. Bacteria become more resistant to antimicrobial agents when in a biofilm (up to 1000-fold decrease in susceptibility), than their planktonic level counterparts in culture liquid media (Costerton 1999; Costerton et al. 1999). Biofilm-related antimicrobial resistance can be classified into two groups; those that are innate (due to growth in biofilm) and those that are induced (resulting as a response to antimicrobial treatment). There are several suggested mechanisms identified for increased antimicrobial resistance of biofilms (Ayukebong et al. 2017). One of the mechanisms is associated with the extracellular polymeric substances (EPS), which supports biofilm formation and structure. However, evidence for this suggestion is conflicting. Since it has been suggested that the biofilm EPS could also be a physical and chemical barrier to antimicrobial agents. For instance, *Pseudomonas aeruginosa* biofilm resistance was correlated with its mucoid properties. It was shown that mucoid biofilms of *P. aeruginosa* were up to 1000 times more resistant to tobramycin than nonmucoid biofilms, despite having similar minimum inhibitory concentrations (MICs) in planktonic states (Ciofu et al. 2012). However, another suggestion related to the biofilm EPS is that the matrix may not inhibit the penetration of antibiotics, but may impede the antimicrobial penetration rate of antimicrobial agents for enough time to induce expression of genes involved in antimicrobial resistance (Jefferson et al. 2005). The conflict of suggested mechanism based on the biofilm EPS is raised from the difference in biofilm formation ability of microbial cells and the difference in

penetration and diffusion ability of antimicrobial agents (Anderl et al. 2000; Stone et al. 2002; Walters et al. 2003).

The second suggested mechanism of increased biofilm resistance is the difference in metabolic state of microorganisms. Biofilms have a three-dimensional architecture and a certain depth depends on the maturation and age of the biofilm. Due to these structural properties, some microorganisms are located in the deepest part of the biofilm, where there is nutrient limitation. This lack of nutrition causes reduces the growth of these microbes (Brown et al. 1988). Deprivation of nutrients and oxygen are well-defined causes of retarded bacterial growth and could cause antimicrobial resistance for many species (Brown et al. 1988; Field et al. 2005). Slow growing or non-growing cells show reduced susceptibility to antimicrobial agents because they are generally in the stationary-phase and rarely divide. As such, antibiotics such as beta-lactams, become less effective as they exert their bactericidal activity on dividing cells (Walters et al. 2003).

Persister cells are another fundamental factor in biofilm antimicrobial resistance (Hobby et al. 1942). Persister cells are a subpopulation of bacterial cells in a dormant state with extreme antimicrobial resistance (Wood et al. 2013). Persister cells have been defined as phenotypic variants of bacteria as they have not undergone genetic alteration (Lewis 2010). Persister cells are protected from elimination by the host immune system due to their location within the biofilm and they contribute towards the pathogenicity of biofilm infections (Lewis 2005, 2010). It has been reported that persister cells remain in the biofilm after treatment with antibiotics, and these remaining persister cells support biofilm formation and pathogenicity. The repopulated biofilm does not show additional antimicrobial resistance compared to the eradicated original biofilm cells and this has been accepted as supporting evidence for persister cells being a phenotypic variant, rather than a mutant (Lewis 2001).

Another suggested resistance mechanism is the genetic adaptation of microbes to different conditions. Microbes in a biofilm have a higher mutation rate than planktonic counterparts (Driffied et al. 2008). In addition, several microbial genes play a role in biofilm formation and some are expressed by biofilm-growing microorganisms (Yadav et al. 2012; Szczuka et al. 2013). Microbes exhibit phenotypic differentiation

when adapting to biofilm style and this phenotypic differentiation was described as a biologically programmed response of cells on a surface (Costerton et al. 1994, 1999).

1.3 Oral microbiome related oral diseases

1.3.1 Dental caries

Dental caries/tooth decay is one of the most common global diseases, which causes dissolution of the tooth structure due to increased acid levels by oral bacteria. This increased acidic level, resulting in a decreased pH level, is a consequence of the fermentation of carbohydrates by oral bacteria. In individuals who consume an excess amount of carbohydrates, the increased acid production causes erosion to the tooth structure, as the host becomes insufficient at increasing pH levels. Moreover, in this situation, the oral cavity becomes more favourable for aciduric species and this causes changes in the microbial composition of dental plaque (Tkahashi and Nyvad 2011). Caries of enamel surfaces are generally common in ages up to 20 years and in later years root surface decay is a growing concern because with the gingival recessions the susceptible cement is exposed to microbial colonisation. The development of lesions means disintegration of the enamel and the transfer into the surrounding environment of calcium and phosphate ions. The earliest phases of caries are reversible and can be remineralised, especially when fluoride is present (Marsh et al. 2009).

Enamel caries is linked to a change in the equilibrium of dental plaque bacteria, especially the acid-tolerant species. Particularly streptococci and lactobacilli species exerted a higher isolation frequencies and proportions in areas with carious diseases. *S. mutans* has been widely studied because of its cariogenic properties including biofilm formation ability in oral cavity, ability to survive in acidic environment and specific interaction with other microorganism (Krzyściak et al. 2014). *S. mutans* has been considered the primary pathogenic bacterium for dental caries. However, in addition to *S. mutans,* there are many other microbes that produce acid from dietary carbohydrates and could have roles in dental caries pathology. These species include *S. mitis*, *S. anginosus*, *S. salivarius*, *Enterococcus faecalis, Actinomyces naeslundii, A. viscosus* and lactobacilli. However, there is no evidence that one single species is uniquely responsible for dental carious lesions, since dental caries can occasionally

develop when the *S. mutans* is absent. Moreover, sometimes these organisms can be present without any signs of demineralisation (Marsh et al. 2009; Gross et al. 2010).

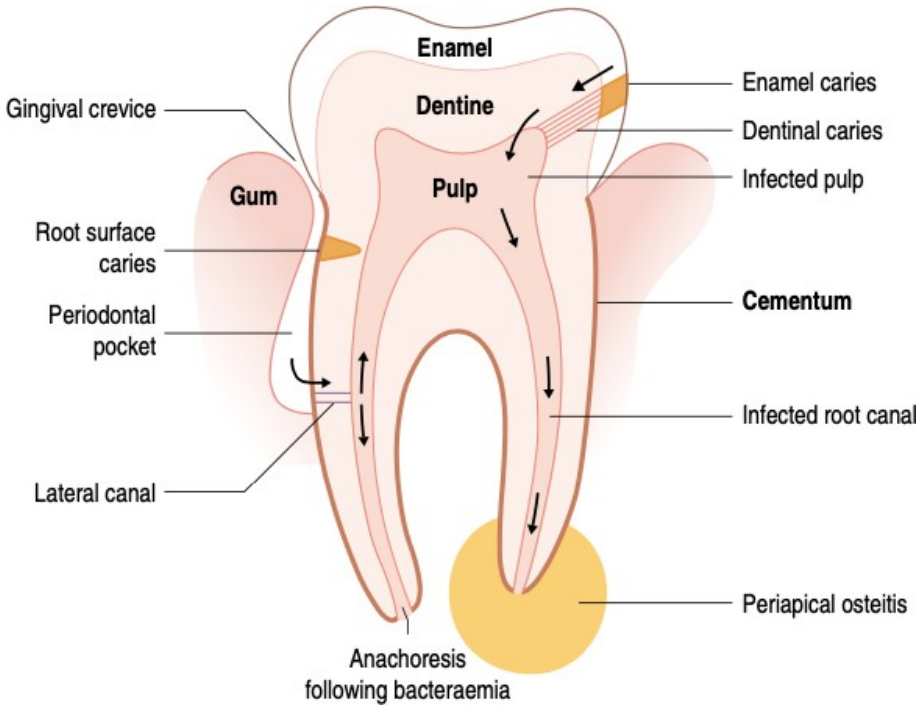


Figure 1.6: Progress of infections of the tooth and supporting structures. Adapted from Marsh et al. (2009).

1.3.2 Gingivitis

Chronic marginal gingivitis is probably the most common microbial infectious disease, with a greater than 90% prevalence in adults worldwide (Coventry et al. 2000). This disease is defined as a non-specific, reversible response to dental plaque involving the gingival margins. The excessive accumulation of dental plaque is considered the primary reason for development of gingivitis (Nyvad and Kilian 1987). In addition to excessive plaque accumulation, systemic factors also play a role in gingival diseases. There is a diverse microflora at the sites of gingivitis, although none of the taxa have been specifically associated with gingivitis. However, studies have shown that certain microorganisms are found more commonly in subjects with

gingivitis, with a rare existence in healthy subjects (Marsh et al. 2009). Gingivitis can be reversed with regular oral hygiene and a variety of oral hygiene practices have been used historically to remove the dental plaque, including brushing teeth with toothpaste (Stuart 1997). When oral hygiene practices are done regularly, they maintain the dental plaque in an immature state and in a relatively small quantity by preventing accumulation. However, in the absence of oral hygiene, the proportion of pathogenic microbes can increase and cause the dental composition to change. This situation results in irritation and inflammation, due to increased endotoxins and other virulence factors from microbes. Swollen, bleeding and inflamed gingivae are the symptoms of gingivitis, which can be reversed with regular oral hygiene (Loe et al. 1965; Socransky 1977).

Not all cases of gingivitis progresses to more severe forms of periodontal disease, although gingivitis is considered a precursor of periodontitis (Figure 1.7). A minor proportion of the gingivitis microflora has been detected as being predominant in periodontitis, and absent in healthy gingiva. This could indicate that the environmental components that arise during gingivitis (such as blood ingestion, increased crevicular fluid flow) may promote the growth of species that can induce periodontal diseases (Marsh et al. 2009).

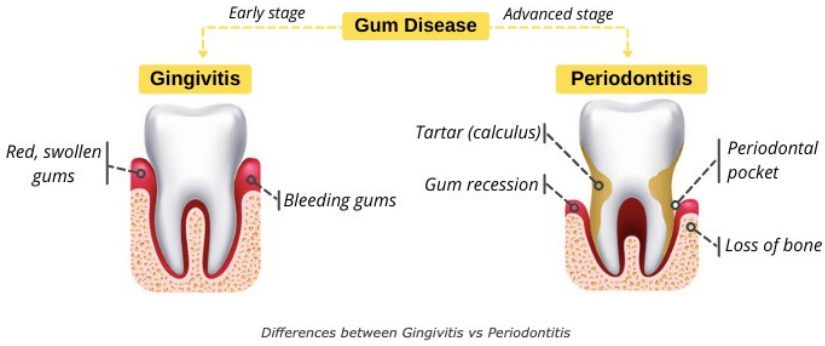


Figure 1.7: An illustration showing the progression from gingivitis to periodontitis. Periodontitis occurs when gums are inflamed or infected (gingivitis) and are not treated. Infection and inflammation spreads to the periodontal ligament and bone, which support the teeth from the gums (gingiva). Generally, the loss of this support means that teeth are loosened and ultimately decay. Adopted from a dental blog, Share

Dental Care, under the title of gum disease, available at: https://sharedentalcare.com/gingivitis-vs-periodontitis/.

1.3.3 Chronic periodontitis

Chronic periodontitis is the most common form of advanced periodontitis and is considered the primary reason for tooth loss after the age of 25 years. Individuals with untreated gingivitis may progress to experience periodontitis, which is an irreversible inflammation of the periodontal tissue, resulting in loss of attachment between the gingivae and teeth. In contrast to gingivitis, chronic periodontitis is an irreversible inflammatory disease. Factors such as subgingival plaque accumulation, over- hanging restoration and crowded teeth that improve plaque retention or hinder plaque clearance may lead to chronic periodontitis (Marsh et al. 2009). The loss of attachment results in the formation of periodontal pockets, which become colonised with primarily anaerobic bacteria. The host immune response cannot induce an efficient response, although host-derived factors such as proteases and reactive oxygen species (ROS) cause extensive tissue damage (Waddington et al. 2000; Darveau 2010). The progression of inflammation continues into the alveolar bone which supports the teeth, inducing enhanced bone resorption by osteoclasts, leading to increased tooth mobility and loss. In addition to environmental and genetic factors, a specific microbiota has been associated with periodontitis (Van Dyke and Sheilesh 2005). These specific microbiota include members of the 'red complex' (*Tannerella forsythia, Porphyromonas gingivalis,* and *Treponema denticola*) and the 'orange complex' (*Fusobacterium spp, Prevotella spp, Parvimonas micra, Eubacterium spp,* and *Streptococcus constellatus*) and have been linked to periodontal disease. Many studies have been performed to identify plaque-associated microorganisms in chronic periodontitis. It was shown that there was a diverse cultivable microflora, including a large proportion of obligately anaerobic Gram-negative rods and filament-shaped bacteria, many of which were asaccharolytic (unable to gain energy from the catabolism of carbohydrates) but proteolytic (Marsh et al. 1994; Socransky et al. 1998; Neilands et al. 2015). Although it is difficult to identify the primary pathogens responsible for periodontitis, some bacteria are associated with periodontitis due to their frequency in deeper periodontal pockets. These species include *P. gingivalis, Treponema denticola,* and Tannerella *forsythia,* and this group of bacteria is referred

to as the “red complex” (Figure 1.8). The association of these bacteria was investigated with culture-based techniques and confirmed via whole-genomic DNA probes (Socransky et al. 1998). The appearance of red complex bacteria was frequently preceded by members of the orange complex, typically located in deeper pockets, but more varied in their membership. Species of the yellow, green and purple complexes, in contrast, were deemed 'host compatible', with *A. naeslundii* typically linked with healthy locations (Marsh et al. 2009) (Figure 1.8).

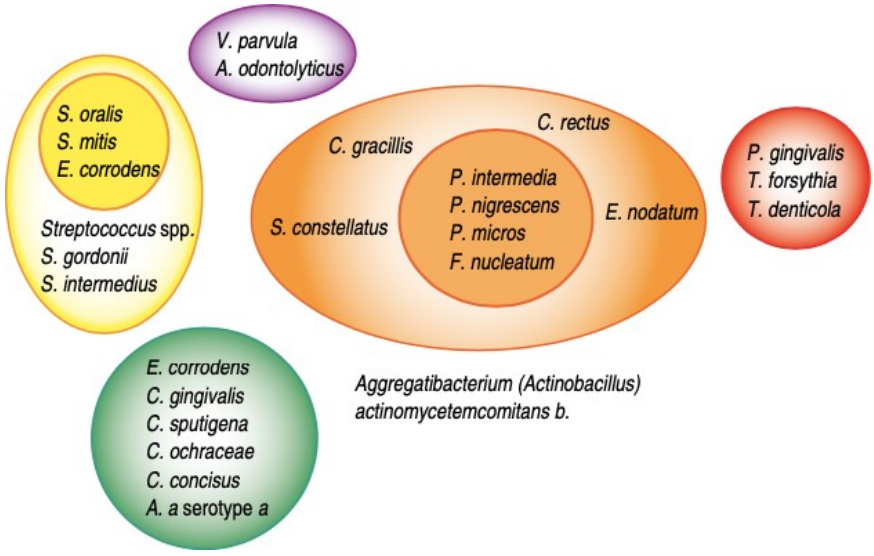


Figure 1.8: Bacteria grouping into complexes to represent their health and periodontal diseases relationships with the host. "Red complex” is most often seen in deep periodontal pockets, with members of the "orange complex" generally preceding it. Members of the "yellow," "green" and "violet" complexes are usually linked to healthy locations. Adapted from Marsh et al. (2009).

These kinds of culture-independent research change the perception of the importance of bacteria in disease. These investigations have shown that complex consortia may be isolated from sites with severe diseases and that deep pockets can are predominated by poorly categorised species that are frequently unculturable in the laboratory. Therefore, it has been difficult to determine whether these organisms are active in disease (Marsh et al. 2009).

1.3.4 Oral candidosis

Oral candidosis is an opportunistic infection in the oral cavity and caused by a yeast-like fungi, such as *Candida* species (Guida 1988; Odds 1988; Epstein 1990). *Candida* species are commonly found in the oral cavity of healthy individuals and generally considered normal residents of the oral microflora. While *Candida* species are generally considered non-harmful and part of the normal oral microflora, when oral conditions alter, increased proliferation of *Candida* species can increase. As a result of this increase in proliferation, *Candida* species can become pathogenic (Marsh et al. 2009). *Candida albicans* is considered the most pathogenic and the most isolated *Candida* species. In addition to *C. albicans,* other species such as *C. glabrata, C. krusei, C. tropicalis, C. guilliermondii, C. kefyr* and *C. parapsilosis* are also found in the oral cavity (Fidel et al. 1999; Tintelnot et al. 2000; Ghannoum et al. 2010). Oral candidosis is one of the most common human fungal infections and is especially seen in elderly and immunosuppressed people. *C. albicans* has the potential to turn into a pathogenic (disease-causing) hyphal form under particular circumstances such as temperature, serum, carbon dioxide and oxygen tension, and neutral pH. In addition, the presence of not only other *C. albicans* cells, but also bacterial cells, which are both recognised by quorum sensing chemicals, regulates the morphological switch (Sudbery 2011). Several factors relate to, but are not limited to, the pathological colonisation of *Candida* species. These include extreme age, malnutrition, metabolism, concomitant infections, antibacterial treatment, immunocompromising conditions, radiotherapy, transplanted patients, hypopfunctional salivary glands and long-term steroid use (Odds 1988). In immunosuppressed patients, oral candidosis can spread through to the bloodstream or the upper gastrointestinal tract and could cause a systemic infection. Often systemic candidoses are associated with high mortality rates (Dupont et al. 1992; Fraser et al. 1992).

1.4 Inflammatory and immune pathways in periodontal diseases

Periodontal diseases are associated with a complex immuno-inflammatory pathway that is induced by dental plaque. The oral microbiome is considered an aetiological factor of periodontal diseases and this microbiome comprises a substantial and constantly evolving community of microbial species. Contrary to many infectious

diseases, periodontal diseases are generally seen instead of as an endogenous infection arising from an over proliferation of commensal microorganisms (Trowbridge and Emling 1997; Gemmel et al. 2000). It is critical to understand how immune and inflammatory responses are regulated during periodontal diseases to find effective solutions for them.

Gingivitis and periodontitis are the most common diseases that affect the periodontium (Fiorellini et al. 2006; Preshaw et al. 2020). The initial inflammation commonly starts with formation of subgingival and supragingival plaque and results in gingivitis (Novak and Novak 2006). This initial inflammation and gingivitis could be reversed with the removal of plaque. However, if plaque is not removed, this inflammation could lead to persistent lesions and pathology (Abusleme et al. 2021).

During the initial lesion, while there is no indication of clinical inflammation, there are alterations in tissue which can be observed histologically. The initial lesion results in a response to dental plaque from resident leukocytes and endothelial cells. Junctional epithelium cells and neutrophils generate cytokines and neuropeptides as a response to bacterial metabolic products. These released cytokines and neuropeptides cause vasodilation of blood vessels, and facilitate neutrophils migration to the inflammation site. Early stage lesion exhibit increase in neutrophils, macrophages, lymphocytes, plasma cells and mast cells in the connective tissues. Complement proteins are also triggered. The clinical signs of inflammation such as bleeding and gingival crevicular fluid flow increases are also observed. The established lesion considered a transition between the innate and acquired immune response. This means that blood flow is impeded and that macrophages, plasma cells, T-lymphocytes and B-lymphocytes become dominant (Panagakos and Scannapieco 2011; Fiorellini et. al 2006). In the final lesion, namely periodontitis, irreversible attachment loss and alveolar bone loss, is observed clinically. The inflammatory area the extends to the alveolar bone (Fiorellini et. al 2006).

The inflammatory and immune responses in periodontal diseases is a wide topic and includes many facets, such as complement cascade system, neuropeptides, cells and mediators and Toll-like receptors (Pan et al. 2019). Attention is given to below cytokines and lipid mediators, which is a focus of this submitted.

1.4.1 Cytokines

Cytokines and chemokines are the communication signals between cells and they regulate the immune response to infections. Cytokines are low-molecular-weight proteins, and play roles both in initiation and later stages of inflammation (Figure 1.9). The secretion of pro-inflammatory cytokines from different cells is generally maintained by activation of nuclear factor kappa-B (NF-κB) (Baldwin 1996; Hanada and Yoshimura 2002). NF-κB is a protein transcription factor that regulates innate immunity and coordinates cellular resistance against invading pathogens (Salminen et al. 2008; Baltimore 2009). The NF-κB pathway is triggered by pathogen-associated molecular patterns, such as lipopolysaccharide (Hanada and Yoshimura 2002).

The source of cytokines are resident cells, such as epithelial cells and fibroblasts. In acute and early chronic phases of inflammation, cytokines are produced by phagocytes (neutrophils and macrophages), and in the established lesion and in periodontitis, cytokines are produced by immune cells (lymphocytes) (Ara et al. 2009). In addition, antigen-representing cells, and specific T cell subsets (such as type 17 helper T [TH17] cells) are recruited during the continuous inflammation of tissue (Pan et al. 2019). Interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) are the cytokines involved in the initial progression of periodontal diseases in response to the recognition and presentation of microbes to appropriate cells (Garlet 2010). Specifically, IL-1β and IL-6 is considered the most common cytokines in inflammation and have been associated with migration of inflammatory cells and osteoclastogenesis (Graves et al. 2008; Fonseca et al. 2009). TNF-α has many functions, including coordinating cell migration and tissue destruction and stimulating production of IL-1β and IL-6 (Dinarello 2000; Wajant et al. 2003; Garlet et al. 2007; Graves et al. 2008). In addition, TNF-α has a role in ECM degradation and bone resorption. TNF-α can also induce the secretion of matrix metalloproteinases (MMPs) and activate receptors of nuclear factor kappa-B ligand (RANKL) (Garlet et al. 2003; Graves and Cochran 2003; Graves et al. 2008).

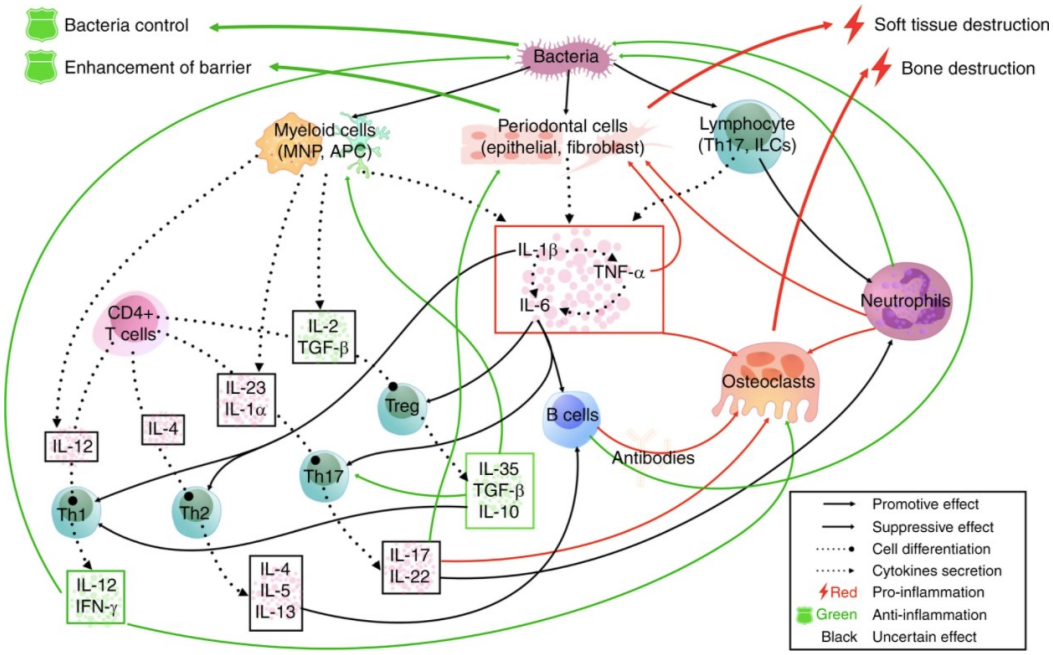


Figure 1.9: The cytokine network in pathogenesis of periodontitis. Full lines show interaction between cells and cytokine effects on cells. Dashed lines show the secretion of cytokines. Adapted from Pan et al. (2019).

These cytokines (IL-1β, IL-6 and TNF-α) are representative of the first wave of cytokines from interaction between the microbiome and host cells. These cytokines are well recognised pro-inflammatory cytokines and have multiple effects on immuno-inflammatory responses and tissue destruction. Secretion of these pro-inflammatory cytokines leads to specific immune cell subsets being activated and recruited to the inflammation site, and this could cause direct tissue damage. Differentiation of naive T cells and B cells into mature T cells or plasma cells occurs from the activity of specific cytokines, such as IL- 12 (Glimcher 2007). In addition, these cells stimulate other effector cells such as osteoclasts and neutrophils that can show pro- or anti-inflammatory actions through the secretion of cell-specific cytokines (Pan et al. 2019).

1.4.2 Lipid mediators in periodontal inflammation

Prostaglandins are generated via the hydrolysis of membrane phospholipids. Briefly, phospholipase A2 renders a specific position of membrane phospholipids and produces arachidonic acid, which is an ancestor of a group of small lipids known as eicosanoids (Lewis 1990). Arachidonic acid is metabolised via lipoxygenases or cyclooxygenases 1 and 2 (COX-1 and COX-2). Lipoxygenase activity produces leukotrienes, whilst prostaglandins, prostacyclin and thromboxane are produced by the cyclooxygenase pathway. There are 10 subclasses of prostaglandins and an increased amount of prostaglandins has been reported in inflamed gingiva (Mendieta et al. 1985; Gemmel et al. 1997). Prostaglandin E2 (PGE2) is considered a major inducer of alveolar bone resorption which is mainly found in macrophage-like cells. The secretion of PGE2 increases with bacterial lipopolysaccharide (Loning et al. 1980) and is stimulated by IL- 1β, TNF-α and parathyroid hormone (Richards and Rutherford 1988; Saito et al. 1990a,b). It should be emphasised that PGE2 demonstrates a biphasic pattern in inflammation. As it reduces IgG at higher doses and increases IgG level at low doses. Moreover, when it is combined with IL-4, an anti-inflammatory cytokine, synergistic enhancement IgG production is observed (Harrell and Stein 1995).

1.5 Prevention and control of dental diseases by antimicrobial agents

Antimicrobial agents used to improve oral hygiene are normally formulated into oral care products, such as toothpastes and mouthwashes. The oral cavity has advantages and disadvantages, in terms of drug delivery. When antimicrobial agents are applied to oral surfaces they have an initial high concentration, but this concentration decreases rapidly due to continuous salivary flow and swallowing (Vanderouderaa and Cummins 1989). However, continuous salivary flow provides transportation of antimicrobial agents between different regions of the mouth and could increase exposure time of potential antimicrobial activity. There are many antimicrobial agents which have different mode of actions for oral health, and they have generally been used as adjunctive therapies with physical dental plaque removal (Baehni and Takeuchi 2003; Rmaile et al. 2014).

1.5.1 Chlorhexidine

Chlorhexidine, which is a cationic biguanide antimicrobial agent, was first introduced as an antiseptic in 1954 (Davies et al. 1954). In 1969, chlorhexidine was suggested for use as an efficient antiplaque agent (Schroeder and Shanley 1969) and has since been widely used for plaque and gingivitis control. Chlorhexidine is a broad-spectrum antimicrobial agent and proposed to exert its antimicrobial activity by disrupting the cell membrane of bacteria (Hugo and Longworth 1965). Chlorhexidine was found to be effective against both Gram-positive and Gram-negative bacteria and yeasts and reduces dental plaque, caries and gingivitis (Fejerskov et al. 2015). In higher concentrations, chlorhexidine is bactericidal and in sub-lethal concentrations, inhibits sugar transportation and acid production in cariogenic bacteria and also inhibits of major proteases (gingipain) of *P. gingivalis* (Stoeken et al. 2007). Chlorhexidine was also effective in gingivitis control by using 0.1% or 0.2% chlorhexidine mouth rinse for children as an adjunct therapy with regular tooth brushing (Lang et al. 1982). Similarly, 0.12% chlorhexidine gluconate was found to be effective in gingivitis control for adults (Grossman et al. 1986).

1.5.2 Zinc salts

Metal salts have been used widely in oral care products since they have a long substantivity in the oral cavity and effective against both Gram-positive and Gram-negative bacteria (Cummins 1992). For instance, at sub-lethal doses, zinc can interfere with the transportation of sugar, acid production and protease activity (Brading and Marsh 2003). It was also suggested that zinc can inhibit bacterial adherence to surfaces by changing the bacterial surface charge. Zinc can also interfere with bacterial protein synthesis (Stephen et al. 1988). The high substantivity of these metals provides a prolonged time of antimicrobial activity. For instance, zinc citrate remains on the tooth surface after brushing and retained zinc citrate inhibits regrowth of bacteria and control dental plaque accumulation (Gilbert and Williams 1987; Ingram et al. 1992).

1.5.3 Triclosan

Triclosan is a bisphenol, broad spectrum antimicrobial agent which can inhibit dental plaque formation/accumulation and also reduce gingival inflammation (Brading and

Marsh 2003). It has been used in toothpaste formulations and is one of the most common compounds in dentifrice formulations (McBain et al 2003). The suggested antimicrobial mechanism of triclosan is disruption of bacterial cell membranes and blocking fatty-acid synthesis at the envoy-acyl carrier protein reductase step (McMurry et al. 1998; Russell 2004). In addition, it was reported that sub-lethal concentrations of triclosan can inhibit acid production by oral streptococci and production of proteases by *P. gingivalis* (Brading and Marsh 2003). Moreover, it was found that triclosan has high substantivity and is retained in the oral cavity (8 h in dental plaque and 3 h in oral mucosa) after tooth brushing (Gilbert and Williams 1987). Combination of zinc salts and triclosan exerted an enhanced anti-plaque and anti-gingivitis activity. In addition, triclosan presented a potentiated anti-inflammation activity when it was used in combination with a fluoride copolymer delivery system (Moran et al. 2001; Phan and Marquis 2006).

1.5.4 Fluoride

Fluoride is a well-known oral therapeutic due to its benefit on tooth demineralisation/remineralisation cycles. Fluoride was initially used in toothpastes along with anti-caries and cleaning products in the second half of the 20th century. Fluoride has been used in formulations with sodium fluoride, stannous fluoride, sodium mono fluorophosphates and amine fluoride (Paraskevas 2005; Hara et al. 2013). The anti-caries activity of fluoride has been attributed to its effect on demineralisation/remineralisation cycles of tooth enamel. The relatively low concentrations (0.02 ppm and 0.04 ppm) of fluoride in salivary or dental plaque was found to be beneficial in remineralisation of tooth enamel (Gibbs et al. 1995; Leverett et al. 1997; Hughes et al. 2004). In addition, the antimicrobial activity of fluoride was reported to exert cariostatic properties and control gingivitis (Binney et al. 1995; Miller et al. 1999; Ganss et al. 2004). Fluoride concentration and regular brushing (at least twice daily) are important factors to obtain optimal activity from fluoride (Davies et al. 2003).

1.5.5 Natural products, plant extracts and probiotics

Phytochemicals from plants have been used in the treatment of dental caries and the control of dental plaque, and found to be effective and economic treatments for oral infections (Chinsembu 2016). Catechin, emetine, quinine and flavone are the most reported phytochemicals to have shown effectiveness in clinical trials (Salehi et al. 2019; Chauhan et al. 2020). Although these were found to be effective in the treatment of dental caries, they could cause toxicity to host cells, and thus safety tests and dose controls are necessary for their use in oral health (Bodiba et al. 2018). It was found that a 0.5% extract of *Stevia rebaudiana* leaves inhibited cariogenic microorganisms and enhanced salivary buffering capacity in patients with high caries levels (Usha et al. 2017). Probiotics have been investigated for their beneficial effects in the oral cavity, and it was found that certain probiotics inhibit cariogenic microorganisms via the production of microcin (Zschüttig et al. 2012). Natural products could provide huge benefits as adjunctive therapeutics in dental care products, and their wide availability and cost effectiveness would benefit the general population (Chauhan et al. 2020).

1.6 Pomegranate

1.6.1 Pomegranate in history

The scientific name of pomegranate is *Punica granatum* and was derived from the name *Pomum* (apple) and *granatus* (grainy) or seeded apple. Persia and surrounding areas have been thought to be the habitat of pomegranate, and it is believed that pomegranate was spread to the rest of the world from Central Asia, especially to parts of Iran (Simmonds 1976; Harlan 1992; Levin 1994; Levin 2006; Verma et al. 2010). Another view for the origin of pomegranate is that it was cultivated in ancient Egypt and early in Greece, Italy, and Iraq, and then it spread to other parts of the world including Turkmenistan, Afghanistan, India and China, along with Iran, North Africa and Mediterranean Europe (Melgarejo and Martinez 1992). Pomegranate is considered to be one of the oldest domesticated fruit and was first planted between 4000BC and 3000 BC. Moreover, the pomegranate fruit has been mentioned in ancient documents, such as the Bible and Koran (Chandra and Tarachand 2010). Another claim was that

the probable pomegranate progenitor was very similar in appearance to its domesticated form. However, fruit size with larger seeds or colour could be primary differences between progenitor and domesticated pomegranate (Harlan 1992; Hancock 2004; Still 2006). The pomegranate family is a single genus, *Punica*, and only comprises two species, *P. granatum* and *P. protopunica*. *P. protopunica* has been considered an ancestor of pomegranate and it is endemic to Socotra Islands in Yemen. It might have a contribution to the evolution of the cultivated form of pomegranate (Zukhovskij, 1950; Moriguchi et al. 1987; Guarino et al. 1990).

During the domestication process, pomegranate selection has been made according to flower, rind, aril colour, fruit size, sugar and acid contents, resistance to environmental and other stress factors, yield and storage stability (Harlan 1992; Hancock 2004; Levin 2006; Holland et al. 2009).

Pomegranate has been shown to exist in many cultures over hundreds of years. In Judaism, Christianity, Islam, and Buddhism it has taken a place in religious beliefs and rituals, and it was a symbol of life, health, spirituality, morality and mortality (Mahdihassan 1984). It has been used as symbol of fertility in many cultures (Mahdihassan 1984). Furthermore, it has been used in traditional medicine by different cultures. The fruit was used as a blood tonic and pomegranate hulls used in the treatment of dysentery and diarrhoea, and as an antiparasitic agent (Boukef et al. 1982; Caceres et al. 1987; Nagaraju and Rao 1990; Naqvi et al. 1991). There are also reports about its traditional use for snakebites, diabetes, burns and leprosy (Siang 1983; Jain and Puri 1984; Singh 1986).

In the present time, pomegranate is cultivated throughout the world including tropical and sub-tropical areas. However, Mediterranean and Asian countries are the primary areas for commercial cultivation of pomegranates. In addition, it is cultivated in Argentina, Australia, Brazil, Chile, South Africa and the United States (La Rue 1980; Frison and Servinsky 1995). It has been reported that there are more than 500 pomegranate cultivars around the world, but around 50 of them are commercially cultivated (Hooks et al. 2021). The cultivation process has caused a great decrease in genetic diversity of modern pomegranate cultivars. However, progenitors still have a

huge variety in genetic diversity and it is important to have genetic tools to improve the cultivated pomegranate in future (Rana et al. 2007).

Today, it is estimated that around 1.5 million tonnes of pomegranate fruits are produced annually across the world (Holland and Bar-Ya'akov 2008). Iran has the highest rate of pomegranate export with almost 60,000 tonnes, followed by India with 35,176 tonnes (Holland and Bar-Ya'akov 2008; Chandra and Jadhav 2009).

1.6.2 Pomegranate in modern industry

Pomegranate has a long history of being used for its nutritional value. The fruit has been consumed raw, juice, and in jams and salad dressing. In addition to this nutritional usage, it has been shown that pomegranate fruit can be used to prepare numerous products in nutritional, cosmetic and health-related products, due to its high content of phytochemicals. Pomegranate rind is a by-product of the pomegranate juice industry and found to have prominent use in the food and medicinal industries, due to its high phenolic contents (Viuda-Martos et al. 2010, 2013). The antioxidant activity of pomegranate fruit is well known, and addition of 0.5% dried pomegranate peel extract can enhance the antioxidant capacity of other juices, such as tomato and orange juices (Salgado et al. 2012). In addition, pomegranate fruit has been used to make ink and dyes since ancient times (Bruni et al. 2010). Recently, it has been found that cotton textile can be dyed with a natural dye extracted from pomegranate peel (Kulkarni et al. 2011). Moreover, pomegranate fruit has been used for nanoparticle formation due to its high capacity as a reducing agent. Synthesising nanoparticles with pomegranate and other plants which have reducing capacity is considered better for the environment. Thus, nanoparticle synthesis with plant extracts has been called green synthesis (Chauhan et al. 2011; Li and Gu 2011; Li et al. 2011; Ahmad and Sharma 2012; Ahmad et al. 2012). Similarly, pomegranate has been used for removal of wastes, hazardous materials and metals from natural environments (El-Ashtoukhy et al. 2008; El Nemr 2009).

1.6.3 Pomegranate functional phytochemicals

Many phytochemicals have been identified in different pomegranate tissues using methods such as diode array detection (DAD), electron spin resonance (ESR), fluorescence detection (FD), flame ionisation detection (FID), infrared spectroscopy (IR), mass spectrometry (MS), nuclear magnetic resonance (NMR), high pressure liquid chromatography (HPLC) and thin layer chromatography (TLC). The knowledge about pomegranate phytochemicals is important to elucidate the biological activities of pomegranate fruit. However, it should be noted that there might be variations between the type of chemicals and their quantity in the fruit because of different pomegranate cultivars (Melgarejo et al. 2000; Kaufman and Wiesman 2007; Tzulker et al. 2007; Pande and Akoh 2009; Qiu et al. 2013). Polyphenols phytochemicals mainly found in the rind of pomegranate fruit (Amri et al. 2017) (Figure 1.10). Total phenolic content was found to be greater in pomegranate rind than arils, seeds of pomegranate fruit, juice, leaves and flowers in different cultivars including Israeli, Turkish, Tunisian and Persian cultivars (Gozlekci et al. 2011; Elfalleh et al. 2012; Orak et al. 2012; Orgil et al. 2014).

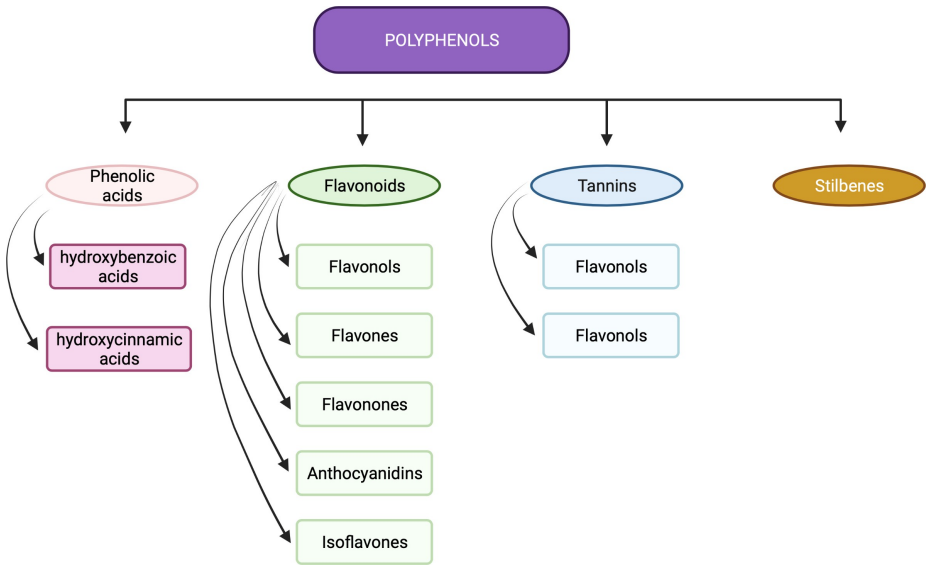


Figure 1.10: Classification of major polyphenolic compounds (Reproduced from Tsao 2010).

1.6.3.1 Tannins

Tannins are polyphenols with a high molecular weight ranging from 500 Da to more than 3000 Da. Tannins can be found in different parts of plants, including leaves, bark, fruit, wood and roots. Tannins have been associated with the plant defence mechanisms against mammalian herbivores, birds and insects (Hagerman and Buther 1981; Hassanpour et al. 2011). Tannins are divided into two main groups, based on their chemical structures; namely, hydrolysable tannins (gallotannins and ellagitannins) and condensed tannins (or proanthocyanidins) (Athanasiadou et al. 2001; Chaichi Semsari et al. 2011; Hassanpour et al. 2011; Maheri-Sis et al. 2011). Hydrolysable tannins (HTs) are the most studied phytochemicals in pomegranate and can be classified into ellagitannins (ETs) and gallotannins (GTs), according to types of phenolic acids that make an esterification with the core cyclic polyol molecule, which is often a glucose molecule. More than 60 HTs have been identified in pomegranate using MS and/or NMR (Figure 1.11). Pomegranate peel/rind is one of the most studied tissues of pomegranate and it has been found to be particularly rich in ETs. Punicalagin isomers were found to be the main ETs in pomegranate peel with up to 85 % (w/w) of total tannins from pomegranate fruit peel (Seeram et al. 2005). Punicalagin is also a major tannin in pomegranate root, but found in a lower amount (Ono et al. 2012). Pomegranate rind has a high content of ellagitannins and ellagic acid derivatives, including punicalagin (hexahydroxydiphenoyl (HHDP)-gallagyl-hexoside), punicalin (gallagyl- hexoside), ellagic acid hexoside and ellagic acid pentoside. Moreover, granatin A, granatin B, punicalin, punicalagin, corilagin, gallagyldilactone, pedunculagin and tellimagrandi tannins have been identified in pomegranate rind (Satomi et al. 1993). Punicalagin is the most studied bioactive polyphenol, due to its high proportion in pomegranate rind, and it has been associated with the biological activities of pomegranate rind itself (Cam and Hisil 2010).

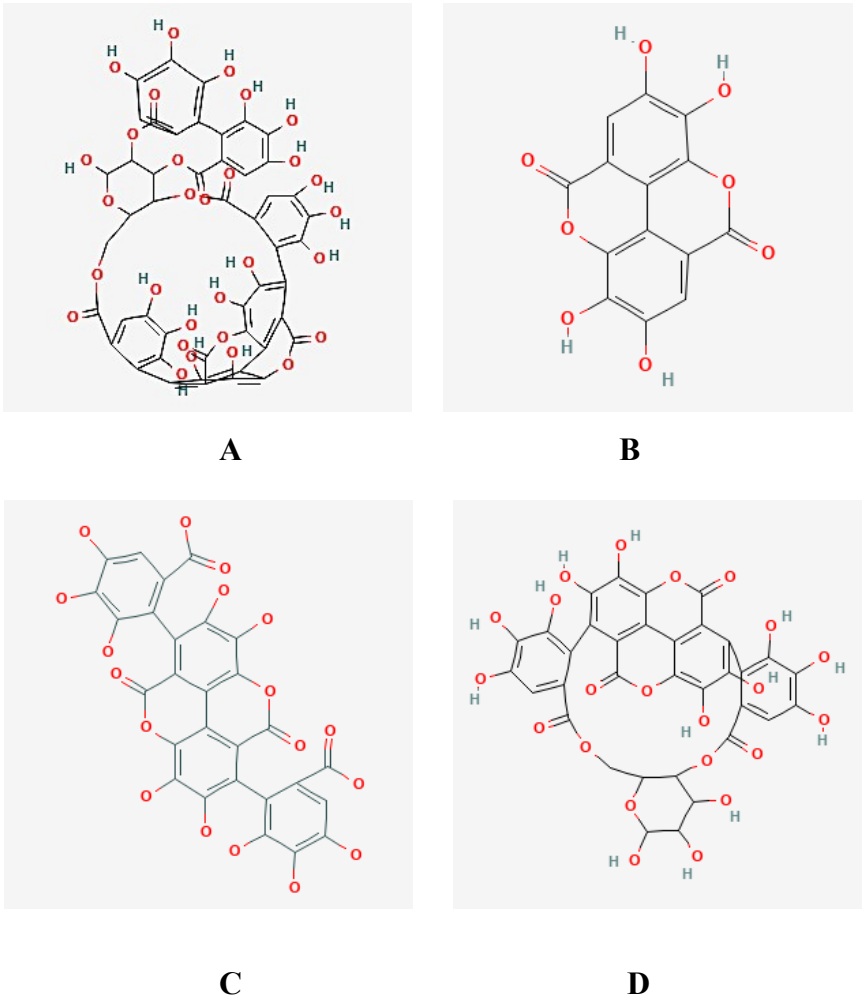


Figure 1.11: The chemical structures of selected compounds (A; punicalagin, B; ellagic acid, C; gallagic acid, D; punicalin) in pomegranate rind (National Library of Medicine, PubChem, 2021, available at https://pubchem.ncbi.nlm.nih.gov).

1.6.3.2 Flavonoids

Flavonoids are found in most edible plants, and include the polyphenolic flavan skeleton substitutes with hydroxy, methyl, galloyl, glucosyl and acyl moieties. Flavonoids can form complexes with other flavonoids, metal ions and other molecules (Asen et al. 1972; Brouillard et al. 1989; Haslam 1998; Harborne et al. 2013). Pomegranate peel/rinds are abundant with flavonoids, including catechin, epicatechin, quercetin, anthocyanins and procyanidins. Flavonoid composition and concentration show differences based on the fruit harvesting and the type of cultivars (Yuan and

Fang 2018). Flavonoids (mainly anthocyanins) are the compounds which give pomegranate its colour (Lawrence 1950; Bateson 1965; Barritt and Einset 1969; Sparvoli et al. 1994; Holton and Cornish 1995; Boss et al. 1996). Pomegranate rind contains almost 30% of the total anthocyanins of pomegranate fruit (Zhao et al. 2013). The content and composition of pomegranate rind anthocyanins is highly variable based on the pomegranate rind colour. For instance, anthocyanins, cyanidin 3,5-diglucoside (157.8mg/kg), pelargonidin 3,5-diglucoside (145.8 mg/kg), pelargonidin 3- glucoside (56.7 mg/kg), cyanidin 3-glucoside (41.2 mg/kg), cyanidin 3-rutinoside (18.4 mg/kg), delphinidin 3-glucoside (13.3 mg/kg), delphinidin 3,5-diglucoside (10.8 mg/kg), cyanidin hexoside (1.7 mg/kg) and cyanidin-pentoside (1.4 mg/kg), were identified and quantified in rind of Peruvian pomegranate cultivar (Fischer et al. 2011). In a Tunisian cultivar, pelargonidin-3-glycoside, pelargonidin-3,5- diglycoside, delphinidin-3-glycoside, delphinidin-3,5-diglycoside, cyanidin-3- glycoside, cyanidin-3,5-diglycoside, cyanidin-3-pentoside and cyanidin-3- rutinoside were identified as anthocyanins in pomegranate rind by high- performance liquid chromatography-mass spectrometry (HPLC-MS) analysis (Wafa et al. 2017). Catechin, epicatechin, quercetin and rutin (especially in fresh pomegranate rind) were identified in different cultivars, including South Africa, China, Georgia and Iran. However, the level of these flavonoids showed variability between different cultivars and developmental stages of the fruit (Fawole et al. 2012; Mansour et al. 2013; Zhao et al. 2013; Mphahlele et al. 2017). It should be noted that environmental factors, such as soil quality, temperature, climatic differences and variation in the genetics of different cultivars can affect the composition and level of flavonoids in plants including pomegranate (Mullins et al. 1992; Reid et al. 2005).

1.6.3.3 Phenolic acids

Phenolic acids are a group of compounds comprised of two subgroups: namely, hydroxybenzoic and hydroxycinnamic acids. Gallic, p-hydroxybenzoic, protocatechuic, vanillic and syringic acids, which have the C6–C1 structure in common, are found under the subgroup of hydroxybenzoic acid. Hydroxycinnamic acids, an aromatic compound with three carbon side chains, include caffeic, ferulic, p-coumaric and sinapic acids, being the most common and hydroxycinnamic acids are a

major class of phenolic compounds (Balasundram et al. 2006; Gonthier et al. 2006). Phenolic acids, chlorogenic, caffeic, syringic, sinapic, p-coumaric, ferulic, vanillic, ellagic, gallic and cinnamic acid have all been identified in pomegranate rind (Elfalleh et al. 2011). It has been reported that the ellagic acid content comprises more than 50% of total phenolic compounds, including polyphenols, in pomegranate rind (Fawole et al. 2012).

1.6.3.4 Other compounds

In addition to polyphenols, pomegranate rind comprises minerals, such as calcium, phosphorus, magnesium, potassium and sodium. Furthermore, sugars including glucose, fructose, sucrose and maltose, along with protein, crude fibres, fatty acids, organic acids and alkaloids have all been identified in pomegranate rind (Van Elswijk et al. 2004; Rahmani et al. 2017). Recently, a pyrrolidine-type alkaloid, punigratane (2,5-diheptyl-N-methylpyrrolidine), was characterised in pomegranate rind (Rafiq et al. 2016). Punicic acid, linoleic acid and oleic acids are found in a higher amount than other fatty acids in pomegranate seed oil and it was reported that punicic acid levels range from 73.4% to 83.5% of total fatty acids (Khalili- Dehkordi et al. 2011; Fernandes et al. 2015).

In pomegranate rind, lignans have also been identified, such as isolariciresinol and pomegralignan (Fischer et al. 2012; Ito et al. 2014). Lignans are defined as phytoestrogens, and they can be metabolised by gut microbiota (Fischer et al. 2012). Triterpenoids and phytosterols are also found in pomegranate rind (Wahab et al. 1998).

1.6.4 Pomegranate medicinal properties

1.6.4.1 Antimicrobial activities

While herbal antimicrobials formed the origin of modern medicine, it should be remembered that almost 80% of the world's population continues to use traditional medicine as a primary health care resource. Because of the mass expense of developing and testing new drugs, the induction of new antimicrobials has decreased

since 1980. Additionally, conventional antimicrobials kill beneficial bacterial types such as gut flora alongside the targeted bacteria with prolonged use, requiring patients to take prebiotics to replace the beneficial gut flora (Khan and Hanee 2011). Furthermore, with the increasing resistance of bacteria to conventional antimicrobial drugs, the antimicrobial bioactivity of natural drugs has gained greater importance and popularity. In recent decades, several studies inspired by the problems of over-dependence on antibiotics have demonstrated the antimicrobial activity of plants on different microbes (Grosvenor et al. 1995; Valsaraj et al. 1997). *Punica granatum L.* has been widely used in traditional medicine for the treatment of different diseases. Pomegranate has been used to treat microbial infections and pomegranate extracts have demonstrated anti-viral activity against herpes and influenza viruses (Zhang et al. 1995; Sundararajan et al. 2010).

1.6.4.1.1 Antibacterial activity

The antibacterial activity of pomegranate has been studied by many scientists across the world, with conclusions indicating that pomegranate extracts prepared from different parts of the pomegranate fruit have an antibacterial activity on Gram- positive and Gram-negative bacteria. The antibacterial mechanism of pomegranate extracts has been associated with a reaction between bacterial cell membrane proteins and the pomegranate phenolic content. This reaction causes bacterial cell lysis. Consequently, there could be a reaction between phenolic compounds of pomegranate and protein sulfhydryl groups that makes them unavailable for microbial growth by generating phenolic toxicity (Haslam et al. 1997). Additionally, the hydrophilic properties of the compounds also has a role in antimicrobial activity. Pomegranate polyphenols, such as ellagic acid and punicalagin, are hydrophilic and have good solubility in hydrophilic solvents. However, hydrophobic solvents yield extracts with relatively weaker or no microbial activity (Al-Zoreky 2009). A study of pomegranate peel extract (rind), seed extract, juice and whole fruit on selected bacterial types showed that peel extract has the highest antibacterial activity, compared to other extracts, and the rind extract presented the highest activity against *Staphylococcus aureus* (Dahham et al. 2010). Negi and Jayaprakasha (2003) prepared pomegranate rind extracts using different solvents containing ethyl acetate, acetone, methanol and water against some Gram-

positive and Gram-negative bacteria types using the *in vitro* pour plate method. Acetone extract showed the highest antimicrobial activity followed by the methanol and water extracts. It was found that the methanol extract of pomegranate rind has antibacterial activity against *S. aureus, E. coli* and *Pseudomonas aeruginosa.* Abdollahzadeh et al. (2011) found that methanolic extract pomegranate peel was effective against both Gram-positive and Gram- negative bacteria, such as *S. epidermidis, S. aureus, L. acidophilus, S. mutans, S. sanguinis, S. salivarius* and *E. coli* but was not effective against *A. viscosus* and *C. albicans*. Vasconcelos et al. (2006) showed that pomegranate gel was active against *S. mutans, S. sanguis, S. mitis*, and *C. albicans*. Moreover, the study also used pomegranate gel on the combination of studied microbes, and it was found effective.

Overall, the antibacterial activity of pomegranate compounds has been studied by many scientists in different fields such as oral diseases, food preservation and intestinal flora. The general suggested mechanism for pomegranate extracts is the relation between the molecular structure of pomegranate tannins and their toxicity, and strong astringent properties. Further studies are needed to determine the precise antibacterial mechanism of pomegranate, to find the active compounds and whether there is a synergistic effect between pomegranate secondary compounds (Celiksoy and Heard 2021).

1.6.4.1.2 Antifungal activity

Pomegranate has antifungal activity alongside its other antimicrobial activities, mentioned above. *In vitro* studies revealed that oral bacteria and *C. albicans* are sensitive to pomegranate extracts. According to a clinical study by Vasconcelos et al. (2003), pomegranate could be a promising topical agent for the treatment of candidosis associated with denture stomatitis. This study tested 60 patients with denture stomatitis, divided into groups A and B. Group A were treated with miconazole, the standard conventional drug for candidosis, while group B was treated with pomegranate gel obtained from bark of fresh fruit. Both groups took the medicines three times per day for 15 days. The results showed a satisfactory and regular response from 27 and 21 patients from groups A and B, respectively. Absence of yeasts was

observed in 25 subjects from group A and 23 from group B (Vasconcelos et al. 2003). A study of six different cultivars of pomegranate from the Mediterranean region of Turkey found that these pomegranate extracts showed positive results regarding the inhibition of *C. albicans*, Gram-negative and Gram- positive bacteria (Duman et al. 2009). Anibal et al. (2013) evaluated the antifungal activity of *Punica granatum* extracts to identify the compounds that have a role in the inhibition of fungal activity. Along with mass spectrometry, Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) were used to determine the structural and morphological effects produced by extracts. SEM results revealed that the pericarp and peel extracts of pomegranate caused morphological alterations to the yeast tested, *C. albicans* and *C. krusei*, in addition to cell aggregation and growth inhibition. Additional observations noted an irregular cell wall, with viscous material on cell surface, in addition to rupture of the hyphae with desquamation.

It is concluded that pomegranate extracts, especially rind extracts due to their high phenolic contents, have antifungal activity against some *Candida* species. The extracts could be used as alternative antifungal drugs for microbial diseases and infections in light of supported studies, which show the antifungal activity of pomegranate compounds.

1.6.4.1.3 Antiviral activity

Plant extracts have become popular and important for their use as alternative antivirals. Pomegranate extracts could be used to inhibit the activity of viruses transmitted via infected foods, bodily fluids and other different routes of transmission (Howell and Souza 2013). The antiviral activity of pomegranate extracts has been attributed to pomegranate polyphenols, as extracts without tannins did not show any antiviral activity (Neurath et al. 2004). Haidari et al. (2009) showed that pomegranate polyphenol extract inhibited the replication of human influenza A/Hong Kong (H3N2) *in vitro*. The study evaluated four major polyphenols from pomegranate (ellagic acid, caffeic acid, luteolin and punicalagin), and demonstrated that punicalagin was the polyphenol responsible for the antiviral activity. Furthermore, it identified that the replication of virus ribonucleic acid (RNA) was blocked, which inhibited the

agglutination of chicken red blood cells by the virus as well as exhibiting general virucidal effects (Haidari et al. 2009). In another study using SEM, it was indicated that viral inactivation by pomegranate polyphenols was mainly structural damage on the virion and the direct anti-influenza activity of pomegranate polyphenols was substantially modulated by small changes in envelope glycoproteins (Sundararajan et al. 2010). Experiments revealed that punicalagin targeted and inactivated herpes simplex virus 1 (HSV-1) viral particles and prevented binding, penetration, cell-to-cell spread and secondary infection (Lin et al. 2011). A more recent study demonstrated the antiviral activity of pomegranate rind extract and punicalagin against HSV and acyclovir-resistant HSV (Houston et al. 2017b). Pomegranate juice and polyphenols also induce antiviral activity against foodborne viral surrogates FCV-F9, MNV-1 and bacteriophage MS2 (Su et al. 2010).

1.6.4.1.4 Anti-parasitic activity

Parasitic infections are a significant problem across the world and especially in countries with low economic and social status. Pomegranate has commonly been used as vermifugal and also taenicide agents (Tagboto and Townson 2001; Prakash and Prakash 2011). This activity of pomegranate has been determined in folkloric medicine, with pomegranate used as anthelmintic for diarrhoea against tapeworms (Das et al. 1999; Asres et al. 2001). Recent studies reported that methanolic extract pomegranate leaves were found to be effective against *Shistosoma mansoni* in both *in vitro* and *in vivo* studies (Fahmy et al. 2009). It was shown that pomegranate extracts caused inhibition in motor activity, lethality and morphological alterations in adult worms (Yones et al. 2016). Moreover, the activity against *T. vaginalis* was found during both *in vitro* and clinical studies (El-Sherbini et al. 2010).

1.6.4.2 Anti-inflammatory activity

Inflammation manifests as a multifactorial network of chemical signals that initiate and maintain the host response towards healing in response to tissue injury (Coussens and Werb 2002). Inflammation is a natural response of the immune system to infection. However, prolonged inflammation causes discomfort, pain and tissue

damage in the infectious and surrounding tissues. Furthermore, conventional anti-inflammatory drugs may cause serious side effects, which could be lethal in some situations (Gautam and Jachak 2009; Pountos et al. 2011). Therefore, it is important to develop novel and alternative drugs with minimal side effects for inflammatory diseases. Pomegranate has long been used for its wide range of healing effects, including treating inflammation. Recent empirical studies have demonstrated anti-inflammatory activity of a wide range of pomegranate extracts. While the mechanism of the anti-inflammatory activity of pomegranate extracts has not been clearly identified, studies show that pomegranate extracts influence eicosanoid enzyme inhibition, cytokines, eicosanoid/cytokine crosstalk and matrix metalloproteinases (Lansky and Newman 2007). It has been suggested that pomegranate and derived extracts supress mast cells and basophils, which have a key role in activation of inflammatory disease. Pro-inflammatory cytokines produced by mast cells, basophils and certain other cell types that promote inflammation have a substantial role in acute and late-phase inflammatory reactions (Rasheed et al. 2009). Nitric oxide ($NO^{\cdot}$), a pro-inflammatory mediator, is excessively produced in pathological or abnormal conditions and induces inflammation. Ellagic acid, gallic acid and punicalagin anomers potentially inhibit lipopolysaccharide (LPS)-induced $NO^{\cdot}$, PGE2 and IL-6 production in the human basophilic cell line KU812 (Ben Saad et al. 2017). Furthermore, another study showed $NO^{\cdot}$ inhibition by pomegranate extracts using *ex vivo* methods (Rasheed et al. 2009). COX, an enzyme that produces prostaglandins from arachidonic acid, has two isoforms: COX-1 and COX-2. COX-1 maintains normal physiological functions, while COX-2 has a role in the inflammation process and is responsible for a high level of PGE2 at inflammation sites (Fletcher et al. 1992; Meade et al. 1993; Prescott and Fitzpatrick 2000). Therefore, identification of COX-2 inhibitors is an important and promising step to prevent inflammatory diseases (Zeilhofer and Brune 2006; Jachak 2007). Recently, several studies have demonstrated that pomegranate extracts inhibit COX-2. For instance, topically applied pomegranate rind extract (PRE) and total pomegranate tannin exerted a significant anti-inflammatory activity by affecting COX-2 expression on viable epidermis (Houston et al. 2017a,c). Pomegranate juice, total pomegranate tannins and punicalagin significantly suppressed TNF-α-induced COX-2 protein expression by 79%, 55% and 48%, respectively (Adams et al. 2006). Conventional anti-inflammatory drugs have

been used for decades, but their side effects on the human body could cause more serious problems with long-term use. It is reported that gastrointestinal problems related to the use of non-steroidal anti-inflammatory drugs (NSAIDs) are the most common problems associated adverse drug reaction (Singh and Triadafilopoulos 1999). The side effects of NSAIDs have been reported for systemic and topical use. NSAIDs can cause upper gastrointestinal bleeding, perforation and peptic ulcers especially for elderly patients in both systemic and topical use (Rainsford et al. 2008). Based on the above, pomegranate extracts could be a promising drug candidate due to their beneficial effects on inflammation pathways and pro-inflammatory mediators.

Pomegranate extracts are also effective at repealing multiple signal transduction pathways and inhibiting inflammatory mediators play role in the initiation and induction in inflammatory diseases. In an *in vivo* study on arthritis, pomegranate-fed mice had significantly lower IL-6 in arthritic joint lysate compared to the control group. In the same study, pomegranate suppressed IL-1b at low dose feeding (Shukla et al. 2008). Pomegranate also showed similar anti-inflammatory activity in high fat diet rats. In this *in vivo* model, high fat diet rats exhibited a significant increase in serum C reactive protein (CRP), IgA, IgG, IgM, IL-1b, IL-6, IL-4 and TNF-α levels. Pomegranate treatment caused a significant reduction in all these pro-inflammatory markers which were induced via high fat diet, and efficiently restored these markers to their normal levels (Zhao et al. 2016). It could be suggested that pomegranate may show anti-inflammatory activity by several mechanisms, including suppression of COX-2 and inducible nitric oxide expression, inhibition of activation of nuclear factor kappa B (NF-κB), inhibition of phosphorylation of mitogen-activated protein kinases (MAPKs) proteins and inhibition of pro-inflammatory cytokines (Afaq et al. 2005; Adams et al. 2006; Lansky and Newman 2007; Zhao et al. 2016).

1.6.5 Potentiated antimicrobial activity of pomegranate rind extract and metal ions

Almost two decades ago, it was suggested that PRE showed higher antiviral activity when used with ferrous sulphate (FeSO4) (Jassim et al. 1995; Stewart et al. 1998). An iron-based antiviral agent was subsequently developed with the addition of plant

extracts such as pomegranate rind, tea and maple leaves. It was indicated that ferrous sulphate, either alone or in combination with certain plant extracts, provides a potent broad spectrum virucidal activity. The free radical system was proposed to play a role in this synergistic mechanism (Gould et al. 2009). Redox reactions are defined as any chemical reactions that include an alteration in oxidation number and/or state (Kohen and Nyska 2002). The synergistic virucidal activity of PRE and FeSO4 was associated with the reaction between PRE and the Fe (II) ion. It has been claimed that this interaction occurs due to conversion of Fe (II) ions to bound Fe (III) ions. Likewise, tannic acid and Fe (II) exerted a similar reaction. The redox reaction between PRE and FeSO4 was considered the reason of synergistic antimicrobial activity and colour change of the PRE and FeSO4 combination, since a black by-product was observed when PRE and FeSO4 were combined. While the synergistic antimicrobial activity of PRE and iron combination was short term it coincided with a loss of activity (Houston 2011). In addition to iron, other transition metals have also demonstrated antimicrobial activity (Nagar 1990; Singh and Katiyar 2008). PRE was combined with other metals for potentiated antimicrobial activity including Cu (II) and Pb (II), and showed a moderate synergistic bactericidal effect (Gould et al. 2009). However, these metal ions formed a complex with PRE like Fe (II) and caused highly coloured end products. Moreover, throughout the change from Fe (II) to Fe (III), the antimicrobial activity of PRE and iron combination decreased over time (Stewart et al. 1998; McCarell et al. 2008; Gould et al. 2009).

Moreover, existence of the black product could cause problems from a patient's aesthetic perspective, as this colour change can be seen in a commercial product of PRE and transition metal combination. Using salts of alternative transition metals which have similar d orbital electrons and maintaining the +2 state without any alteration in colour could have potential for combinational use with PRE for synergistic antimicrobial activity. Zinc has been considered as a promising candidate in this regard. Zinc is found in group 12 of the periodic table and does not show oxidation states higher than +1 and +2, as d-valence shells of zinc are always full and there is no available place for bonding. However, most transition metals form two or more ions as they have free space for bonding in their 3d-orbital. The oxidation chemistry of zinc generally exists as +2 oxidation state and it can be found in the Zn

(II) form (Deming et al. 1976). Hence, Zn (II) ions are in a thermodynamically stable state and available in salt forms of zinc and zinc salts are generally water soluble and uncoloured (Nagar 1990; Singh and Katiyar 2008). PRE extracts were combined with Zn (II) instead of ferrous sulphate for use in further potentiated virucidal activity, since this combination did not exhibit colour or, redox change or new compounds. Houston (2011) was found that a combination of PRE and zinc sulphate (ZnSO4) generates a new mechanism in which free Zn (II) ions, irrespective of the anion, significantly potentiate the antiviral activity of punicalagin against HSV-1 and HSV-2 (Houston 2011). PRE in combination with Zn (II) ion showed virucidal activity against HSV-1, HSV-2 and acyclovir resistant HSV-1, and caused up to a 6-log reduction (Houston et al. 2017b). Moreover, PRE and Zn (II) ion combination in a hydrogel formulation demonstrated this synergistic antiviral activity in an *ex vivo* model study (Houston et al. 2017a).

1.7 Aims of study

The aim of this research was to investigate the potential of PRE and a PRE/Zn (II) combination for microbial diseases associated with the oral cavity. PRE has been widely investigated for its antimicrobial activity and previous studies revealed a synergistic bactericidal and virucidal activity for a PRE/Zn (II) combination (Houston et al. 2017b; Alrashidi 2020; Celiksoy et al. 2021). However, the potential of PRE or a PRE/Zn (II) combination for infectious diseases of the oral cavity has not yet been extensively explored.

Therefore, the specific aims of this research were to:

- Determine whether PRE and a PRE/Zn (II) combination had antimicrobial or anti-adhesive activities against a range of oral commensal and pathogenic microbes found in both planktonic and biofilm states.
- Determine whether synergistic antimicrobial activity exists when PRE and Zn (II) are combined.

- Determine the potential mechanism behind the synergistic antimicrobial activity of the PRE/Zn (II) combination.
- Determine whether PRE, punicalagin and their Zn (II) combinations modulate inflammatory, antioxidant and fibroblast and keratinocyte viability, proliferation and cell migration *in vitro*.
- Determine whether PRE and the PRE/Zn (II) combination exhibit anti-microbial, immune-modulatory and tissue repair capabilities using an *ex vivo* infected rat mandible co-culture model.

The hypothesis of this PhD Thesis was that PRE and/or a PRE/Zn (II) combination could be promising oral care products due to their antimicrobial, anti-inflammatory and wound healing activities. Through this research, it was envisaged that a more comprehensive understanding of PRE and PRE/Zn (II) combination activities against oral microbes and their additional immuno-inflammatory and repair response in relevant oral tissues would be determined. Such findings could subsequently support their future development as pharmaceuticals to use in the prevention and treatment of infectious oral diseases.

Chapter 2: General Materials and Methods

2.1. General Materials

General materials, consumables and equipment used in this research are listed in Tables 2.1, 2.2, and 2.3, respectively.

List of consumables used for experiments	
Plastics (6, 12, 24 and 96 well plates, and pipette tips, tubes)	Sarstedt (Sarstedt Ltd. Leicester, UK)
24 well plate (for scratch wound assay)	Falcon microtiter plates (BD Biosciences)
qPCR plates	Primer Design, Chandler's Ford, UK
Agar plates (90 mm)	ThermoFisher Scientific (Loughborough, UK)
0.45 µm Millex-FG syringe driven filter unit	ThermoFisher Scientific (Loughborough, UK)
Whatman 0.45 µm nylon membrane filter	Sigma-Aldrich Company Ltd. (Poole, UK)
50 mL and 15 mL centrifuge tubes, blue cap	Greiner Bio-One Ltd. (Stonehouse, UK)
Microbank beads	Pro-Lab Diagnostics, Birkenhead, UK
Microcentrifuge tubes (1.8 mL)	Sigma-Aldrich Company Ltd. (Poole, UK)
Microscope slides	ThermoFisher Scientific (Loughborough, UK)
Millex®HA syringe-driven filter unit	Millipore (Watford, UK)
Syringe (5 mL, Sterile)	Sigma-Aldrich Company Ltd. (Poole, UK)
Syringe needles	Sherwood-Davies and Geck (Gosport, UK)
Whatman filter paper	ThermoFisher Scientific (Loughborough, UK)
T 75 tissue culture flasks	Sarstedt (Sarstedt Ltd. Leicester, UK)
Glass bottom 96-well plates	Greiner Bio-One Ltd. (Stonehouse, UK)

Spectrophotometer microcuvettes	ThermoFisher Scientific (Loughborough, UK)
Sterile pipettes 10 mL and 25 mL	Sarstedt (Sarstedt Ltd. Leicester, UK)
ELISA plates	Peprotech, UK
8-well chamber soda-lime glass slides	Falcon Culture Slide, Corning Incorporated Life Sciences, New York, USA
25 mm diameter, mixed cellulose esters (MCE) membrane, hydrophilic, white, 100 discs	Sigma-Aldrich Company Ltd. (Poole, UK)
25 cm cell scraper	Sarstedt (Sarstedt Ltd. Leicester, UK)

Table 2.1: List of consumables used for experiments

List of chemicals and reagents used for experiments.	
Antibiotic (100 µg/ mL streptomycin sulphate, 0.25 µg/mL amphotericin B and 100 U/mL penicillin G sodium)	Sigma-Aldrich Company Ltd. (Poole, UK)
Dulbecco's Modified Eagle Medium (Gibco DMEM 1X) contains 4.5 g/L glucose and 0.11 g/L sodium pyruvate	Sigma-Aldrich Company Ltd. (Poole, UK)
Trypsin (Gibco)	Sigma-Aldrich Company Ltd. (Poole, UK)
Foetal calf serum (FCS)	Sigma-Aldrich Company Ltd. (Poole, UK)
Trypan blue stain (0.4%)	Sigma-Aldrich Company Ltd. (Poole, UK)
Ethanol (HPLC grade)	ThermoFisher Scientific (Loughborough, UK)
Pomegranate	Lidl (Cardiff) (Maindy Rd, Cardiff CF24 4HQ) (original source from Spain) (purchased in September, 2017)
Punicalagin	Sigma-Aldrich Company Ltd. (Poole, UK)
Zinc sulphate ($ZnSO_4$) heptahydrate	ThermoFisher Scientific (Loughborough, UK)
Sodium hydroxide	Sigma-Aldrich Company Ltd. (Poole, UK)
3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT)	Sigma-Aldrich Company Ltd. (Poole, UK)
Trifluoracetic acid (TFA)	Sigma-Aldrich Company Ltd. (Poole, UK).
Potassium hydrogen phthalate	ThermoFisher Scientific (Loughborough, UK)
Mueller Hinton Broth (MHB)	Oxoid, Sigma-Aldrich Company Ltd. (Poole, UK)
Brain Heart Infusion Agar	Oxoid, Sigma-Aldrich Company Ltd. (Poole, UK)
Brain Heart Infusion Broth	Oxoid, Sigma-Aldrich Company Ltd. (Poole, UK)
Fastidious Anaerobe Broth	Oxoid, Sigma-Aldrich Company Ltd. (Poole, UK)

Brucella Agar	Oxoid, Sigma-Aldrich Company Ltd. (Poole, UK)
Defibrinated sheep blood	TCS Bioscience Ltd. (Buckingham, UK)
Rats	28 days old, male, Wistar rats, Charles River, Portishead, UK
Folin ciocalteu reagent	Sigma-Aldrich Company Ltd. (Poole, UK)
Tannic acid	Sigma-Aldrich Company Ltd. (Poole, UK)
Ascorbic acid	Sigma-Aldrich Company Ltd. (Poole, UK)
Sodium carbonate (Na2CO3)	Sigma-Aldrich Company Ltd. (Poole, UK)
2,2-diphenyl-1-picrylhydrazyl (DPPH)	Sigma-Aldrich Company Ltd. (Poole, UK)
2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic-acid (ABTS)	Sigma-Aldrich Company Ltd. (Poole, UK)
Potassium persulphate	Sigma-Aldrich Company Ltd. (Poole, UK)
Trolox	Sigma-Aldrich Company Ltd. (Poole, UK)
Gram-staining kit	ThermoFisher Scientific(Loughborough, UK)
Live/Dead Baclight Kit	ThermoFisher Scientific(Loughborough, UK)
Potassium hydrogen phthalate	ThermoFisher Scientific(Loughborough, UK)
Sodium hydroxide (NaOH)	ThermoFisher Scientific(Loughborough, UK)
Phosphoric acid	ThermoFisher Scientific(Loughborough, UK)
PureLink™ RNA Mini Kit	ThermoFisher Scientific(Loughborough, UK)
RNase/DNase Free Water	ThermoFisher Scientific(Loughborough, UK)
High-Capacity cDNA synthesis kit	ThermoFisher Scientific(Loughborough, UK)
Taqman Universal Fast Master Mix	AppliedBiosystems, ThermoFisher Scientific (Loughborough, UK)
qPCR primers and probes	AppliedBiosystems, ThermoFisher Scientific (Loughborough, UK)

Formalin	Sigma-Aldrich Company Ltd. (Poole, UK)
Pierce LDH Cytotoxicity Assay Kit	ThermoFisher Scientific(Loughborough, UK)
Rat TNF-α Mini ABTS ELISA Development Kit	PeproTech (London, UK)
Rat IL-1β Mini ABTS ELISA Development Kit	PeproTech (London, UK)
Rat IL-6 Mini ABTS ELISA Development Kit	PeproTech (London, UK)
Prostaglandin E2 Parameter Assay Kit	R&D Systems (Abingdon, UK)
Ethylenediaminetetraacetic Acid (EDTA)	Sigma-Aldrich Company Ltd. (Poole, UK)
Ammonium hydroxide	ThermoFisher Scientific(Loughborough, UK)
Sodium thiosulphate	ThermoFisher Scientific(Loughborough, UK)
Peptone	Sigma-Aldrich Company Ltd. (Poole, UK)
Lecithin	ThermoFisher Scientific(Loughborough, UK)
Tween-80	ThermoFisher Scientific(Loughborough, UK)
Hydrochloric acid (HCl)	Sigma-Aldrich Company Ltd. (Poole, UK)
H&E Staining Kit (Haematoxylin and Eosin)	Abcam (Cambridge, UK)
TUNEL Assay Kit - HRP-DAB	Abcam (Cambridge, UK)
Glutaraldehyde	Sigma-Aldrich Company Ltd. (Poole, UK)
SYPRO ruby ready to use protein gel stain	ThermoFisher Scientific(Loughborough, UK)
Calcofluor White staining	Sigma-Aldrich Company Ltd. (Poole, UK)
DCFH-DA probe	Sigma-Aldrich Company Ltd. (Poole, UK)

Table 2.2: List of chemicals and reagents used for experiments.

List of equipment used for experiments	
Plate reader	FLUOstar Omega, BMG Labtech (Ortenberg, Germany)
Confocal laser scanning microscope	AOBS Spectral Confocal Microscope, Leica SP5 (Heidelberg, Germany)
Scanning electron microscopy	Tescan VAGA SEM & XRMA System, Cambridge, UK
Freeze dryer	Scanvac CoolSafe Freeze Dryer, SLS Ltd., Wilford, Nottingham, UK
Ax70 Upright Fluorescent Microscope	Olympus UK Ltd. (Bolton, UK)
Quant Studio 6 QPCR	ThermoFisher Scientific (Loughborough, UK)
Cell IQ	Chip-Man Technologies Ltd. (Tampere, Finland)
Nanodrop	ND-8000 Spectrophotometer, ThermoFisher Scientific (Loughborough, UK)
High Pressure Liquid Chromatography (HPLC)	Thermo LCQ classic LCMS with ESI source
HPLC Agilent Series 1100	Agilent Technologies LDA UK Ltd., Didcot, Oxford, UK
Centrifuge	Heraeus Multifuge 3S/3S-R centrifuge
Cryovials for cell stock	Greiner Bio-One Ltd., Stonehouse, UK
Isopropanol freezing container	Sigma-Aldrich Company Ltd. (Poole, UK)
Microbank beads	Pro Lab Diagnostics, Birkenhead, Wirral, UK
Neubauer Improved Haemocytometer	ThermoFisher Scientific (Loughborough, UK)
Incubator -CO_2 cabinet (5 % v/v CO_2, 20 % v/v H2 , 70 % v/v N2	Don Whitley Scientific Ltd. (Shipley, UK)
Aerobic cabinet	LTE scientific LTD, Oldham, Manchester, UK
Spectrometer	Sherwood Scientific (Cambridge, UK)
Sputter coaters	DSR1 desk sputter coater; VacTechniche (Hastings, East Sussex, UK)
Light microscope	CK2 Inverted Microscope, Olympus UK Ltd. (Bolton, UK)
Paraffin wax embedding machine	Shandon Pathcentre, ThermoFisher Scientific (Surrey, UK)
Microtome	Leica (Buckingham, UK)

Table 2.3: List of equipment used for experiments.

2.2. General methods

2.2.1. Preparation of solutions

2.2.1.1. Phthalate buffer pH 4.5

Potassium hydrogen phthalate (0.1 M) and sodium hydroxide (0.1 M) were prepared in deionised water. Potassium hydrogen phthalate (250 mL) and sodium hydroxide solutions (87 mL) were adjusted to 1 L with deionised water and sonicated for 5 min. The pH was adjusted to 4.5 with phosphoric acid.

2.2.1.2. Zinc sulphate (ZnSO4)

ZnSO4 (1 M) solution was prepared in phthalate buffer pH 4.5. Briefly, 2.876 g zinc sulphate heptahydrate was added to 10 mL phthalate buffer pH 4.5 and sonicated for 10 min at 50-60 hertz (Hz). Finally, the solution was filtered through a 0.45 µm Millex-FG syringe driven filter unit.

2.2.1.3. Punicalagin solution

Punicalagin stock solution (1 mg/mL) was prepared by adding standard punicalagin into phthalate buffer pH 4.5 and the solution was sonicated at 50-60 Hz for approximately 15 min to obtain fully dissolved solution. For further required concentrations, dilutions were made from this solution using phthalate buffer pH 4.5.

2.2.2. Preparation of pomegranate rind extraction (PRE)

The rind of the purchased pomegranates (Spanish origin, September 2017) was peeled with a scalpel and cut to pieces around 2 cm length and 1 cm width. The net weight of the rind was 300 g. Pomegranate rind was blended in 25% w/v deionised water and boiled for approximately 10 min. To ensure that the rind extract was cold, it was centrifuged using a Heraeus Multifuge 3S/3S-R centrifuge machine at 5980g at 4°C for 30 min. The centrifugation process was repeated four times and the supernatants

of each tube filtered through a Whatman 0.45 µm nylon membrane filter. 550 mL of filtered PRE was obtained, which was placed in 50 mL centrifuge tubes, wrapped with aluminium foil to protect from light and kept at -20°C until required. After 18 h at -20°C, three tubes were freeze-dried using a Scanvac Freeze Dryer (Houston 2011). An illustration for PRE preparation is presented in Figure 2.1.

2.2.2.1. Freeze-dried pomegranate rind extract (PRE) reconstitution

The required amount of freeze-dried PRE was weighed and dissolved in the required volume of phthalate buffer pH 4.5. The solution was sonicated at 50-60 Hz until fully dissolved. The solution was then filtered through a 0.45 µm Millex-FG syringe driven filter unit and frozen at -20 ℃.

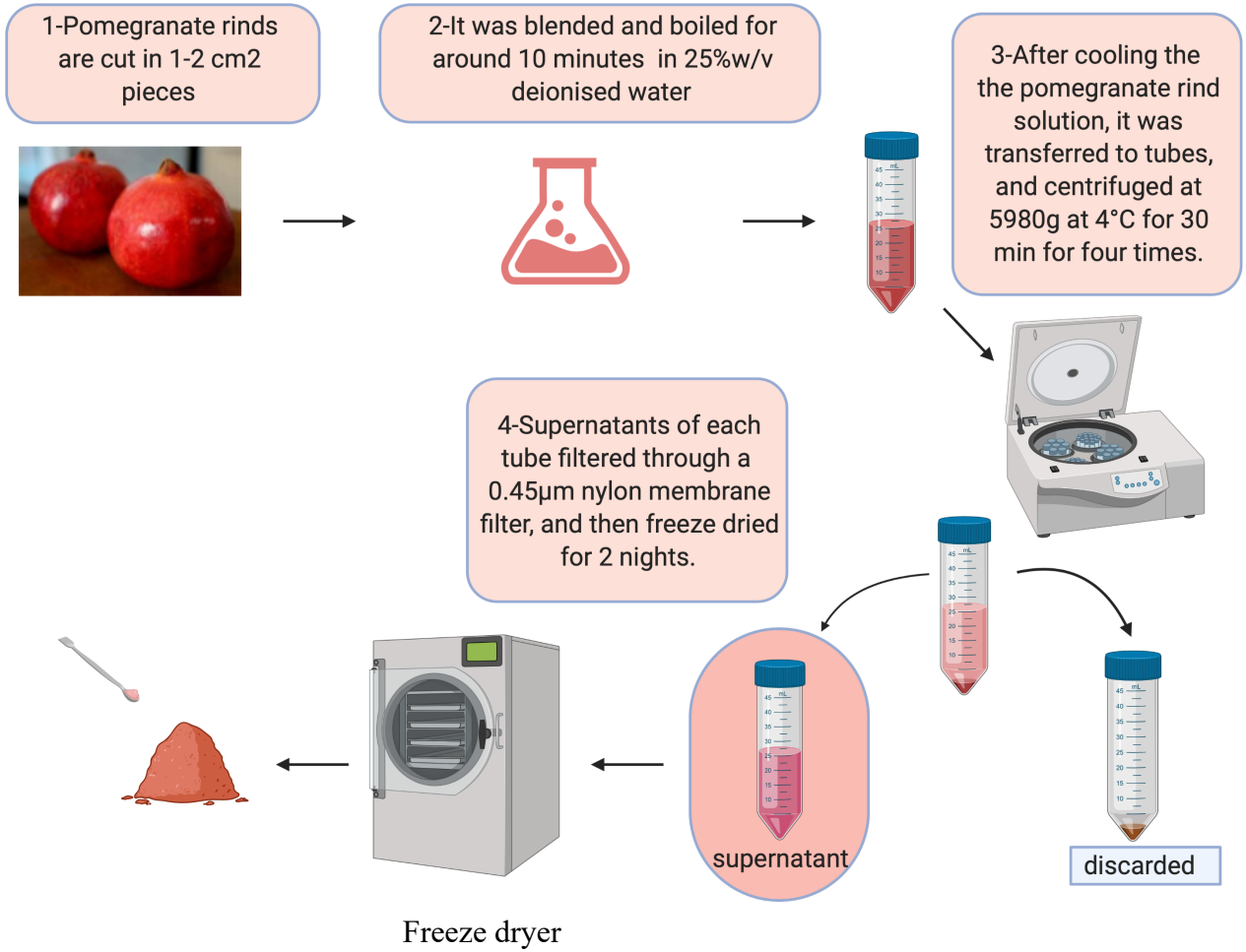


Figure 2.1: An illustration of the steps involved in PRE preparation.

2.2.3. General methods for microbiology studies

2.2.3.1. Growth media and bacterial diluents

Fastidious anaerobe agar (FAA) with 5% v/v defibrinated sheep blood, Brucella agar (BA) supplemented with haemin and vitamin K, fastidious anaerobe broth (FAB), Mueller Hinton broth (MH broth), Brain heart infusion broth (BHI broth), Brain heart infusion agar (BHI agar) and Mueller Hinton agar (MH agar) were used as growth media. All agar and broth were purchased from Oxoid Ltd. (Basingstoke, UK) and prepared according to the manufacturer's instructions. For sterilisation, media were autoclaved at 121℃ for 15 min by moist heat sterilisation.

2.2.3.2. Bacterial species and strains

Bacteria and *C. albicans* used in this study were taken from the culture collection of Cardiff School of Dentistry, and the list of microbes evaluated are presented in Table 2.4. Only *S. gordonii* NCTC 7865 was purchased from the Public Health England culture collection.

2.2.3.3. Bacterial storage

Bacterial stock cultures were stored in special cryotubes with bacterial preserver beads (Microbank beads, Pro Lab Diagnostics). For the freezing process, the manufacturer's instruction was followed, and tubes were stored at -80 ℃.

2.2.3.4. Preparation of agar plates

Agar plates (90 mm diameter) were filled with approximately 20 mL of agar and kept upright without lids in a sterile cabinet with open flow at room temperature, for 2 h. Then agar plates were labelled according to the experiment and microbe culture.

Microorganisms	**Reference source and ID**	**Gram +/-**	**Oxygen requirements**
S. gordonii	NCTC 7865, ATCC 10558T	+	Facultative
S. sanguinis	NCTC 7863T	+	Facultative
P. gingivalis	W50	-	Anaerobe
F. nucleatum	ATCC® 49256™	-	Anaerobe
E. coli	NCTC 10418	-	Facultative
S. aureus	NCTC 8325	+	Facultative
S. mutans	DSM 20523	+	Facultative
C. albicans	ATCC 90028		Facultative
S. salivarius	DSM 20560 (ATCC 7073, NCTC8618)	+	Facultative
S. anginosus	NCTC 10713	+	Facultative

Table 2.4: Microbial isolates assessed in this study. NCTC: National Collection Type of Cultures; DSM: German Collection of Microorganisms and Cell Cultures; ATCC: American Type of Cell Culture +: Gram-positive bacterium; -: Gram-negative bacterium.

2.2.3.5. Preparation of bacterial master plates

For bacterial culture, two microbank beads were placed on appropriate agar. For anaerobes, FAA with 5% sheep blood or BA supplemented with haemin and vitamin K were used. For other bacterial strains, MH agar and/or BHI agar supplemented were used. Agar plates were incubated in the anaerobic cabinet for anaerobes for at least 48 h and the aerobic incubator was used for other studied bacterial strains for 24 h. Stock plates for anaerobes were stored in the anaerobic cabinet and every week colonies were sub-cultured once or twice, according to condition of the bacterial cultures. For the remaining aerobic and facultative bacterial strains, stock plates were stored at 4 ℃ and each bacterial species were sub-cultured at three-weekly intervals.

Bacterial sterility and contamination risk was assessed via the streak plate method, Gram staining and visualisation of colonies every week for studied microbes.

2.2.3.6. Preparation of bacterial experimental culture

Broth cultures was prepared by taking one colony from the cultured agar plates. FAB was used for overnight culture of obligate anaerobic bacteria, *P. gingivalis* and *F. nucleatum*. BHI broth was used for overnight culture of aerobic and facultative bacteria, and *C. albicans*. Obligate anaerobic strains were then incubated in the anaerobic cabinet for 48 h and facultative strains incubated in an aerobic incubator at 37 ℃ for 24 h for optimal growth. The optical density (O.D.) of cultures was measured and correlated with colony forming units (CFU) using a standard curve according to procedure described in 2.2.3.7. Microbial suspensions were centrifuged at 12000 rpm for 5 min at room temperature and supernatants were discarded. An appropriate volume of specific broth was added to the microbial pellet until an O.D. of 0.2 was achieved.

2.2.3.7. Preparation of standard curves of optical density against colony forming units

A calibration curve was prepared of O.D. correlation with colony forming units (CFU) of test microorganisms. For this, the Miles and Misra viable count dilution method was

used (Miles and Misra 1938). Test bacterial cultures were prepared in a suitable broth, as described above. Seven different optical density values (range between 0.05, and 0.2) were prepared by diluting the cultured broth. 20 µL broth was taken from each diluted broth and serially decimal diluted. 20 µL of each dilution was then deposited on appropriate agar media Agars were incubated under suitable environmental conditions and incubation times.

For the calculation of CFU of bacteria, only appropriate dilutions which had countable numbers of colony, in the range of 5 to 75, were used to plot the cell density (CFU/mL) against corresponding optical density O.D.600. This experiment was repeated twice independently for each bacterial strain and mean values were used in the graph to obtain O.D.600 values corresponding to approximately 10^8 CFU/mL of microbe. For aerobic and facultative bacteria, 0.1 O.D.600 was found to be the optimal value to obtain 10^8 CFU/mL of microorganism. However, for anaerobic bacteria, *P. gingivalis* and *F. nucleatum*, the optimal O.D.600 was found to be 0.2 to obtain 10^8 CFU/mL of bacteria.

2.2.3.8. Preparation of test treatments

PRE and Zn (II) stock solutions were prepared in phthalate buffer pH 4.5 and diluted in MH broth for desired concentration and sterilised using a 0.45 µm Millex-FG syringe-driven filter to use in experiments. Only MH broth was added to all microbiology tests for sterility control and 10% phthalate buffer in MH broth were added to all microbiology tests to see its antimicrobial effect on tested bacteria.

2.2.4. General cell culture and treatment method

In the present study human primary gingival fibroblast cells (HPGFs) and a spontaneously immortalized, cultured human keratinocyte line (HaCaTs) were used. The general cell culturing method is described in the below sections.

2.2.4.1. General cell culture

The tissue culture safety cabinet and the necessary equipment used in the cabinet were disinfected using 70% of ethanol before initiating any experiment. Additionally, either all equipment and materials were sterilised according to standard autoclaving procedures, or they were purchased sterile and for single usage. The water bath and cabinet were manually sterilised each week with suitable disinfectant detergents. Incubators were sterilised each month with automatic heat decontamination (180℃ overnight).

2.2.4.2. Preparation of cell culture media

All cell culture media materials, except Dulbecco's Modified Eagle Medium (DMEM), were thawed slowly overnight at 4°C. All media and solutions were placed into a water bath at 37°C for 20 min before use. Routine culture medium was prepared using DMEM supplemented with 10% heat-inactivated fetal calf serum (FCS), 1% L-glutamine, and 1% antibiotics. The freezing solution contained 10% dimethyl sulphoxide (DMSO) and 90% FCS.

2.2.4.3. Preparation of test treatments

PRE, punicalagin, Zn (II), PRE/Zn (II) combination and punicalagin/Zn (II) combination were used in cell culture experiments. Powdered forms of PRE, punicalagin and Zn (II) were solubilised in phthalate buffer pH 4.5 to obtain stock concentrations at 10 mg/mL, 10 mg/mL and 100 mM, respectively. The stock concentrations of compounds were aliquoted 0.5 mL or 1 mL and were stored at -20℃. These aliquots were then used to prepare the desired concentrations of PRE (0.1 µg/mL, 1 µg/mL, 10 µg/mL, 100 µg/mL), punicalagin (0.1 µg/mL, 1 µg/mL, 10

µg/mL), Zn (II) (0.001 mM, 0.1 mM, 1 mM), PRE/Zn (II) combination (0.1µg/mL+0.1 mM, 1 µg/mL+0.1 mM, 10 µg/mL+0.1 mM), and punicalagin/Zn (II) combination (0.1µg/mL+0.1 mM, 1 µg/mL+0.1 mM, 10 µg/mL+0.1 mM) by diluting in 1% serum-containing culture medium. For each experiment, treatment media for each compound and combination were prepared fresh for each experiment. Control culture medium was also supplemented with 1% phthalate buffer pH 4.5 to negate any influences on cellular activities by the buffer itself.

2.2.4.4. Freezing of cells for long-term storage

To maintain and obtain long-term storage, cells were frozen at low passage numbers. The cell pellet was dissolved in freezing solution, which included 10% DMSO and 90% FCS. 1 mL of this suspension was then transferred into the pre- cooled cryovial and frozen gradually over a 24 h period at -80 °C using an isopropanol freezing container, which is designed to freeze cells slowly at 1 °C/min to procure viable cells. After overnight slow freezing at -80 °C the cryovial was placed into liquid nitrogen for long-term storage at -196°C.

2.2.4.5. Thawing frozen cells

Cryovials were removed from liquid nitrogen and warmed to 37°C in a water bath. The cryovial was then taken to the hood as quickly as possible and the cell suspension transferred to a universal tube and 10 mL culture medium was added. The suspension was centrifuged at 1500 rpm for 5 min and the supernatant was discarded. Cells were re-suspended with a suitable volume of media according to flask size used to seed cells. Flasks were placed into the incubator at 37 °C in a 5% CO_2, 95% air humidified incubator. After 24 h, cells were checked and the medium replenished with fresh culture media.

2.2.4.6. Changing culture media and cell passaging

Cells were passaged according to confluence in the flask. When cells had reached 70-90% confluence, they were passaged. If cells were not sufficiently confluent, the medium was changed. Generally, cells were passaged every two or three days. The

used medium was removed, and the cells washed with PBS. Trypsin (3 mL) was added to each flask to detach cells from the growth surface. Flasks containing trypsin were incubated at 37°C in 5% CO_2, 95% air. After 5 min, the anchored cells were dislodged by gently tapping the edge of the flask on the palm of the hand. Media with serum was added to neutralise the trypsin and the cell suspensions were placed into the universal tube and centrifuged at 1500 rpm for 5 min. After centrifugation, the supernatant was removed, and the pellet suspended in culture media with serum. Cells were subsequently seeded into fresh 75 cm^2 flasks, with culture media added to a final volume of 10 mL.

2.2.4.7. Haemocytometer cell counting

A Neubauer haemocytometer was used for cell counting. A coverslip was moistened and placed onto the haemocytometer. A cell suspension (10 µL) was taken from the re-suspended cell solution in universal flask and placed into the well of a 96-well plate. Subsequently, Trypan blue (10 µL) was added into the same well and the contents mixed thoroughly. This mixture (10 µL) was transferred gently between the haemocytometer and coverslip. It is important to do this work gently, to avoid excessive air bubbles which prevent accurate counting. Thereafter, the haemocytometer was placed under the light microscope (CK2 Inverted Microscope, Olympus UK Ltd., Middlesex, UK) at x100 objective lens magnification.

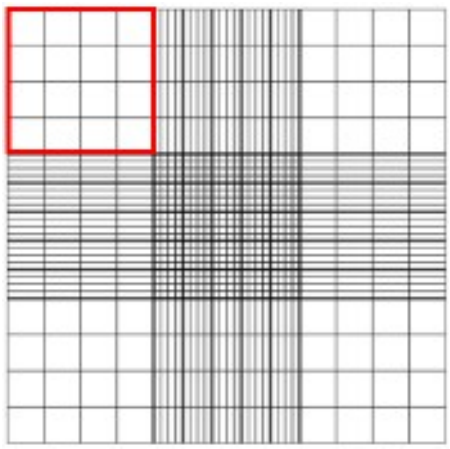


Figure 2.2: Schematic view of haemocytometer squares under the microscope. One of the counted corner squares is outlined in red.

The viable cells were counted in each of the four corners of the square, with one example marked red in Figure *2.2*. Unstained cells were counted using a hand tally. After counting the cells, the haemocytometer and coverslip were cleaned with 70% alcohol. Cell concentrations were calculated as follows:

(Total cells counted)/(4 squares counted)*10^4*initial volume*dilution factor = total number of cells

2.2.4.8. Screening for mycoplasma

To ascertain the presence of mycoplasma contamination, routine checks were undertaken on cells by Dr Maria Stack (School of Dentistry, Cardiff University, UK). When cells were 90-100% confluent, culture media samples were collected and frozen at -20 °C until required. Prior to the assessment, samples were defrosted on ice and then underwent incubation for five minutes at 95°C. Cellular debris was removed by centrifugation and polymerase chain reaction (PCR) Master Mix was established with internal control, GoTaq polymerase, magnesium chloride, nuclease-free water, primer/nucleotide mix, and GoTaq Green buffer, from Promega Ltd., Hampshire, UK) (Lau 2008).

Positive DNA control was used with the negative control (nuclease-free water) to ascertain the presence of contamination by mycoplasma. Samples and controls were assessed by Reverse Transcription Polymerase Chain Reaction (RT- PCR) with PCR reactions prepared at 94 °C for 2 min, followed by 39 cycles of: 30 s at 94°C, 30 s at 55°C, 30 s at 72°C. The samples were maintained at 4°C and run on 2% (w/v) agarose gels at 100 V for 20 min, using Mini-Horizontal Electrophoresis Unit (Jencons-PLS, Bedfordshire, UK) (Lau 2008). The presence of a strong band at 267 bp determined positive mycoplasma contamination. To confirm the reaction had worked, an internal control band at 191 bp was also used. However, in the case of strong mycoplasma contamination, this internal control band at 191bp was not visible. Mycoplasma-positive cells were treated or discarded, as below.

Mycoplasma-positive samples were treated in three cycles over 3 weeks with BM Cyclin (Roche, Burgess Hill, West Sussex, UK). This involved removing contaminated culture medium and replacing it with fresh medium including BM Cyclin 1 (4 µL of stock solution/ml, final concentration 10 µg/mL) (Lau 2008). As described previously, cells were cultured for 3 days, and they were passaged when necessary. Culture medium containing BM Cyclin 1 was then removed and replaced with fresh medium containing BM Cyclin 2 (4 µL of stock solution/mL, final concentration 5 µg/mL). Cells were cultured as previously described for 4 days and passaged when necessary. This treatment cycle over seven days was undertaken three times, prior to reassessing a fresh sample of culture medium to ascertain eradication of the mycoplasma contamination.

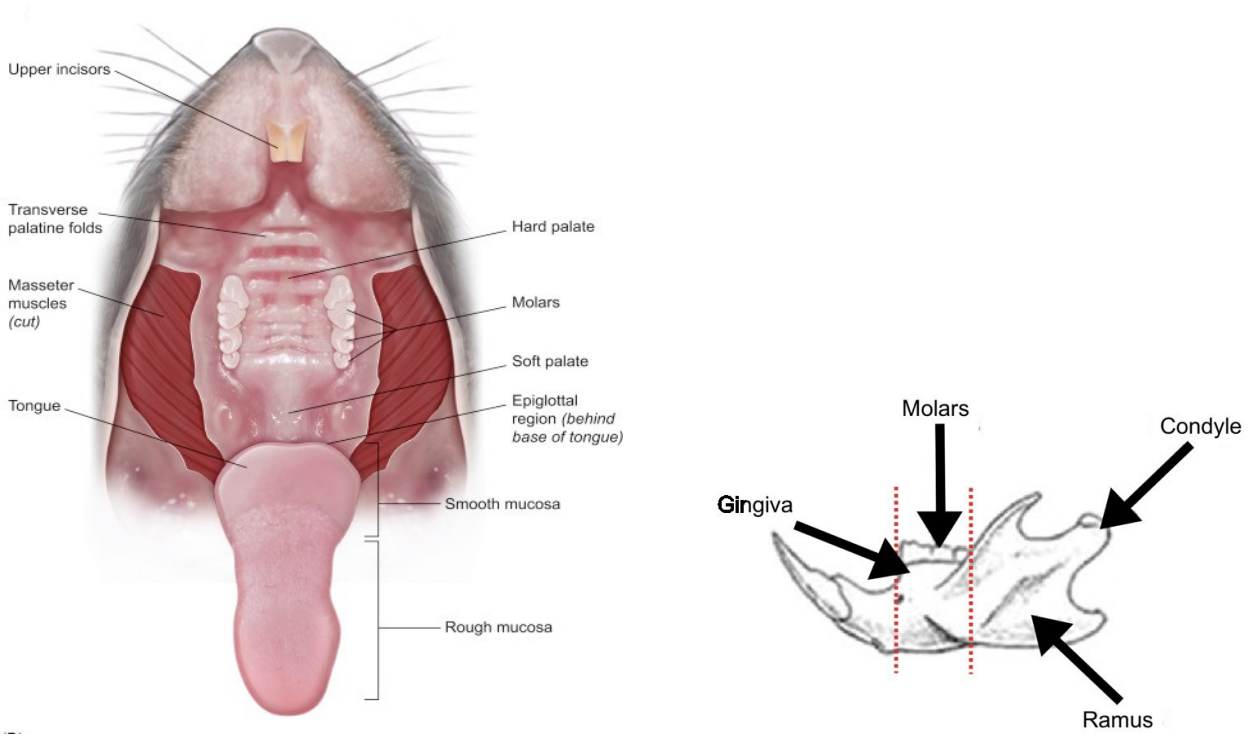
2.2.5. *Ex vivo* rat mandible model preparation

2.2.5.1. Excision of mandible tissue from rat

The rat mandible tissue was excised from 28-day old male Wistar rats, post-mortem (Charles River) (Figure 2.3A). The lower and upper mandible were cut into 2 sections from between its incisors, with each cut section used for each sample condition (Figure 2.3B).

2.2.5.2. Preparation of culture media (Green's media), and treatment media (PRE, Zn (II) and PRE/Zn (II) combination) in Green's media for ex vivo rat mandible model evaluations

Excised mandible tissue from rats was incubated in Green's media, which had been routinely used as a tissue culture media for keratinocyte culture and *ex vivo* skin culture (Table 2.5) (Smith et al. 2010). Stock solutions of PRE and Zn (II) in phthalate buffer pH 4.5 were diluted using Green's media to obtain desired concentrations of PRE, Zn (II) and PRE/Zn (II) combination, and they were applied to the rat mandible tissue for 30 min in an incubator at 37°C in 5% CO_2, 95% air (Rheinwald and Green 1975; Deshpande et al. 2013; Bostan et al. 2016). Green's media was made in 500 mL volumes and stored at 4 ℃ for up to 1 month. The sterility of media was checked by taking 10 mL of media from stock media and left at 37 ℃ for 24 h prior to use and visualising any turbidity in the medium.



A **B**

Figure 2.3: Preparation of the rat mandible model. After dissecting the mandible tissue, the condyle, ramus and incisor were cut by using a sterile scalpel. Figure adapted from Smith et al. (2010).

Component	Volume and stock solution	Final concentration
Dulbecco's modified Eagle's Medium	330 mL	66%
Nutrient Mixture F12 (Ham's F12)	108 mL	21.6%
Fetal Calf Serum	50 mL	10%
Penicillin/Streptomycin	5 mL of 10,000 i.u./mL penicillin and 10,000 µg/mL streptomycin	100 i.u./mL penicillin and 100 µg/mL streptomycin
Amphotericin B	1.25 mL of 250 µg/mL	0.625 µg/mL
Adenine	2 mL of 6.25 mg/mL	0.025 mg/mL
Insulin	2.5 mL of 1 mg/mL	5 µg/mL
3, 3, 5- Tri-iodothyronine/ Apo-Transferrin	0.5mL of 1.36 µg/mL T3 and 5 mg/mL apo-transferrin	1.36 ng/mL T3 and 5 µg/mL apo-transferrin
Hydrocortisone	80 µL of 2.5 mg/mL	0.4 µg/mL
Epidermal Growth Factor	25 µL of 100 µg/mL	5 ng/mL
Cholera Toxin	500 µL of 8.47 µg/mL	8.47 ng/mL

Table 2.5: Composition of Green's media.

2.2.5.3. *Preparation of trowel type culture ex vivo model*

Rat mandible was dissected from 28-day old male Wistar rat and the incisors and condyle sections cut with a sharp scalpel. The *ex vivo* model was prepared using the molars and attached gingiva. A schematic for preparation of experimental plates is shown in Figure 2.4 (Smith et al. 2010).

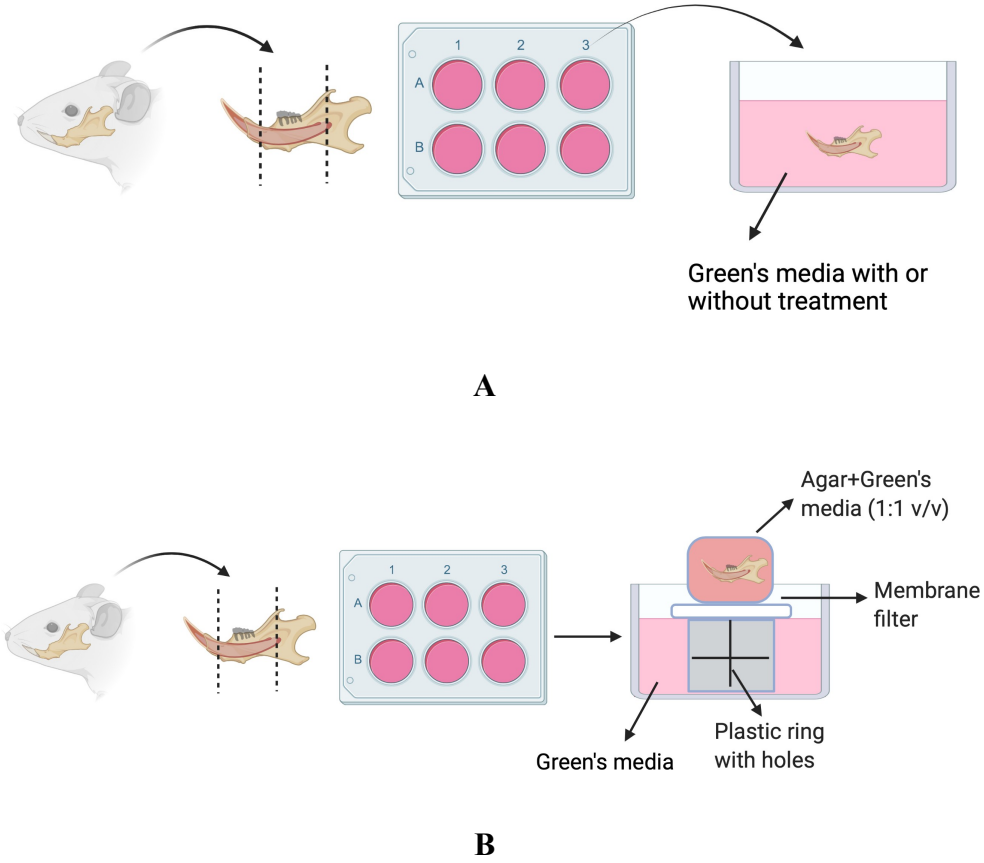


Figure 2.4: A simplified illustration of *ex vivo* rat mandible model preparation. (A) *Ex vivo* rat mandible model used for short time (30 min) incubation of tissue for the determination of toxicity and anti-inflammatory activity of PRE, Zn (II) and PRE/Zn (II) combination. (B) *Ex vivo* model with plastic support ring and semi- solid agar media was used for longer incubation time and used to co-culture *ex vivo* model with bacteria to induce infection (created with BioRender.com).

2.2.6. Histological fixation, processing, and staining methods

2.2.6.1. Fixation and demineralisation

Rat mandible tissues were removed from the treatment and control media and washed with PBS for 5 min. The rat mandible tissues were replaced in 10% (v/v) neutral buffered formalin for 48 h, at 4 ℃ to fix the tissues. After 48 h, tissues were washed with PBS for 5 min and placed in ethylenediaminetetraacetic acid (EDTA) solution to for 7 days at 4°C to demineralise the mandible sections. The quality of the demineralisation process was checked using the calcium oxalate test, as described by Clayden (1952). Briefly, 5 mL of decalcifying solution was taken from each sample and added to 20 mL tubes. Tubes were placed on the pH meter with a magnetic stirrer. Concentrated ammonium hydroxide was then added dropwise and stirred until pH 7 was obtained. After this step, 5 mL of saturated ammonium oxalate was added to each tube, shaken well and left for 30 min. Following these steps, a clear solution was indicative that demineralisation process was complete.

2.2.6.2. Automated tissue processing and paraffin wax embedding

Rat mandible tissues were placed into individual biopsy cassettes, and tissues were processed through different concentrations of ethanol and xylene using an automatic tissue processor to remove water and fixatives from tissue. The step-by- step tissue process is shown below.

1. 70% ethanol, 1 h,
2. 90% ethanol, 1 h,
3. 100% ethanol, 1.5 h,
4. 100% ethanol, 1.5 h,
5. 100% ethanol, 1.5 h,
6. 100% ethanol, 1.5 h,
7. Xylene, 1.75 h,
8. Xylene, 1.75 h,
9. Xylene, 1.5 h,
10. Molten wax, 2.5 h, 60-65 ℃.

This automatic tissue process was followed with paraffin wax embedding (Shandon Pathcentre, ThermoFisher Scientific, Surrey, UK). Tissues were embedded in the paraffin wax in a vertical position to obtain the gingiva sections in all tissue slides.

2.2.6.3. Sectioning of tissue slices

Tissues were cut to 4 µm sections using a Leitz 1400 Microtome (Leica, Buckingham, UK). The cut sections were floated in distilled water to obtain smooth tissue slices. Sections were then mounted on glass poly-lysine tissue slides (ThermoFisher Scientific, Loughborough, UK) and slides were placed in the oven at 60°C for 1 h to allow the sample to adhere to the tissue slides. After this step, the slides were ready for haematoxylin and eosin (H&E) staining and immunohistochemical analyses.

2.2.6.4. Haematoxylin and eosin (H&E) staining of histological sections

An automated tissue stainer was used for H&E staining. After H&E staining, the slides were processed through xylene, ethanol and water washing. These sections were subsequently stained by H&E staining. The slides were gradually processed in a series of different levels of alcohol washes. Finally, slides were immersed in xylene and cover slipped with DPX mountant. All air bubbles were removed and excess of DPX was cleaned with a paper tissue.

Chapter 3: Characterisation of Pomegranate Rind Extract Composition

3.1 Introduction

The general aim of this thesis was to investigate the potential bioactivities of PRE and a PRE/Zn (II) combination for use as a potential agent in oral care products. As a natural extract, PRE is a complex mixture, and it was therefore important to understand PRE's composition. Plants play a crucial role in a human being's life through their daily diet, and they have also been used in folklore medicine in many cultures (Wren et al. 1988; Seeram et al. 2006). In the last few decades, plants have been extensively studied for their nutritional values and bioactivities. Plants are comprised of carbohydrates, lipids and amino acids as essential primary metabolites, but it should be noted that plants also produce a wide variety of low molecular weight compounds called secondary compounds. These secondary compounds play a role in the interaction between plants and their environment. Generally, secondary compounds have a complex and unique structure, and their enhanced production is associated with biotic and abiotic stress of plants against the environment (Dixon 2001).

The plant kingdom comprises 100,000-200,000 metabolites which usually range from 1% to 2% of dry material composition and the analysis of secondary compounds in plant extracts is quite challenging, given their high chemical diversity, low abundance and variability (Oksman and Inze 2004). Only half of the structures of these secondary compounds have been fully determined (Verpoorte 1998; Luca and Piere 2000). These plant secondary compounds exert a high chemical diversity, and each plant comprises unique secondary compounds according to its characteristics. Plant secondary compounds can be structurally classified into five major groups based on their biosynthetic origins: polyketides, isoprenoids (e.g., terpenoids), alkaloids, phenylpropanoids and flavonoids. The biosynthetic pathways of secondary compounds are complex and require multiple synthetic steps, which are catalysed by various enzymes. Hence, most of the biosynthetic pathways of plant secondary compounds are unknown (Facchini et al. 2004). Plants secondary compounds have a role in defence systems and other survival systems in plants. In addition to these roles,

they hold importance for humans in different facets, such as flavours, fragrance, dyes, pesticides and pharmaceuticals (Oksman and Inze 2004).

For the analysis of complex secondary compounds in plant extracts, different analytical techniques have been used. The Folin-Ciocalteu assay is commonly used to elucidate the total phenolic content of plant extracts. Some of these methods are thin layer chromatography (TLC), gas chromatography (GC), high-performance liquid chromatography (HPLC), capillary electrophoresis (CE), and their combination with mass-spectrometry (MS) are also key analytical techniques (Drasar and Marovcova 2004; Liang et al 2004; Liu et al. 2008; Zhou et al 2008). Proton nuclear magnetic resonance analysis (^{1}HNMR) is another method, but its reduced sensitivity is a disadvantage. However, it is the only technique that can produce signals which can be correlated to the number of analytes in the sample (Lewis et al. 2007). This makes use of other chromatographic and electrophoretic techniques in combination with different detectors more common. However, it should be noted that there are sensitivity problems with the CE method, due to the extremely small sample volume. The small sample injection volume (nL) causes detection sensitivity problems because of the small internal diameter of the capillary tubes employed. Therefore, CE is generally considered inferior to HPLC (Ganzera 2008; Masar et al. 2020). GC is also a powerful technique, but it is only for volatile constituents. Therefore, HPLC is becoming the preferred technique for analysis of natural products (Kong et al. 2010). Depending on the investigated constituents, the type of detector changes from Diode-Array detection (DAD) or UV–Vis detectors, evaporative light scattering (ELS) and fluorescence detectors to MS-detection. Thus, in comparison to the above-mentioned techniques, use of liquid chromatography-mass spectrometry (LC-MS) has become more common due to its excellent sensitivity and selectivity in addition with determining or confirming the chemical structures depending on instrument used (Zhou et al. 2009; Gray et al. 2010).

In this project, the major analytical techniques used were HPLC and LC/MS (Dionex Ultimate 3000 HPLC system with Thermo LTQ Orbitrap XL). HPLC is a widely used technique to identify, quantify and purify the individual components in plant extracts (Cannell 1998; Piana et al. 2013). The HPLC is suitable for rapid evaluation of plant mixture in both analytical and preparative scales (Ahuja and Ahuja 2006). Recently,

high resolution mass spectrometry, especially linear ion trap-Orbitrap mass spectrometer (LTQ-Orbitrap), has provided excellent performance for detection in targeted mixtures due to improved data acquisition methods, its detection sensitivity and high speed (Zhang et al. 2014; Zhang et al. 2016). In addition, LTQ-Orbitrap MS has higher resolution power than many other spectrometers and a wide variety of data can be obtained with this instrument, such as molecular weights, elemental composition and fragmentation stages (Zhang et al. 2014; Wang et al. 2014; Li et al. 2015; Chen et al. 2016). These advantages were the reason to choose this method to tentatively elucidate the composition of PRE in this study.

3.1.1 Folin-ciocalteu (F-C) assay for total phenolic content

The Folin–Ciocalteu (F-C) response is an antioxidant measure based on electron exchange, which measures the reductive capacity of an antioxidant. It has been broadly connected in assurance of the full phenol/polyphenol substance of plant derived nourishment and biological samples.Pomegranate has a large variety of phenolic compounds, including hydroxycinnamic acids, flavonoids, anthocyanins, and tannins. These are the major classes of phenolics, and they contain one or more hydroxyl residues linked to a phenyl ring (Hassanpour et al. 2011). Folin-Ciocalteu (F-C) assay is a colorimetric assay to quantify the total phenolic content in food products and biological samples. The F- C assay is a simple and reproducible assay that has been used widely for quantifying the phenolic content in plant extracts (Huang et al. 2005; Everette et al. 2010). The investigation of total phenolic content is important for a preliminary assumption of a plant extract activity, as significant positive correlation has been exhibited between total phenolics and antioxidant activity (Ghasemian et al. 2006; Orak et al. 2012; Derakhshan et al. 2018).

3.1.2 High performance liquid chromatography (HPLC)

The distribution of the analyte (sample) between a mobile phase (eluent) and a stationary phase is the basis for HPLC separation (packing material of the column). The molecules are slowed while passing through the stationary phase, depending on the chemical structure of the analyte. UV-absorption is commonly used in detectors. HPLC is a powerful analytical technique that has been used for qualitative and

quantitative analysis of non-volatile compounds in plant extracts, such as phenolics, terpenoids and alkaloids (Harborne 1998). The HPLC is used for qualitative and quantitative analysis of a selected composition of samples obtained from natural products with a robust analytical separation, even with a higher sample loading volume. In the application of liquid chromatography, the compounds were qualitatively analysed according to their consistent retention times of reference standards. The quantitative analysis is performed using a standard curve generated from the standard reference injected at different concentrations of standard reference compound (Chen et al. 2012; Phang et al. 2016).

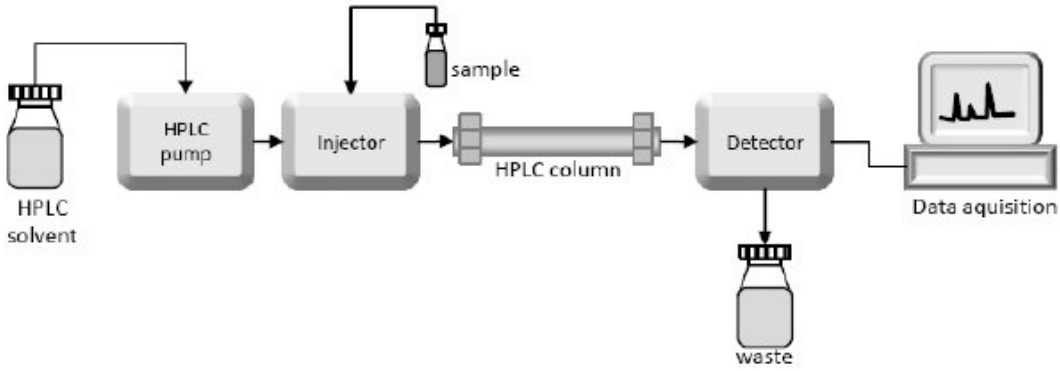


Figure 3.1: A schematic diagram of the HPLC workflow. This figure was adopted from Czaplicki (2013).

3.1.3 Mass spectrometry (MS)

The basic premise of mass spectrometry (MS) is to create ions from inorganic or organic substances using any appropriate technique, separate them utilizing their mass-to-charge ratio (m/z), and detect them qualitatively and quantitatively by their m/z and abundance. MS is used to find the structure characteristics and calculate the mass/charge (m/z) ratio of the known and unknown compounds in plant samples. MS has been considered a standard instrument in analytical laboratories (Devanshu et al. 2010). Qualitative analysis is performed by calculating the mass and relative chemical formula and structure. Quantitative analysis is performed using the relationship

between the peak and compound content of represented peak (Pang et al. 2016). MS comprises three main steps: ionisation, mass analysis and detection. The process starts with sample ionisation and after ionisation the sample is passed to the mass spectrometer and the molecular mass of the investigated compound is calculated based on the m/z ratio (Vacchina et al. 2000).

3.1.4 Liquid chromatography-mass spectrometry (LC-MS)

The separation method of liquid chromatography (LC) is used to isolate the various components of a mixture. A sample is mass transferred is through a polar mobile phase and a non-polar stationary phase in this procedure. The liquid chromatography-mass spectrometry (LC-MS) analytical technique has been used widely in many analytical laboratories for more than 20 years. This technique is a combination of liquid chromatography and mass spectrometry. Therefore, data obtained from this separation instrument provides both retention time data and masses of molecular ions or fragments formed during the process within the mass spectrometer. LC-MS is becoming more popular for natural products studies since exact molecular weight of compounds and structural identification of analytes could be determined, even from small amounts of plant sample extract with low concentrations (Harborne 1998). Developing technology has provided many options and parameters for a better LC-MS analysis, but the basic principles have remained the same. The main components of the system are shown in Figure 3.2.

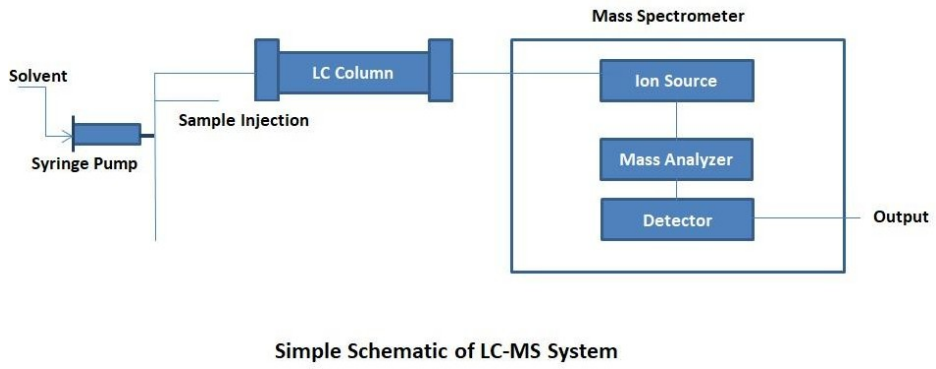


Figure 3.2: Mechanistic flow of the LC-MS system.

3.1.5 Pomegranate

Pomegranate is one of the oldest edible fruits and grows in a wide range of geographical locations around the world, such as Asia, Europe, South and North America, Africa and Australia (Hollan et al. 2009). Recently, the popularity for this fruit has been increased considerably since it comprises secondary compounds which are beneficial for human health with antioxidant, antimicrobial and anticancer properties (Seeram et al. 2005; Lansky and Newman 2007; Akhtar et al. 2015). Pomegranate polyphenolic compounds have been analysed qualitatively and quantitatively by different researchers using a variety of techniques (Seeram et al. 2005; Fischer et al. 2011; Sentandreu et al. 2013). However, it should be noted that there are some variabilities in the secondary compounds in pomegranate extracts (Zaouay et al. 2012; Abid et al. 2017). Therefore, it was necessary in this study to investigate the composition of PRE prior to evaluation of the bioactivities of PRE and its constituents in future chapters.

3.2 Aims and objectives

The aims of this chapter were:

- Determination of the total phenolic content of PRE as tannic acid equivalent using the Folin-Ciocalteu (F-C) assay.
- LC was hyphenated with LTQ Orbitrap MS to identify and characterise the secondary chemicals in PRE based on their m/z value and MS spectra in both negative and positive modes of ionisation.
- Quantify the amounts of punicalagin in PRE, generating a standard curve with a reference standard punicalagin using the HPLC method.

3.3 Materials and Methods

Equipment, glass and plastics, chemical consumables and assay kits used in this chapter were presented in Chapter 2, Section 2.1 in Table 2.1, Table 2.2 and Table 2.3, unless otherwise stated.

3.3.1 Estimation of total phenolic content and other oxidation substrates in PRE using Folin-Ciocalteu reagent

In this study the F-C assay was done according to the method by Ainsworth et al. (2007). PRE (0.5 mg/mL) sample was prepared as previously described (Section 2.2.2), and 200 µL 10% (vol/vol) of F-C reagent in deionised water was added to 100 µL of prepared PRE sample. The suspension was vortexed thoroughly and 800 µL of 700 mM Na2CO3 was added into Eppendorf tubes and incubated at room temperature for 2 h. After incubation, 200 µL of sample was taken from the tube and added to the well of a 96 well plate to read the absorbance at 760 nm using a plate reader.

In this experiment tannic acid was used as a standard, and it was diluted in phthalate buffer pH 4.5 in the range from 25 µM-200 µM (42.5 µg/mL - 340.24 µg/mL) to prepare a calibration curve. The concentration of phenolic compounds in the PRE was expressed as tannic acid equivalents per gram of freeze-dried PRE sample.

3.3.2 LC/DAD and ESI-MS analysis of PRE

LC/DAD and ESI-MS analysis of PRE were performed at the National Mass Spectrometry Facility at Swansea University, UK, using freeze-dried PRE sample in powder form prepared as described in Section 2.2.2.

3.3.2.1 Sample preparation

A few grains of freeze-dried PRE sample were solvated in 300 µL 1:15 methanol (MeOH):H2O then diluted 1: 10 in 100 µL 1:15 MeOH:H2O in a conical glass flask. It was then inserted in an autosampler vial for LC/MS analysis. Blank injections of MeOH in both ionisation modes were included to assess background of MeOH.

3.3.2.2 Instrumentation

A Dionex Ultimate 3000 High Performance Liquid Chromatography coupled with an Orbitrap Mass Spectrometer (Thermo LTQ Orbitrap XL) was used with ESI for the analysis of PRE. The ionisation mode was performed both in positive and negative modes. The mass resolution was set at 60000 and scan range was set at 350-1750 m/z ratio for both ionisation modes. The sheath gas flow (arb), aux gas flow (arb) and capillary temperature (℃) were set at 15, 2, and 325, respectively, again for both positive and negative ionisation modes. The method involves spray voltage (kV) 4.3 and 4.2, capillary voltage (V) 43 and -30 and tube lens voltage
(V) 150 and -100 for positive and negative modes, respectively (Table 3.1). LC was performed using a C18 column (3.5 µm x 2.1 mm x 150 mm, Waters XBridge) with mobile phase comprising A: MeOH: acetonitrile (2:98, v/v) and B: MeOH: acetonitrile with 0.1% formic acid in both phases. The flow rate was 0.2 mL/min and injection volume was 5 µL. The chosen gradients were as follows: 100% A (0-2 min), 50% A (2-28 min), 50%-10% A (28-30 min), 10% A (30-35 min), 10%- 98% A (35-37 min), 98% A (37-45 min) (Table 3.2)

Mobile Phase A	2% MeCN : 0.1% Formic Acid	
Mobile Phase B	MeCN with 0.1% Formic Acid	
LC Column	Waters XBridge C18 3.5µm x 2.1mm x 150mm	
Flow Rate	0.2 mL/min	
Gradient	Time (mins)	% B
	0 – 2	0
	2 - 28	50
	28 - 30	50
	30 - 35	90
	35 - 37	90
	37 - 40	2
	40 - 45	2
Injection	Full loop 5ul	
DAD	190 to 700nm	

Table 3.1: Parameters of the Dionex Ultimate 3000 uHPLC instrument used in this experiment.

	+ve ion	**-ve ion**
OFN Sheath Gas Flow (arb)	15	15
OFN Aux Gas Flow (arb)	2	2
Capillary T (°C)	325	325
Spray Voltage (kV)	4.3	4.2
Capillary Voltage (V)	43	-30
Tube Lens Voltage (V)	150	-100
Scan Range (m/z)	350-1750	350-1750
FT Mode Resolution	60,000	60,000

Table 3.2: Parameters of the LTQ Orbitrap XL MS instrument used in this experiment.

3.3.3 Quantitative analysis of punicalagin in PRE by HPLC

HPLC is a widely used analytical method to quantitatively analyse compounds using absorption spectrophotometry. This method is useful to determine the concentration of a specific compound in a complex sample, such as punicalagin in the PRE sample.

HPLC analysis was performed using an Agilent 1100 HPLC system and embedded with Phenomenex 5µm C18 100A 4.6mm x 150mm column (Kinetex, USA). Separation was achieved using gradient elution and mobile phases comprising (A) HPLC grade water with 0.1% trifluoroacetic acid (TFA) and (B) HPLC grade MeOH with 0.1% TFA. Mobile phases were degassed for at least 2 h before use. The gradient timetable and the percentage of mobile phases are shown in Table 3.3 (Houston 2011). A range of different punicalagin (1 mg/mL to 15.625 µg/mL) concentrations were analysed to determine the punicalagin concentrations in the PRE sample. Punicalagin was dissolved in deionised H2O and the same HPLC method was used to construct a calibration curve for punicalagin.

Time (min)	% MeOH+0.1% TFA	%H_2O+0.1%TFA
0	99	1
2	96.5	3.5
4	95	5
7	92.5	7.5
10	90	10
15	80	20
25	40	60
35	95	5

Table 3.3: Gradient timetable for HPLC analysis.

3.4 Results

3.4.1 Total phenolic content

The quantitative determination of the total phenolic content of PRE was expressed in mg of tannic acid equivalent (TAE) per g of freeze-dried PRE. The results showed that there was approximately 496 mg TAE/g of freeze-dried PRE as a result of three independent experiments.

3.4.2 LC/UV-Vis

Several intense peaks were observed in the UV-Vis chromatograms generated by sample, showing it to be highly complex mixtures (Figure 3.3). Most components eluted within 20 min under the conditions used, and some only just after the solvent front because of chemically similar compounds in PRE. Optimisation of the LC method to improve resolution of co- eluting components would therefore benefit any future studies.

3.4.3 LC/ESI-MS

Analytes were tentatively identified in PRE using the information from MS detectors and by comparison with literature data. As observed in the LC/UV-Vis data, analysis by LC/ESI-MS showed that the PRE sample was a highly complex mixture, and the occurrence of the same ion series repeatedly throughout the analysis suggests several isomeric forms that could match the empirical formulae generated by the measured accurate masses.

Figure 3.4 summarises the base peak chromatographic information for each sample in both ionisation modes, together with printouts of spectra of observed ions in the main peaks and, where possible, theoretical isotope matches were generated. The information is quite extensive, and the main peaks of interest and the associated ions were tentatively characterised and presented in Table 3.4 and Table 3.5. It should be noted that these are only tentative suggestions based on result of studies that were performed with standardized profiling method in the literature, since exact

characterisation requires standards to verify LC retention times to fully characterise PRE using MS and MS/MS and performing complementary analysis using NMR to confirm compound identity with confidence.

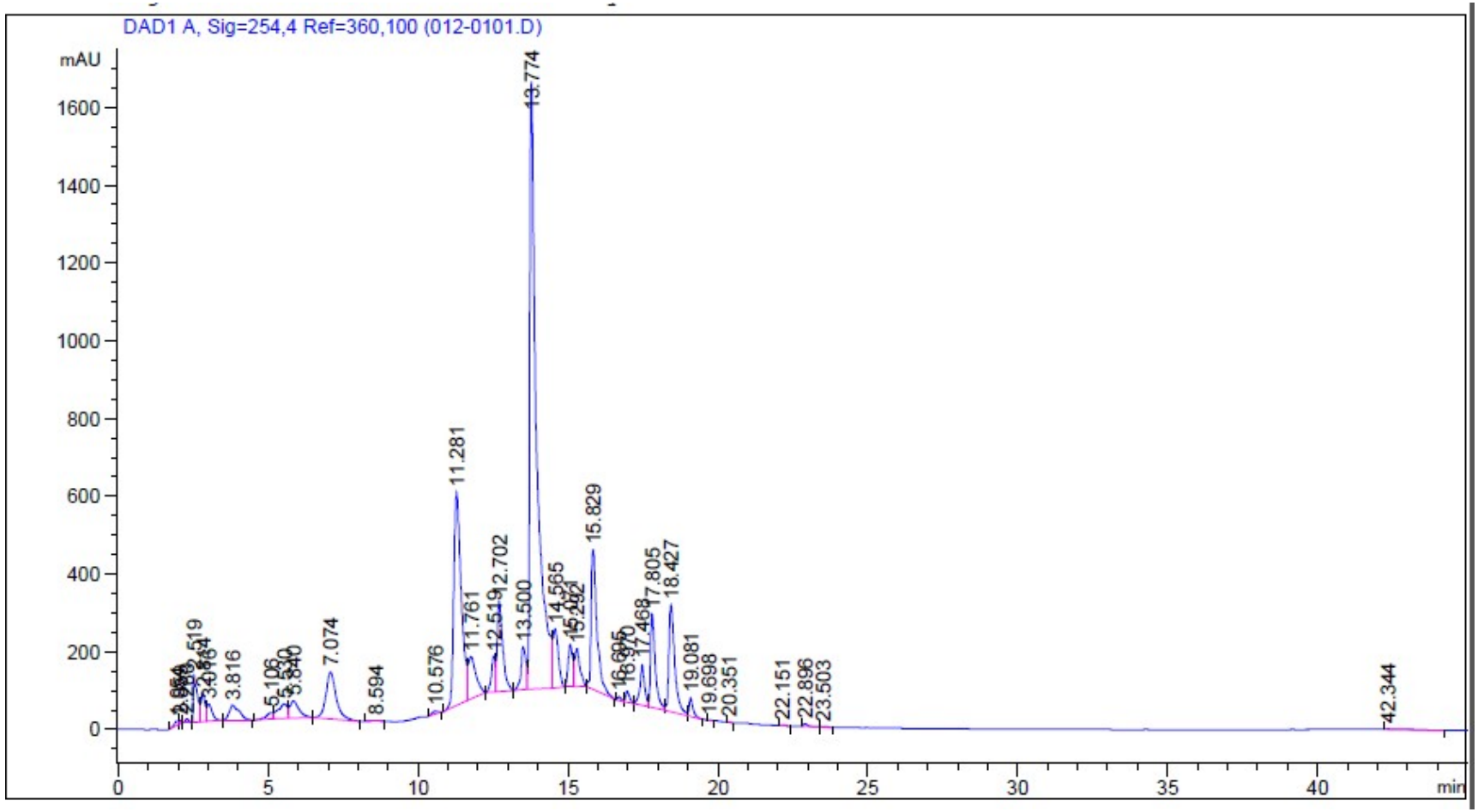


Figure 3.3: LC/UV-Vis profile for PRE.

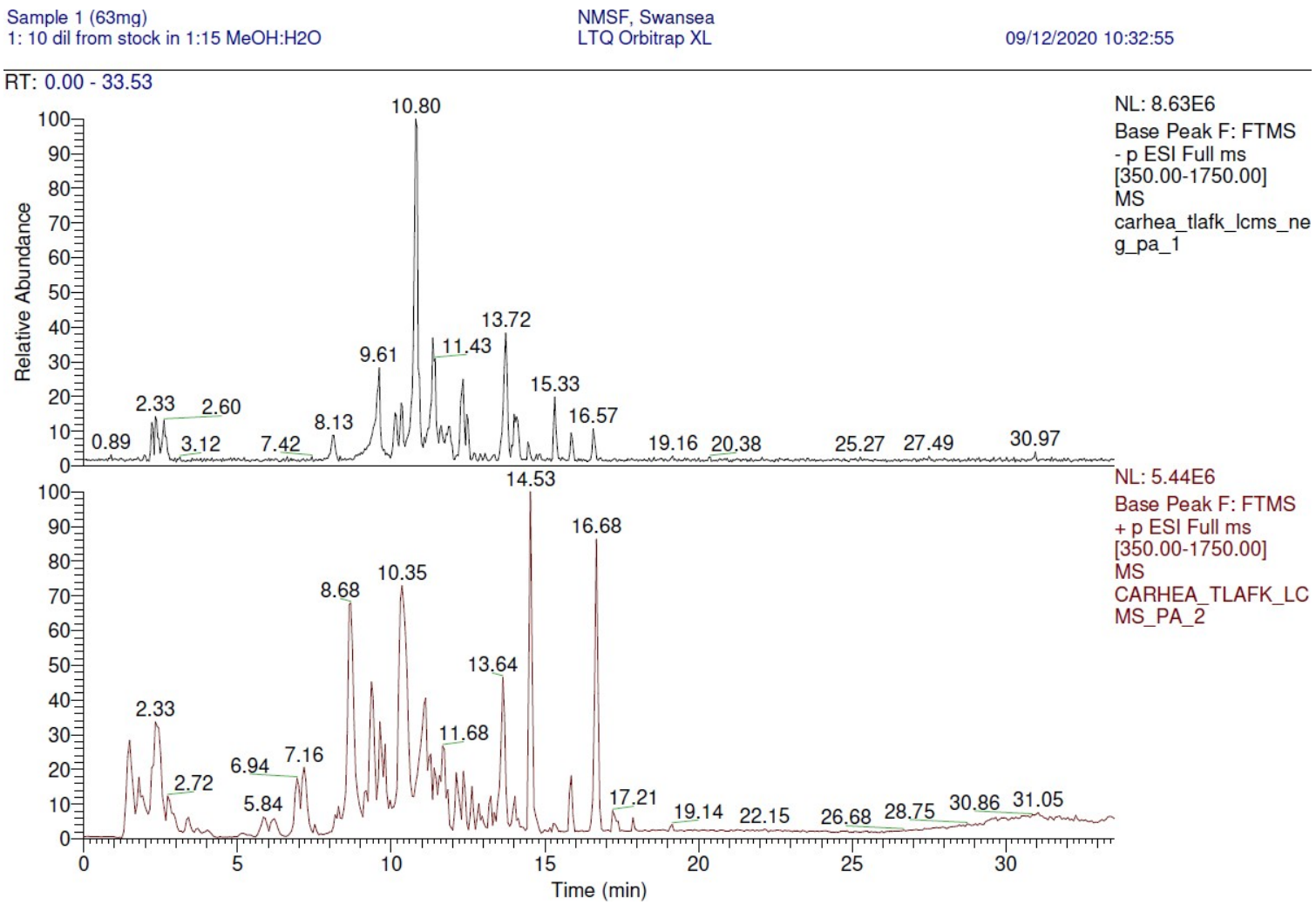


Figure 3.4: Comparison of negative (above) and positive (below) ion LC/ESI-MS chromatograms for PRE.

No.	compound	Retention time (min)	$[M-H]^-$ *(m/z)*	MS^2 ion Fragments *(m/z)*	References
1	**HHDP-hexoside**	2.21/ 2.6	481	463,301,191	(Fischer et al. 2010; Mena et al. 2012; Al-Rawahi et al. 2014; Garcia-Viallalba et al. 2015; Abid et al. 2017; Brighenti et al. 2017; Aguilar-Zárate et al. 2017;Yan et al. 2017; Wafa et al. 2017; Perez-Ramirez et. al 2018; Stefano et al. 2018; Russo et al. 2019)
2	**Bis-HHDP-hexoside (Pedunculagin I)**	8.13/ 10.13/ 10.33	783	391	(Zahin et al. 2010; Fischer et al. 2010; Mena et al. 2012; Garcia-Viallalba et al. 2015; Abid et al. 2017; Brighenti et al. 2017; Aguilar-Zárate et al. 2017;Yan et al. 2017; Wafa et al. 2017; Perez-Ramirez et. al 2018; Stefano et al. 2018; Russo et al. 2019)
3	**Punicalagin derivative**	9.61/ 10.13/ 10.8	1083	541	(Zahin et al. 2010; Mena et al. 2012; Al-Rawahi et al. 2014; Garcia-Viallalba et al. 2015; Abid et al. 2017; Brighenti et al. 2017; Aguilar-Zárate et al. 2017;Yan et al. 2017; Wafa et al. 2017; Perez-Ramirez et. al 2018; Russo et al. 2019; Sorrenti et al. 2019)
4	**(HHDP-galloylglucose)-pentose**	8.13/ 10.33	1415	707	(Mena et al. 2012; Brighenti et al. 2017)
5	**Pedunculagin I der**	10.13/ 10.33	1265	632	(Brighenti et al. 2017)

6	**Galloyl-HHDP-DHHDP-hexoside (granatin B)**	10.13/13.72	951	631, 475, 301	(Fischer et al. 2010; Wafa et al. 2017; Abid et al. 2017; Perez-Ramirez et. al 2018; Stefano et al. 2018; Sorrenti et al. 2019)
7	**Granatin A**	11.35	799	781, 479, 301	(Fischer et al. 2010; Abid et al. 2017; Wafa et al. 2017)
8	**Cyanidin-3-pentoside**	10.33	417		(Abid et al. 2017)
9	**Sanguiin H10**	11.63	1567	783	(Mena et al. 2012)
10	**Galloyl-bis-HHDP-hex (casuarinin) derivative**	11.63	935	783, 633, 467	(Fischer et al. 2010; Brighenti et al. 2017)
11	**Ellagic acid glucoside /Quercetin hexoside**	12.34/ 15.33	463		(Fischer et al. 2010; Mena et al. 2012; Fracasetti et al. 2013; Garcia-Viallalba et al. 2015; Brighenti et al. 2017; Aguilar-Zárate et al. 2017;Yan et al. 2017; Wafa et al. 2017; Perez-Ramirez et. al 2018; Stefano et al. 2018; Russo et al. 2019)
12	**Galloyl-HHDP-hexoside (corilagin)**	12.46	633	463, 301, 275, 229	(Fischer et al. 2010; Mena et al. 2012; Fracasetti et al. 2013; Garcia-Viallalba et al. 2015; Abid et al. 2017; Brighenti et al. 2017; Aguilar-Zárate et al. 2017; Perez-Ramirez et. al 2018; Stefano et al. 2018; Russo et al. 2019)
13	**Diosmetin glucoside**	12.92	461		(Russo et al. 2019)
14	**Dihydrokaempferol-hexoside**	13.05	449		(Fischer et al. 2010; Russo et al. 2019)
15	**Delphinidin 3,5-O-diglucoside**	13.35	627		(Russo et al. 2019)

16	**Cyanidin-3-glucoside**	13.35	449		(Russo et al. 2019)
17	**Ellagic acid deoxyhexose**	14.08/ 16.57	447	301,300,299	(Fischer et al. 2010; Mena et al. 2012; Fracasetti et al. 2013; Garcia-Viallalba et al. 2015; Brighenti et al. 2017; Aguilar-Zárate et al. 2017;Yan et al. 2017; Wafa et al. 2017; Perez-Ramirez et. al 2018; Stefano et al. 2018; Russo et al. 2019)
18	**Ellagic acid pentoside**	14.08	433	301	(Fischer et al. 2010; Mena et al. 2012; Brighenti et al. 2017; Perez-Ramirez et. al 2018; Russo et al. 2019)
19	**Vanillic acid-dihex**	15.33	491		(Brighenti et al. 2017)
20	**Cyanidin-rutinoside**	15.85	593		(Abid et al. 2017)
21	**Quercitrin**	16.57	447		(Russo et al. 2019)

Table 3.4: LC/negative ion ESI/MS results showing retention times, characteristic ions and tentative assignments of compounds in PRE.

No.	compound	Retention time (min)	$[M]^+$	Fragments	References
1	**Cyanidin-3-pentoside**	9.8	419	287	(Fischer et al. 2010; Mena et al. 2012; Sentandreu et al. 2013; Al-Rawahi et al. 2014; Garcia-Viallalba et al. 2015; Abid et al. 2017; Brighenti et al. 2017; Aguilar-Zárate et al. 2017; Yan et al. 2017; Wafa et al. 2017)
2	**Cyanidin-3,5-diglucoside**	14.52	611	449, 287	(Fischer et al. 2010; Zahin et al. 2010; Mena et al. 2012; Sentandreu et al. 2013; Garcia-Viallalba et al. 2015; Abid et al. 2017; Brighenti et al. 2017; Aguilar-Zárate et al. 2017;Yan et al. 2017; Wafa et al. 2017; Stefano et al. 2018; Perez-Ramirez et. al 2018)
3	**Delphinidin-3-glucoside**	15.34	465	303	(Fischer et al. 2010; Zahin et al. 2010; Mena et al. 2012; Sentandreu et al. 2013; Al-Rawahi et al. 2014; Garcia- Viallalba et al. 2015; Abid et al. 2017; Brighenti et al. 2017; Aguilar-Zárate et al. 2017;Yan et al. 2017; Wafa et al. 2017; Stefano et al. 2018; Perez-Ramirez et. al 2018)
4	**Pelargonidin-3,5-diglucoside**	15.86	595	449, 287	(Fischer et al. 2010; Mena et al. 2012; Sentandreu et al.

					2013; Brighenti et al. 2017; Wafa et al. 2017; Stefano et al. 2018)
5	**Pelargonidin-3-glucoside**	17.21	433	271	(Fischer et al. 2010; Mena et al. 2012; Sentandreu et al. 2013; Brighenti et al. 2017; Wafa et al. 2017; Stefano et al. 2018; Perez-Ramirez et. al 2018)
6	**Cyanidin-3-glucoside**	17.21	449	287	(Fischer et al. 2010; Mena et al. 2012; Sentandreu et al. 2013; Wafa et al. 2017; Stefano et al. 2018; Perez- Ramirez et. al 2018)

Table 3.5: LC/positive ion ESI/MS results showing retention times, characteristic ions, possible formula and tentative assignments of compounds in PRE.

3.4.4 Quantification of punicalagin content

HPLC is the most common method to analyse secondary metabolites in plant extracts. Using this method, analysis of the ellagitannin punicalagin gave two distinct major peaks corresponding to punicalagin α and β anomers (Figure 3.5). It has been reported that punicalagin isomers are interconverted and the equilibrium constant K = [β]/[α] was found 1:1 in methanol and 4:1 in water (Doig et al. 1990). In some other studies, this ratio was found to be approximately 2:1 (Satomi et al. 1993; Nigrisa et al. 2011; Houston 2011; Alrashidi 2020). In this thesis, the ratio of punicalagin β and punicalagin α content was found to be slightly lower at 1.76:1 (Figure 3.7). In the study this ratio was consistent during experiments and allowed one anomer of punicalagin to be used for quantification of total punicalagin amount in PRE. For quantification, punicalagin β was used because of its higher concentration.

The calibration curve (Figure 3.7) of punicalagin was constructed using different concentrations of standard punicalagin in serial dilutions. Standard punicalagin solution in deionised water was serially diluted seven times and samples were analysed with the same method of PRE analysis. Using the standard curve, it was calculated that there was 170 mg/g of freeze-dried extract.

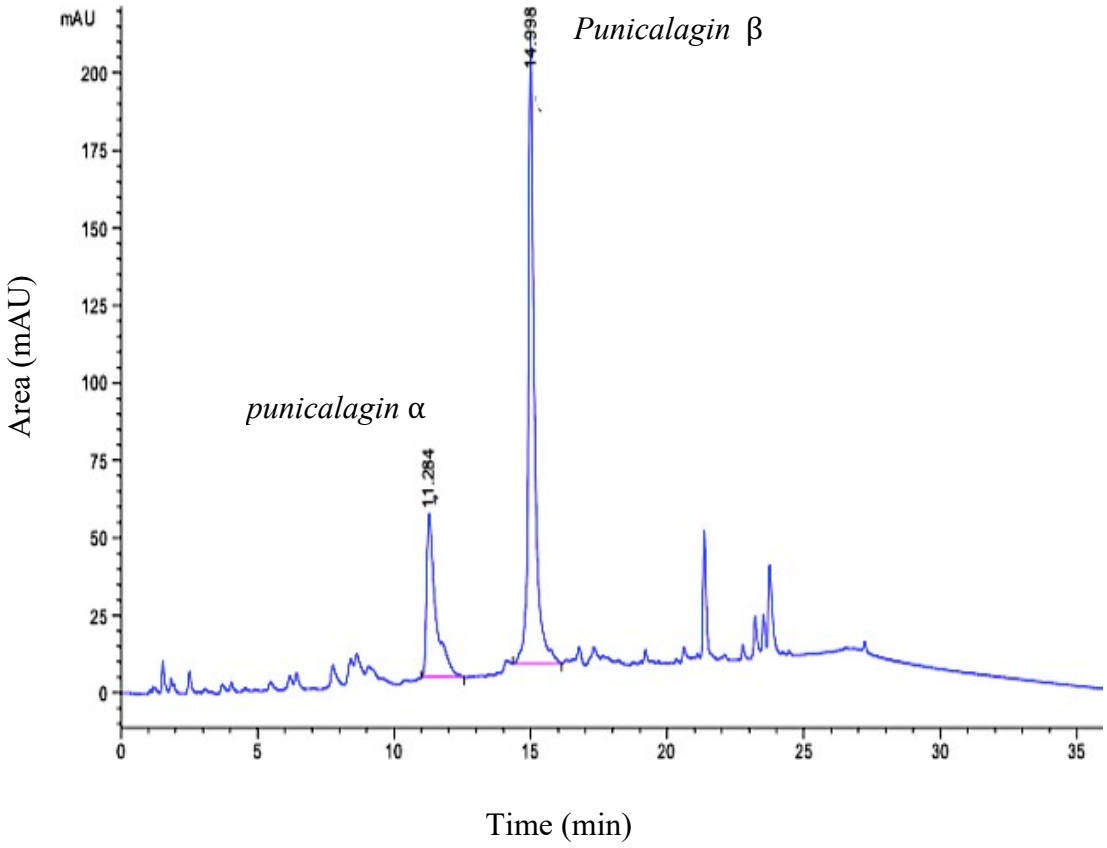


Figure 3.5: A representative HPLC chromatogram sample of 0.1 mg/mL PRE, showing absorption at 258 nm and highlighting the anomers of punicalagin α and β.

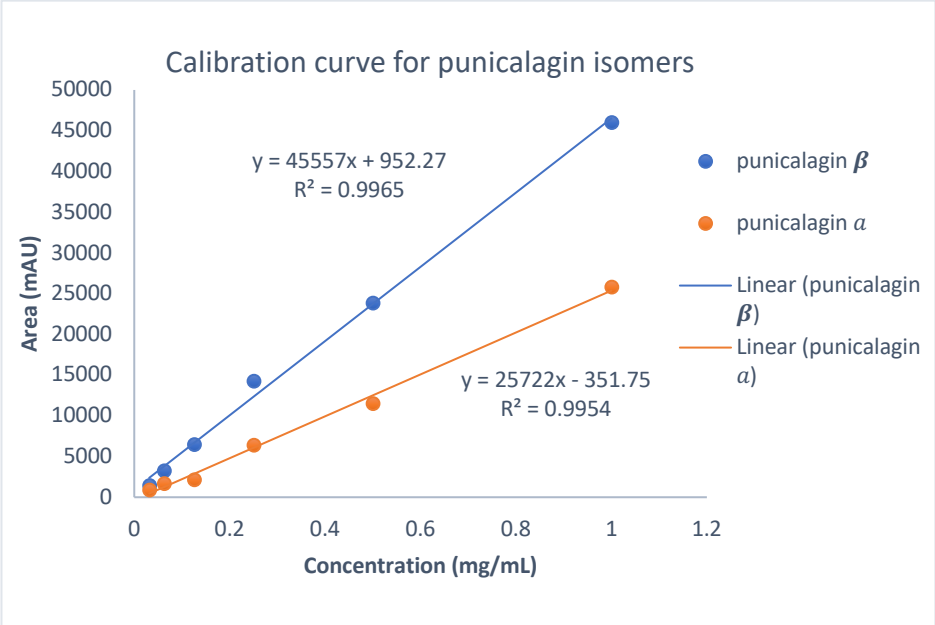


Figure 3.6: HPLC calibration curve for punicalagin anomers α *and* β (N=3±SD).

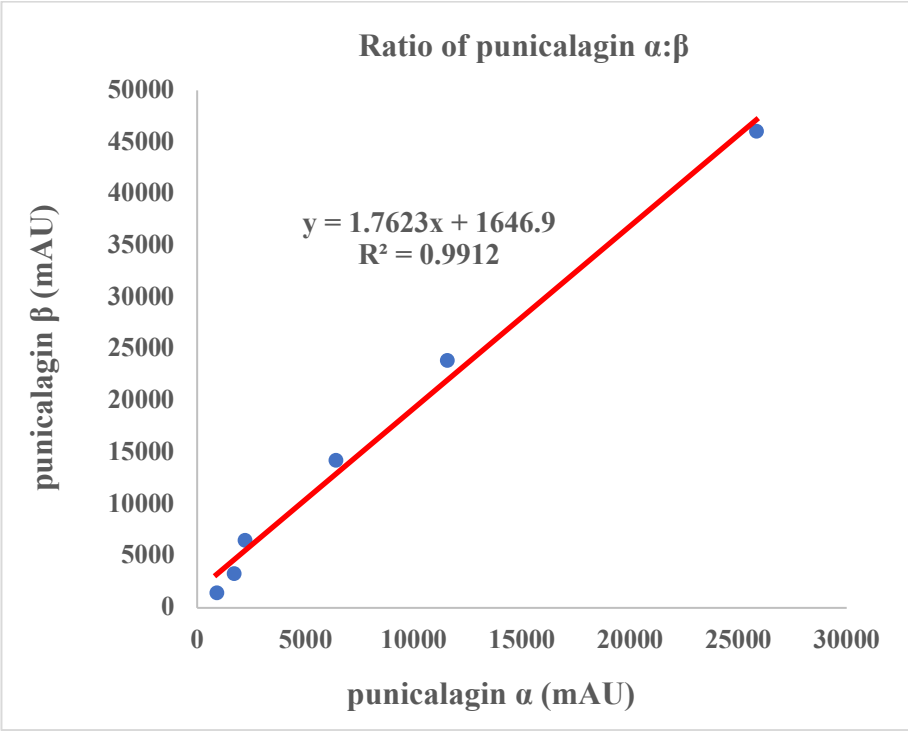


Figure 3.7: Ratio of the area of HPLC analysis between the punicalagin anomers α *and* β. (N=3±SD).

3.5 Discussion

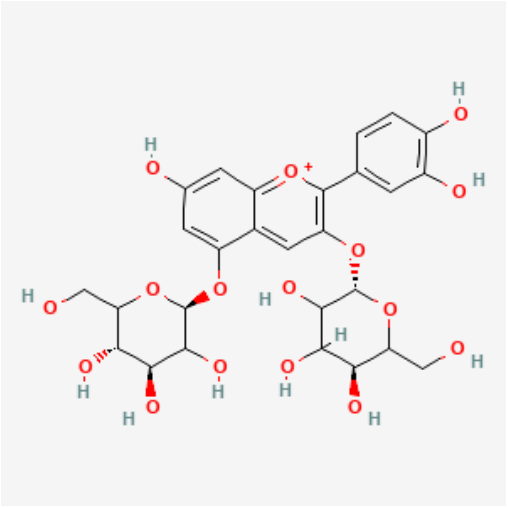
The pomegranate tree is cultivated in tropical and subtropical regions of the world from Middle East countries to the USA, and it is considered a medicinal plant and fruit across the world for its pleasant taste and health benefits (Bar-Ya'akov et al. 2019). Pomegranate peel/rind comprises 40-50% of the total fruit and it has been accepted as a biowaste (Ali et al. 2019). However, pomegranate has recently started to receive increased attention and popularity due to its rich phenolic compound contents. Pomegranate has been proposed as source of economic value to the food and pharmaceutical industries due its excellent health benefits (Kannat et al. 2010; Celiksoy 2020). In this study, it was found that the total polyphenol content of the water extract of pomegranate rind was 496 mg tannic acid equivalent/g freeze dried pomegranate rind. This result was similar to that of the study by Malviya and Jha (2014), where the total polyphenol content of pomegranate rind with different solvents revealed the water extract had highest value (435 mg tannic acid equivalent/ g pomegranate peel) than other solvents, including methanol, ethanol and different ratios of ethanol with water. In this study, extracted PRE was characterized using HPLC-ESI-Orbitrap MS for its phenolic and anthocyanin contents. PRE was found to have similar contents reported in previous studies, and ellagitannins were found to be the major phenolic compounds in PRE (Gil et al. 2000; Fischer et al. 2011; Qu et al. 2012).

Pomegranate peel/rind is rich in terms of phenolic compounds which can be divided in different subgroups such as phenolic acids, flavonoids and tannins. These groups comprise benzene rings and a number of hydroxyl groups attached in their chemical structure (Singh et al. 2018; Andrade et al. 2019). In this study, different secondary compounds were tentatively characterised by interpretation of their fragmentation patterns using the mass spectra and the available literature was also used for a detailed investigation of phenolic compounds.

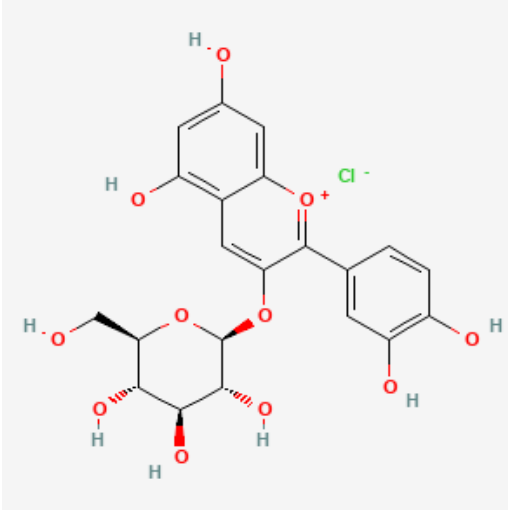
Anthocyanins are the major colour secondary compounds and found in all fruit parts including arils and the peel (exocarp) of the pomegranate (Gil et al. 1996b; Tzulker et al. 2007). The presence of anthocyanins was investigated by MS detection in the positive ion mode since these compounds have an ability to produce $[MH]^+$ ions by ESI in acidic conditions (Brighenti et al. 2017). Mono- and di- glucosides of cyanidin

(red pigments), delphinidin (purple pigments) and pelargonidin (orange pigments) have been identified from pomegranate fruit harvested from different regions of the world (Gil et al. 1996a,b; Ben Simhon et al 2011; Turkyilmaz 2013; Zhao et al. 2013). The tentatively identified anthocyanin compounds with their mass spectral characteristics are summarised in Table 3.5 and their chemical structures are shown in Figure 3.8. In this study, the main anthocyanins were identified by ESI (+) mass spectra were tentatively identified as cyanidin 3- pentoside, cyanidin 3,5-diglucoside, delphinidin 3-glucoside, pelargonidin 3,5- diglucoside, pelargonidin 3-glucoside and cyanidin 3-glucoside. It was observed that monoglycosylated anthocyanins generally had less polar characteristics than diglycosylated compounds, according to elution from the HPLC column. In the study performed by Fischer et al. (2011), 9 anthocyanins were characterised by comparison of UV-Vis spectra, retention times and mass spectra with reference compounds: delphinidin 3,5-diglucoside, cyanidin 3,5- diglucoside, pelargonidin 3,5-diglucoside, delphinidin 3-glucoside, a cyanidin-pentoside-hexoside, cyanidin 3-glucoside, cyanidin 3-rutinoside, pelargonidin 3-glucoside and a cyanidin-pentoside detected. These anthocyanins were identified by ESI (+) ionisation mode in typical mass spectrometric behaviour, and they exerted M^+ ions in the MS^1 experiments. The sequential loss was observed in their saccharide moieties by releasing the aglycones in the MS^2 and MS^3 experiments. Delphinidin 3,5-diglucoside, cyanidin 3,5-diglucoside, pelargonidin 3,5-diglucoside, delphinidin 3-glucoside, cyanidin 3-glucoside and delphinidin 3-glucoside were also identified in other studies (Gil et al. 1996a; Hernandez et al. 1999). In a further study by Hernandez-Corroto et al. (2019), no anthocyanins were detected in the extracts obtained from pomegranate peel. Therefore, it should be noted that anthocyanins variety and content could account for the differences between studies, because of fruit cultivar, geographical location, harvesting time, extraction, purification methods and solvent type used to solvate extract. This variation also shows the more complex profile for anthocyanins in pomegranate fruit (Algihourchi and Bargezar 2008).

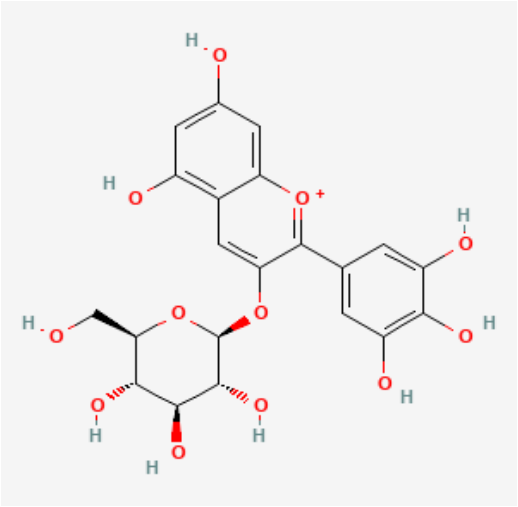
1-) Cyanidin 3,5-diglucoside



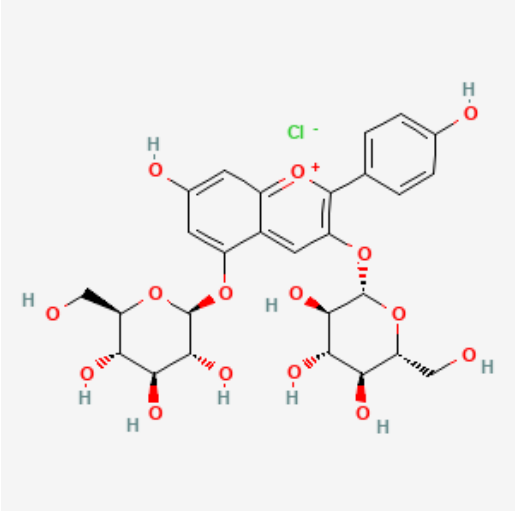
2-) Cyanidin 3-glucoside



3-) Delphinidin 3-glucoside



4-) Pelargonidin 3,5-diglucoside



5-) Pelargonidin 3-glucoside

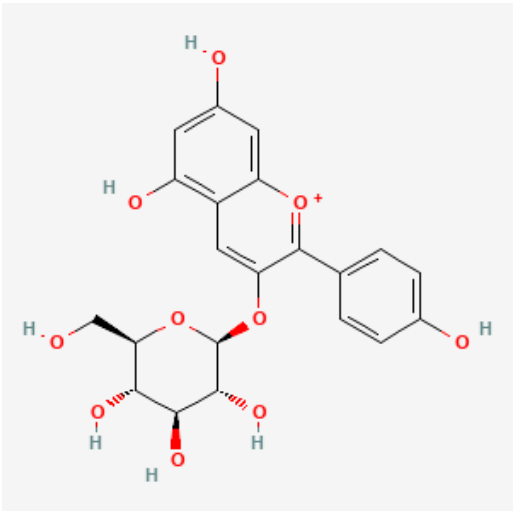


Figure 3.8: The chemical structure of tentatively assigned anthocyanins in PRE, chemical structures obtained from National Library of Medicine, PubChem (2021, available at https://pubchem.ncbi.nlm.nih.gov).

In addition to anthocyanins, other phenolic compounds were detected in PRE and presented in Table 3.4 and Figure 3.9. These compounds were characterised according to their molecular weight, their MS/MS fragmentation patterns and comparison with previously reported data. In Tables 3.4 and 3.5, the previous findings of compound detection in PRE were also shown.

Pomegranate is a rich source of hydrolysable tannins and hydrolysable tannins can be subdivided into gallotannins and ellagitannins depending on the residues to which the ester linkage between glucose and gallic acid. Many hydrolysable tannins (more than 60) have been tentatively identified in pomegranate extract from fruit peel, aril, juice and seed (Fischer et al. 2011a; Mena et al. 2012; Ambigaipalan et al. 2017; Liu and Seeram 2018).

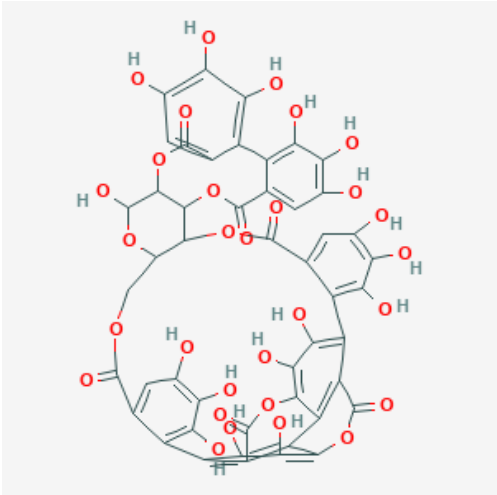
In pomegranate rind, punicalagin anomers (α and β) have been considered the main hydrolysable ellagitannins and account for over 85% of total tannins present (Seeram 2005; Lu et al. 2008). Both ESI (+) and ESI (-) modes exhibited high performance in polyphenolic compounds tentative identification. While there are some common secondary compounds in both MS operating modes, ellagitannins analysis is strictly reliant on the chosen method. Therefore, ionisation mode is critical for the identification of phenolic compounds (De Rijke et al. 2003). MS spectra in negative ionisation mode was chosen for phenolic compounds different from anthocyanins and MS spectra in positive ionisation mode was chosen for anthocyanins, since anthocyanins comprise species that contain the flavylium cation moiety (Lucci et al. 2017).

In the analysis of hydrolysable tannins with ESI, doubly charged ions were reported and larger fragment ions were observed depending on ion in full MS (Arnao et al. 2001; Hukkanen et al. 2007). In these experiments, punicalagin isomers generally generated two ions at m/z 1083 and 541, in singly and doubly charged ions, respectively. Similar ions of punicalagin isomers were generated in previous studies (Abid et al. 2017; Brighenti et al. 2017; Stefano et al. 2018; Russo et al. 2019). Two compounds were detected with a singly charged ion at m/z 481 with close retention times, (2.21 and 2.6). These compounds were tentatively assigned as HHDP-hexoside and were previously found in the pomegranate bark by NMR spectroscopy (Tanaka et

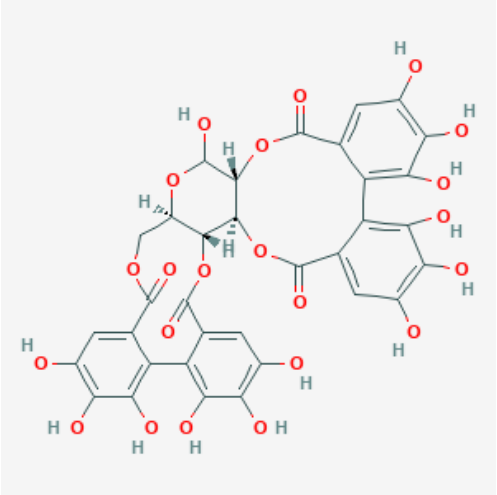
al. 1986). Peaks at m/z 799 and fragments at m/z 781 and at m/z 479 corresponds to a loss of water and ellagic acid, respectively, and this compound was tentatively identified as granatin-A (Fischer et al. 2010; Abid et al. 2017; Wafa et al. 2017).

Other major hydrolysable tannins in pomegranate rind are punicalin, ellagic acid, gallagic acid and ellagic acid glycosides (Seeram et al. 2005; Fischer et al. 2011; Abid et al 2017). Ellagitannins have a polymeric chemical structure which comprises galloyl and HHDP units esterified with glucose (Del Rio et al. 2012). In the PRE extract, studied here, many ellagitannins were tentatively detected and some of them were identified using their characteristic fragment ion spectra (Hukkanen et al. 2007). The tentatively detected ellagitannins had high similarity with previous reported studies (Fischer et al. 2010; Mena et al. 2012; Al-Rawahi et al. 2014; Garcia-Viallalba et al. 2015; Abid et al. 2017; Brighenti et al. 2017; Aguilar-Zárate et al. 2017; Yan et al. 2017; Wafa et al. 2017; Perez-Ramirez et. al 2018; Stefano et al. 2018; Russo et al. 2019). For some mass spectra results, more than one tentative formula was suggested from possible formulae since the fragment ion was not sufficient for definite assumption. Therefore, further studies are necessary with reference compounds and additional NMR could be used for an exact structural determination of detected secondary compounds. For example, in this study, in ESI (-) mode at 8.13 retention time the detected compound revealed an $[M-H]^-$ ion at m/z 1415 and produced fragment ions at m/z 707 (MS^2) and both alnusjaponin A/B and di(HDDP-galloylglucose)-pentose were suggested for tentative assignment. In a previous study with pomegranate juice, the same $[M-H]^-$ ion at m/z 1415 was observed, but an ion fragment at m/z 1397 (MS^2) was seen. This detected compound was tentatively suggested as di(HDDP-galloylglucose)-pentose. Interestingly, di(HDDP-galloylglucose)-pentose and sanguiin H10 isomer were tentatively identified for the first time in pomegranate juice by Mena et al. (2012). In the present study, the sanguiin H10 isomer was tentatively identified with the same fragment ions.

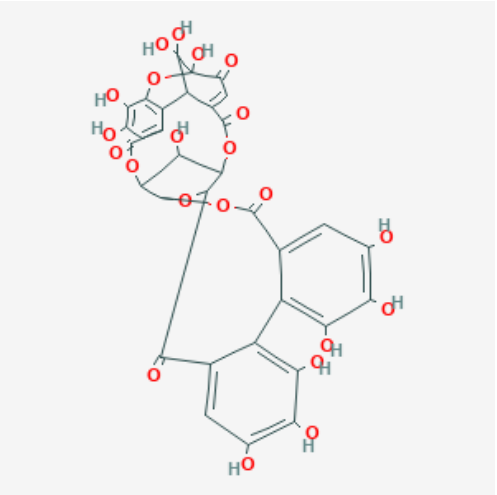
Punicalagin



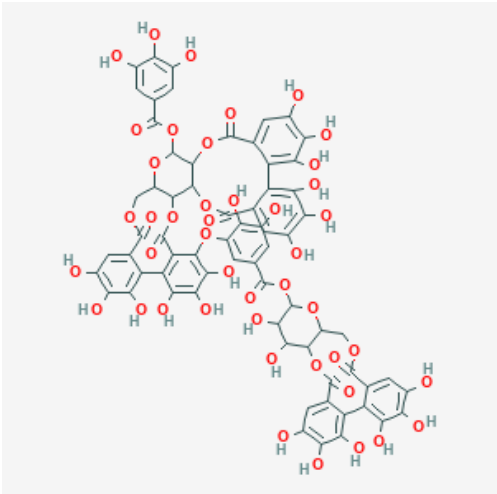
Pedunculagin I



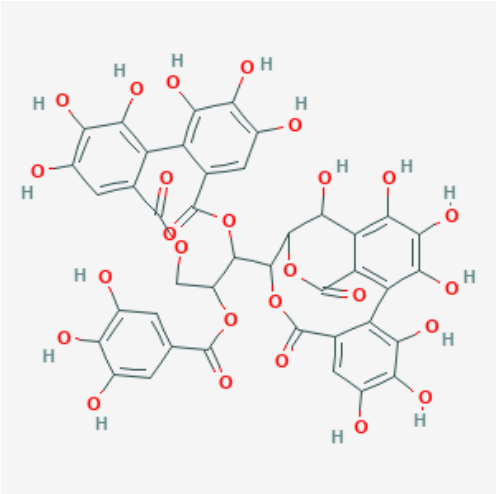
Granatin A



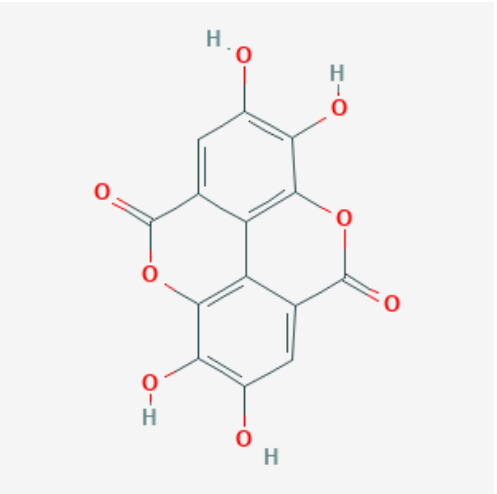
Sanguiin H10



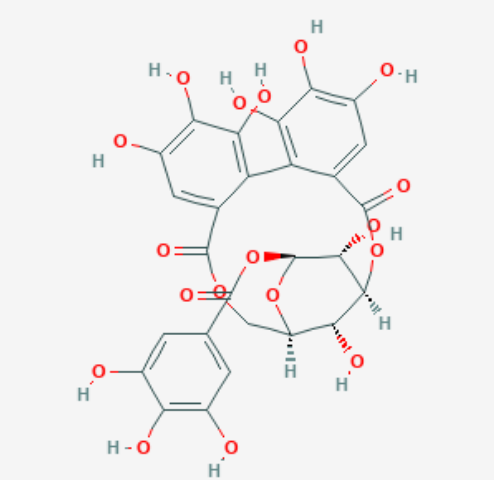
Casuarinin



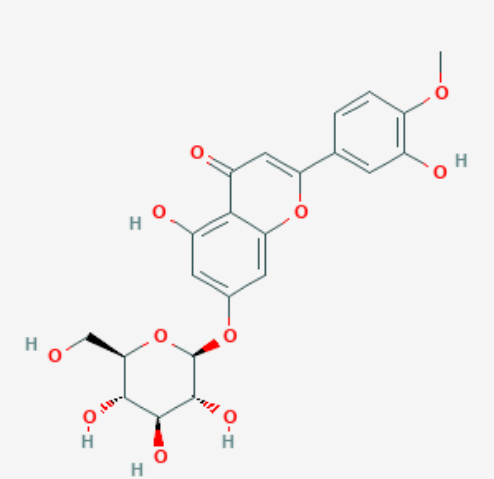
Ellagic acid



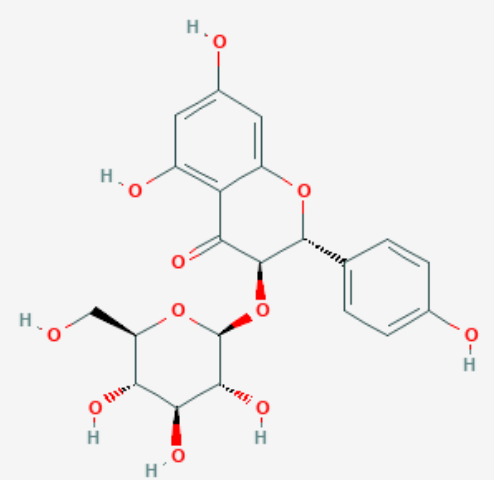
Corilagin



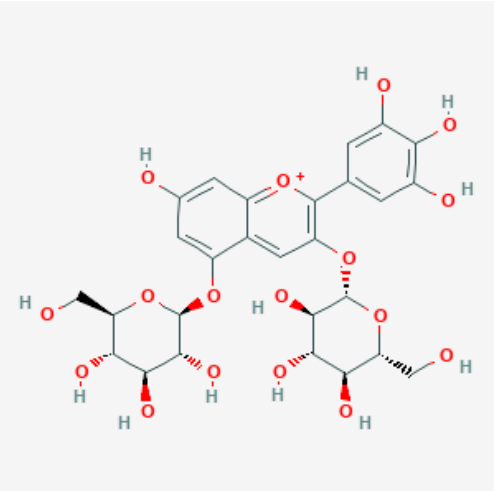
Diosmetin-glucoside



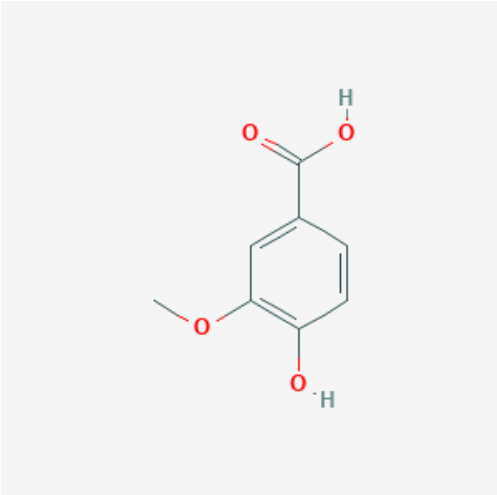
Dihydrokaempferol-glucoside



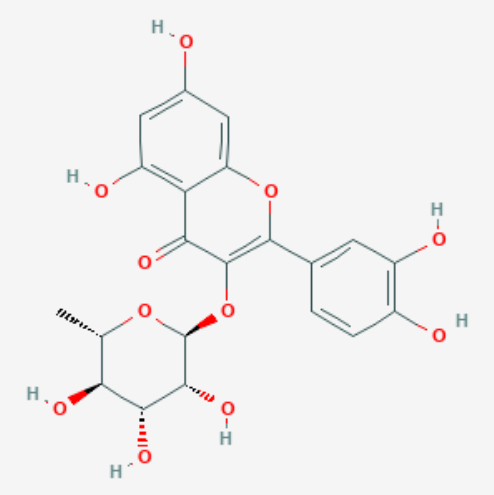
Delphinidin 3,5-O-diglucoside



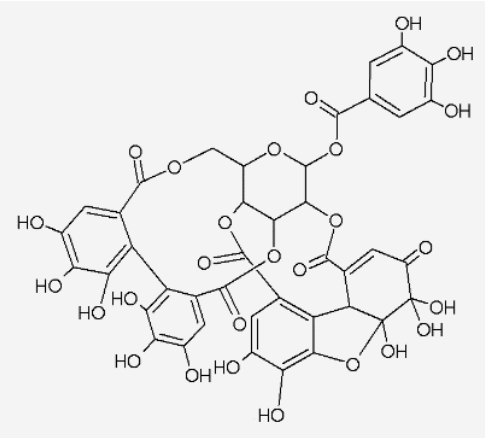
Vanillic acid



Quercitrin



Granatin B



HHDP-hexoside

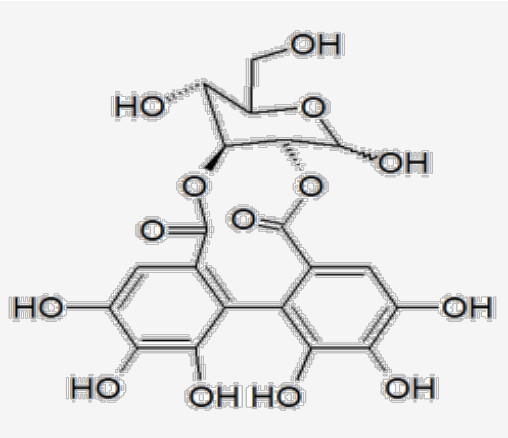


Figure 3.9: The chemical structure some of tentatively assigned ellagitannins in PRE (Chemical structures were obtained from National Library of Medicine, PubChem (2021, available at https://pubchem.ncbi.nlm.nih.gov).

Punicalagin was considered the major and the most abundant hydrolysable ellagitannin in pomegranate extracts (Aguilar-Zarate et al. 2017; Rongai et al. 2019). Thus, a calibration curve was generated using commercially available punicalagin (Sigma Aldrich, UK). The amount of punicalagin in the PRE sample was calculated using the generated standard curve and it was found that around 20% of the PRE sample comprised of punicalagin (170 mg/g). Similar results were also obtained in previous studies. For example, 16 Chinese originating pomegranate husk extracts were analysed for their punicalagin content, which varied from 39.8 mg/g to 121.5 mg/g (Lu et al. 2008). In another study, three kinds of pomegranates from different regions of Pakistan were compared for their punicalagin content, and it was found that this content varied from 98.7 mg/g to 118 mg/g (Khalil et al. 2017).

The results of the present study showed that the main compounds in PRE were anthocyanins and especially ellagitannins. These findings are consistent with recent studies that have investigated the main phenolic compounds in Spanish, Greek and Chinese pomegranate juice, flowers and peels (Zhang et al. 2011; Fischer et al. 2012; Mena et al. 2012; Gomez-Caravaca et al. 2013; Lantzouraki et al. 2015).

Overall, the PRE used in the present study was tentatively characterised by HPLC-ESI/MS for its phenolic and anthocyanin contents by using both negative and positive modes. Our results agreed with previous studies indicating that ellagitannins were the major phenolic compounds in pomegranate rind, since they represent over 99% of the total content of pomegranate phenolics (Fischer et al. 2011; Qu et al. 2012; Romeo et al. 2015). Moreover, punicalagin was the main ellagitannin in pomegranate rind (Gil et al. 2000; Fischer et al. 2011; Qu et al. 2012).

3.6 Conclusion

Phenolic compounds in PRE were tentatively investigated tentatively by using LC-ESI/MS method in both negative and positive ionisation modes. The results showed a variety of phenolic compounds in PRE that were mainly ellagitannins and anthocyanins. In ellagitannins, punicalagin was found to be predominate in PRE, comprising almost 20 % of the total PRE. Results were compatible with most previous studies that have investigated the phenolic contents of pomegranate extracts. PRE

characterisation allows a more in-depth evaluation of the bioactivities of PRE in future chapters of this thesis. However, future studies are still necessary to characterise all these secondary compounds in PRE and to find the approximate quantities of each in PRE.

Chapter 4: Antimicrobial and Microbicidal Activity of PRE, Zn (II) and PRE/Zn (II) Against a Panel of Oral Microbes

4.1 Introduction

Oral diseases are one of the major health problems in the world, with dental caries and periodontal diseases affecting 90% of school aged children, along with a significant proportion of the adult population in industrialised countries (Petersen et al. 2005). There is also evidence of a relationship between poor oral health and chronic or systemic diseases, such as diabetes, cardiovascular diseases and rheumatoid arthritis (Petersen et al. 2005; Rautemaa et al. 2007). One of the most important barriers to providing good oral health to populations is the economy. In developed countries, 10% of the public health expenditure comprises dental care treatments. However, in developing countries, where public health expenditure is low and it is not easy to access to dental healthcare, it mostly consists of emergency or pain relief treatment (Petersen et al. 2005).

The oral microbiota is a complex community that comprises more than 700 microbial species and a key component is the biofilm called dental plaque (Jenkinson and Lamont 2005). The general aetiology of oral infectious diseases is correlates with accumulated dental plaque, which could increase the complexity of biofilm matrix and proportion of pathogenic microbes over commensal microbes (Bowen et al. 2018; Chen et al. 2020). The oral microbiota has a critical role in health and disease situations, and the balance between pathogenic and commensal microbes is an important factor for a healthy oral cavity. However, under certain conditions, the imbalance between invading and commensal bacteria in the oral microbial community can result in oral infectious diseases. Both Gram-positive and Gram- negative bacteria play a role in dental diseases. Primarily, the increased *S. mutans* population in dental plaque has been highly associated with dental caries, as *S. mutans* biofilm can produce acid and extracellular polysaccharides. In addition, *S. mutans* biofilms are acid-resistant, since the acidic environment enhances the metabolising range of carbohydrates into organic acids that provide low pH conditions (Zhang et al. 2017). However, recent studies showed that treatment strategies which just focus on *S.*

mutans biofilms are not sufficient to prevent dental caries, since organic acid components produced by other microorganisms e.g., *Actinomyces, S. mitis,* and *S. gordonii* can also cause dental caries (Bowen et al. 2018; Fakhruddin et al. 2019). In addition to *S. mutans*, the other Gram-positive streptococci bacteria play a role in the metabolism of sucrose to organic acids, which causes demineralisation and leads to tooth decay in supra-gingival tissues (Loesche 2007). In sub-gingival regions, however, Gram-negative bacteria such as *P. gingivalis* and *F. nucleatum*, play important roles in initiating infection of the gingival crevice, which affects the gingiva and its surrounding tissue by causing a change in inflammatory cellular responses (Tichy and Novak 1998; Jenkinson and Lamont 2005). This inflammation can cause gingivitis (reversible), which without appropriate treatment can lead to periodontitis (irreversible) (Loesche 2007).

There is an increasing need for new, alternative preventative and treatment methods or products which are safe, effective and economically affordable for periodontal diseases. While there are some commercially available antimicrobial agents in clinical use such as chlorhexidine, they have adverse side-effects (Serrano et al. 20009; Xie et al. 2014; Ardila and Bedoya-Garcia 2020). Antimicrobial resistance is a significant problem in treating pathogenic bacteria and limits the use of current antibiotics for inflammatory diseases (Badria and Zidan 2004; Marston et al. 2016; Hofer 2019). Chlorhexidine, which is commonly used in oral care products, is an example of this due to its reported toxicity and causing staining of teeth. Ethanol, which is commonly used in mouthwashes, is another example and is linked to oral cancer (Knoll-Köhler and Stiebel 2002; Rodrigues et al. 2007). These reasons are making it vital to search for alternative oral care products. It is also important to develop affordable dental care products for economically disadvantaged populations in order to provide sustainable dental healthcare.

Pomegranate has been used since ancient times, due to its beneficial effects on health. Extracts of this fruit have been reported to have antimicrobial activity against oral pathogens in *in vitro* and clinical studies (Jurenka 2008). It has been shown that pomegranate extracts induce antimicrobial effect through inhibiting bacterial growth, preventing adherence and quorum sensing and inactivating *S. aureus* pathogenic bacterial products (Prasad and Kunnaiah 2014).

While most bacteria have a protective mechanism against heavy metal ions, there are many reports showing that metal compounds act as an effective antimicrobial agent (Silver 1996; Stewart et al. 1998; McCarrell et al. 2008). Stewart et al. (1998) reported that there is a significantly increased anti-bacteriophage activity when PRE is combined with ferrous salts. In another study, the antimicrobial activity of PRE was studied in combination with other metal salts, Cu (II), Fe (II), Mn (II), or Zn (II) and vitamin C against some Gram-positive and Gram-negative bacteria. It was found that an enhanced antimicrobial activity was occurred with the addition of these metal ions (McCarrell et al. 2008; Gould et al. 2009). In this study, PRE was combined with Zn (II) only, rather than other metallic ions. Zn (II) has also been widely used in oral care products and it has relatively lower toxicity compared to Cu (II). It was also shown that there was enhanced viricidal activity when PRE was combined with zinc salts (Houston et al. 2017b).

4.2 Aims and objectives

The objectives of this chapter were to investigate the antimicrobial activities of PRE, Zn (II) and a PRE/Zn (II) against selected oral microbes. The specific aims of this chapter were:

- To investigate the antimicrobial activities of PRE or Zn (II) against selected oral microbes namely *S. gordonii, S. mutans, S. salivarius, S. sanguinis, S. anginosus, P. gingivalis, F. nucleatum, C. albicans*, *S. aureus* and *E. coli*
- To investigate the PRE/Zn (II) for antimicrobial activity against oral microbes.
- To investigate the effect of PRE, Zn (II) and PRE/Zn (II) on *S. sanguinis, S. gordonii, S. mutans, C. albicans,* and *P. gingivalis* 24 h single- species bacterial/fungal biofilm formation and biofilm eradication.

4.3 Materials and methods

The agar plates, broth and bacterial cultures were prepared as described in Chapter 2 (Section 2.2.3). All bacterial strains and their origin were similarly described in Chapter 2, Table 2.4. Equipment, glass and plastics, chemical consumables and assay kits used in this chapter were presented in Section 2.1 in Table 2.1, Table 2.2, and Table 2.3, unless otherwise stated.

4.3.1 Preparation of PRE and Zn (II) solutions for antimicrobial susceptibility assay

Stock solutions of PRE and Zn (II) were prepared in phthalate buffer pH 4.5, as described in Chapter 2 (Section 2.2.1.2 for Zn (II) and Section 2.2.2 for PRE). Stock solutions of PRE and Zn (II) were then diluted to desired concentrations in Mueller Hinton (MH) broth. These solutions were used for all antimicrobial susceptibility assays except for agar well diffusion assays and were prepared fresh for experiments day as previously described in Section 2.2.3.8.

4.3.2 Agar well diffusion (zone of inhibition) assay

Antimicrobial activity of PRE and Zn (II) was preliminarily studied by agar well diffusion (Devillers et al. 1989). Agar plates, FAA with 5 % sheep blood and BA supplemented with haemin and vitamin K were used for anaerobes, and MH agar was used for facultative bacteria. An overnight experimental bacterial culture broth (100 µL) containing 10^8 CFU/mL was plated on the surface of the agar using a sterile cotton swab and swabbed across the plate in different directions to obtain an homogenous distribution of microbial culture. Then, six wells (6 mm) were punched in the plates using a heat sterilised stainless-steel cork borer. The wells were filled with 50 µL of two-fold dilutions of PRE (6.25-50 mg/mL) and Zn (II) (62.5-500 mM) and incubated for 48 h in an anaerobic cabinet for obligate anaerobic bacteria and for 24 h in an aerobic incubator for facultative bacteria and *C. albicans*. Chlorhexidine digluconate (0.2 %) and potassium phthalate buffer (pH 4.5) were used as positive and negative controls, respectively.

4.3.3 Determination of minimum inhibitory concentration (MIC)

PRE and Zn (II) were first assessed to determine their MIC against planktonic suspensions of studied microbes. The microbroth dilution method was used. The general protocol of the Clinical and Laboratory Standards Institute (CLSI) was performed with small differences (CLSI Guidelines M07-A09; CLSI Guidelines M11-A08). In these experiments, an overnight culture of each bacterial species was prepared in suitable liquid medium, and 100 µL containing approximately 10^6 CFU/mL bacteria was added into appropriate wells of 96-well plates. Then, 100 µL of PRE (0.25-32 mg/mL) and Zn (II) (0.78-50 mM) were added onto the bacterial solution in the 96-well plates. Additionally, a growth control (appropriate broth + bacteria), sterility control (only broth) and positive control (chlorhexidine 0.2%) were included in each experiment.

96-well microtitre plates were incubated for 24 h and 48 h at 37°C in an aerobic incubator and anaerobic cabinet for facultative and obligate anaerobes, respectively. After incubation, the MIC value for each microbe was determined visually choosing the lowest concentration without turbidity.

4.3.4 Determination of minimum lethal concentration (MLC)

Minimum lethal concentrations (MLC) was determined by evaluating the contents of the wells at MIC or higher for viable cells according to National Committee for Clinical Laboratory Standards Guideline (NCCLS) (Wayne et al. 1999). The 96-well microtitre plate was prepared as for the MIC assay. The liquid was taken from selected wells showing microbial growth inhibition and added to appropriate 20 mL liquid culture media. This suspension was incubated in a suitable environment according to assayed microbe type. After incubation, control groups and treatment groups were visually observed, and results recorded. The clear suspensions and control suspensions were centrifuged at 4000 x g for 5 min and the supernatants discarded. Then, 50 µL of suitable broth was added to the remaining pellet and plated on suitable agar media. It was incubated for a further 24 h or 48 h, and then the MLC value was recorded as the lowest concentration where no growth was observed.

4.3.5 Assessment of antimicrobial activity interaction between PRE and Zn (II)

4.3.5.1 Fractional inhibitory concentration (FIC) via checkerboard test

The existence of synergism between PRE and Zn (II) was evaluated using a checkerboard assay, according to a previously described method by Endo et al. (2012). Different concentrations of PRE and Zn (II) were applied as two-fold dilutions, starting from 32 mg/mL PRE and 25 mM Zn (II) in different 96-well plates. The two-fold dilutions for PRE and Zn (II) combination were prepared in a new 96-well plate that was used as the experiment plate. Also, growth, sterility, and PRE or Zn (II) only controls were included. A 100 µL volume of experimental bacterial culture was added at a final concentration of 10^6 CFU/mL, except for the sterility control group. An illustration of a 96-well microtiter plate layout for the checkerboard assay provided in Figure 4.1. The plate was incubated for 24 h in incubator at 37 ℃ and after 24 h the wells were visually examined and MICs recorded for PRE, Zn (II) and PRE/Zn (II). The observed MIC values were used to calculate the fractional inhibitory concentration (FIC). The FIC for the combination doses was calculated by dividing the MIC value of the compound to MIC value in the combination and then the FICindex values were calculated by adding FIC value of each compound to each other.

FIC value of compound A, FICA= (MICA in combination)/ (MICA alone)
FIC value of compound B, FICB= (MICB in combination)/ (MICB alone) FICindex= FICA + FICB
FICindex was used to classify the combination of antimicrobial agents as synergistic when FICindexes were ≤ 0.5; additive when FIC >0.5 but < 1; indifferent when values were >1 and < 4; antagonistic when values were ≥ 4 (Eliopolus and Moellering 2000).

Assays were performed in triplicate and results expressed as calculated FIC value and shown for each microbe. The FIC value was then calculated according to the above equation, and the FICindex and interaction of antimicrobial activity of PRE/Zn (II) for each microbe were calculated.

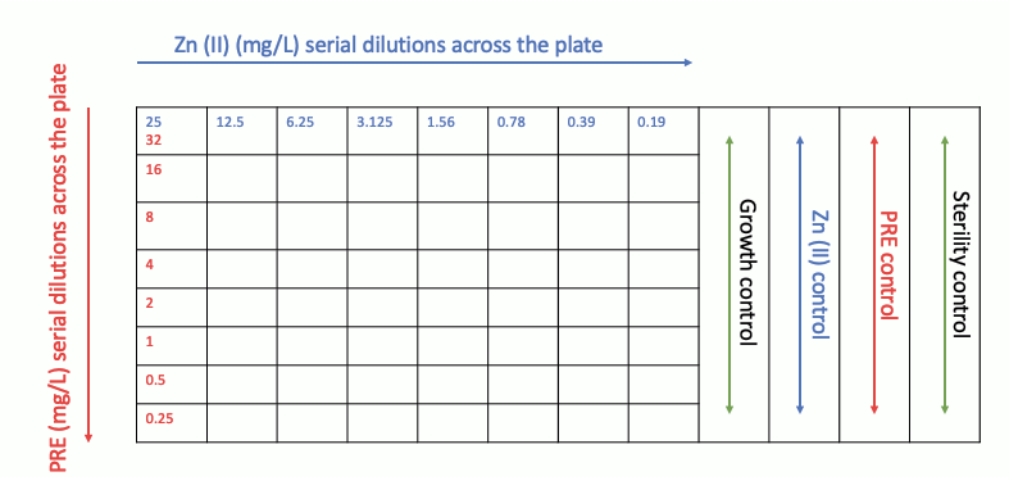


Figure 4.1: An illustration of checkerboard assay layout for studied microbes.

4.3.5.2 Time kill (log reduction) assay

A time kill kinetic assay was used to determine and confirm the relationship between the antimicrobial activities of PRE in combination with Zn (II). This assay was performed only with *S. mutans, S. gordonii* and *C. albicans* that showed synergistic or additive activities in the checkerboard assay. The MIC and half MIC values of PRE, Zn (II) and PRE/Zn (II) were used for this assay. The overnight culture of selected microbes with inoculum of 10^8 CFU/mL were added to microcentrifuge tubes, containing 990 µL PRE, Zn (II) or PRE/Zn (II) and incubated for 10 min, 30 min, 60 min and 240 min. After the specified contact times, 1 mL sample inoculum with treatment agent was added to 9 mL universal quenching agent (UQA which comprised 0.1% peptone, 0.1% sodium thiosulphate, 0.5% Tween 80, and 0.07% lecithin w/v at pH 7) to terminate the antimicrobial activities of PRE, Zn (II) and PRE/Zn (II). Then, 20 µL samples were taken from the UQA tubes and diluted 10-fold in 96-well plates which had 180 µL of PBS in each well. 20 µL of diluted samples were plated on the appropriate agar and incubated for 24 h, colonies were counted, and CFU/mL were calculated and plotted against specified contact times (Miles et al. 1938).

The log10 values were calculated for control and treatment groups to obtain the colony forming units per one millilitre (CFU/mL), as follows:

(Number of colonies x dilution factor)/volume of culture medium

Then, log10 values were determined for each treatment and control group. The log reduction value was found by using the formula:

Log10(A)-Log10(B)

A represents the CFU/mL of the control (phthalate buffer) and B the CFU/mL of test sample. Assays were performed in triplicate and results were expressed as mean ± SEM.

4.3.5.3 Bacterial growth curves

The growth of bacteria under the PRE (MIC, MIC/2), Zn (II) (MIC, MIC/2) and PRE in combination with Zn (II) (MIC+MIC, MIC/2+MIC/2) treatments were studied in 96-well microtiter plates for *C. albicans, S. gordonii* and *S. mutans*. Plates were prepared by the same method, but only MIC or MIC/2 of compounds and combinations for each bacterial species with 10^6 CFU/mL final bacterial suspension. Then, plates were placed in a microtitre plate reader at 37 °C and absorbance read at 600 nm every hour over 24 h. Untreated growth (broth + bacteria) and sterility (only broth) controls were included in each experiment. Experiments were conducted in triplicate and each 4 replicates per condition.

4.3.6 *In vitro* assessment of PRE, Zn (II) and PRE/Zn (II) on biofilm formation and pre-formed biofilm

4.3.6.1 Biofilm formation assessment

This assay was performed to investigate the studied microbes' adherence and biofilm forming capacities. A crystal violet assay was used to quantify biofilm biomass. The experiment was set up in a 96-well microtiter plate by adding 10^8 CFU/mL of overnight bacterial suspension to each well and subsequent incubation for 24 h at 37 °C in an aerobic environment (Abdallah et al. 2009). After 24 h, the supernatant from each well was carefully discarded without disrupting the biofilm in the bottom of the well. Wells were then washed twice with distilled water (100 µL/well) to

remove planktonic bacteria. Plates were air dried for 15 min to remove wash solution (distilled water). The microtiter plates were stained with 1% crystal violet (100 µL/well) prepared in distilled water and incubated for 30 min at room temperature. The unbound crystal violet was removed by washing the wells three times with distilled water (100 µL/well) followed by air-drying 15 min. The bounded crystal violet was dissolved by adding 95 % ethanol (100 µL/well) and incubated for 20 min for total solubilisation of crystal violet. The absorbance of wells was read at 570 nm with a plate reader (Fluostar Optima, BMG Labtech, Aylesbury, UK). The results were presented as mean O.D.570 value for each strain ±SEM and assays were repeated three times.

4.3.6.2 Assessment of PRE, Zn (II) and PRE in combination with Zn (II) on biofilm formation inhibition and eradication

Bacterial suspensions were prepared as previously described. Biofilm inhibition assays were performed in a 96-well plate based on a modified spectrophotometric assay (Abdallah et al. 2009). Plates were prepared with 100 µL of treatment compounds at MIC and MIC/2 of PRE, Zn (II) and PRE/Zn (II) and 100 µL bacterial suspension (10^8 CFU/mL). Background plate was prepared for specified concentrations of compounds and combinations without bacterial inoculation. Plates were incubated for 24 h at 37 ℃ under aerobic conditions. After 24 h, the liquid suspensions were removed and 100 µL of 1 % (v/v) aqueous solution of crystal violet added and incubated for 30 min at room temperature. The dye was removed, wells washed thoroughly, and 95 % ethanol added and incubated for 20 min. The crystal violet solution was read spectrophotometrically at 570 nm. The background O.D. value of compounds (without microbial inoculation) was subtracted from O.D. treatment and O.D. control to account for any non-specific binding of crystal violet. The results were presented as mean O.D.570 value for each strain ±SEM and assays were repeated on four separate occasions.

For biofilm eradication, a similar method was employed. Briefly, bacterial inoculation was added to the plate and incubated at 37 ℃ for 24 h under aerobic conditions to create a biofilm. After 24 h, freshly prepared compounds and combinations were

added as described above for 24 h. The supernatant was discarded, and biofilm biomass assessed using crystal violet staining as previously described.

The results were presented as mean O.D.570 value for each strain ±SEM and assays were repeated on four separate occasions.

4.3.6.3 Live/dead imaging of biofilms treated with PRE, Zn (II) and PRE in combination with Zn (II)

A Live/Dead BacLight™ bacterial viability assay was performed to investigate effect of PRE, Zn (II) and PRE in combination with Zn (II) on biofilms in glass bottom 96-well plates. The assay was performed using the method previously described by Powell et al. (2018). Briefly, a bacterial suspension of 10^8 CFU/mL was prepared from an overnight culture of test microorganism. For the obligate anaerobe *P. gingivalis* W50, a bacterial suspension of 10^7 CFU/mL was prepared from a 48-h culture. Then prepared bacterial suspension was added the wells of a glass bottom 96-well plate (100 µL/well). For facultative bacteria, the plate was incubated on a shaker at 20 rpm at 37℃ in 5 % CO_2 for 24 h. *P. gingivalis* incubated in an anaerobic cabinet at 37℃ for 48 h. After incubation, supernatants from each well were carefully discarded. Freshly prepared PRE, Zn (II) and PRE in combination with Zn (II) solutions were added at the MIC values of PRE and Zn (II) and MIC+MIC for PRE/Zn (II) for each studied microbe and incubated in an appropriate atmospheric condition at 37 ℃ for 24 h (Table 4.1). After incubation, the supernatant was gently aspirated from the plate. Each well was immediately stained with 4 µL/well Live/Dead stain (Live/Dead Bacterial Viability kit; prepared by mixing 2 µL of SYTO 9 [LIVE] and 2 µL of propidium iodide [DEAD] in 1 mL of PBS). The stain was carefully added to the centre of well to avoid biofilm disruption. Plate was incubated for 10 min under appropriate atmospheric conditions at 37 ℃ and covered with aluminium foil to prevent quenching by ambient light. Then, 47 µL of PBS was added to each well, prior to imaging by confocal microscopy, using Leica TCS SP5 Confocal Microscope (Leica Microsystems (UK) Ltd, Buckinghamshire, UK).

Microorganism	**PRE (100 μL)**	**Zn (II) (100 μL)**	**PRE +Zn (II) (50 μL +50 μL)**
S. mutans	1 mg/mL	1.56 mM	2 mg/mL +3.12 mM
S. gordonii	2 mg/mL	0.78 mM	4 mg/mL +1.56 mM
C. albicans	4 mg/mL	6.25 mM	8 mg/mL +12.5 mM
S. sanguinis	2 mg/mL	0.78 mM	4 mg/mL +1.56 mM
P. gingivalis	2 mg/mL	3.125 mM	4 mg/mL +6.25 mM

Table 4.1: Concentrations of PRE, Zn (II) and PRE/Zn (II)s used for the treatment of 24 h biofilms of streptococcus species and *C. albicans* and 48 h *P. gingivalis* biofilms and subsequent assessment by live/dead imaging.

Images were visualised using a 60 x 1.8 oil objective with a z-step of 1 μm. Four fields of view were chosen for each well and obtained images were analysed using COMSTAT2 with ImageJ to develop image sequences and for the quantification of biofilm microorganisms and unconnected microorganisms. Otsu automatic thresholding was applied to each image slice, and this facilitated separation of bacterial fluorescence from background noise. Image sequences were analysed for different parameters in COMSTAT 2 such as biomass, roughness coefficient, mean thickness of biofilm, and dead/live ratio.

Another program, IMARIS (v4.1.3, Bitplane AG, Zurich, Switzerland), was used to visualise structure of biofilm images. The COMSTAT2 analysis results were presented as mean ± SEM for each parameter and assays were repeated three separate ocassions.

4.3.6.4 Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) was used to assess the effect of PRE, Zn (II) and PRE/Zn (II) on 24 h biofilms of *C. albicans, S. mutans* and *S. gordonii*. Biofilms (24 h) were prepared on a glass round coverslip in a 6 well-plate. Overnight cultures of microbes were adjusted to 10^8 CFU/mL in BHI broth and 1 mL of each suspension to glass coverslips in 6-well plates. After aerobic incubation at 37 °C for 24 h, the

supernatant from each well was carefully aspirated and 1 mL of freshly prepared PRE, Zn (II) and PRE/Zn (II)s in MH broth (Table 4.2) were added for another 24 h aerobic incubation at 37°C. Only 1 mL of MH broth was added for an untreated growth control group and 0.2% chlorhexidine di-gluconate was used as a positive control. Samples were then prepared for SEM imaging using a protocol adopted from that of Powell et al. (2018). Briefly, samples were immersed in 1 mL of 2.5-3 % of glutaraldehyde for 1.5 h at room temperature and washed 4 times with distilled water. After washing, biofilms were immersed in distilled water and then completely frozen. Frozen samples were placed in a freeze-dryer to remove all liquid. Biofilm samples were then sputter coated with gold (2-20 nm thick) using a rotary pump and gold-palladium target (DSR1 desk sputter coater; Vac Technique, East Sussex, UK), and imaged on a Tescan VAGA SEM system, at 5-10 kV.

Microorganism	**PRE (1 mL)**	**Zn (II) (1 mL)**	**PRE +Zn (II) (500 μL +500 μL)**
S. mutans	1 mg/mL	1.56 mM	2 mg/mL +3.12 mM
S. gordonii	2 mg/mL	0.78 mM	4 mg/mL +1.56 mM
C. albicans	4 mg/mL	6.25 mM	8 mg/mL +12.5 mM

Table 4.2: Concentrations of PRE, Zn (II) and PRE/Zn (II)s for the treatment of 24 h biofilm on round glass slides for SEM of studied microbes.

4.3.7 Statistical analysis

Each experiment was repeated on at least three independent occasions. Two- way analysis of variance (Two-way ANOVA or mixed model) in grouped analysis with multiple comparisons was used to calculate the statistical differences in crystal violet staining. One-way analysis of variance (one-way ANOVA) with post-test Tukey analysis was used to analyse statistical differences for results from the COMSTAT2 analysis. $p<0.05$ was considered as statistically significant and statistical significance further indicated by $*p<0.05$, $**p<0.01$, $***p<0.001$, and $****p<0.0001$.

4.4 Results

4.4.1 Agar-well diffusion (zone of inhibition) assay

The zone inhibition assay was performed as a preliminary assay to evaluate the antimicrobial activity of PRE (6.25-50 mg/mL) and Zn (II) (6.25-50 mM). Results are presented as susceptible (S) and resistant (R) for studied concentrations of compounds and not detected (ND) for obligate anaerobes for PRE (Table 4.3). For obligate anaerobes, the antimicrobial activity of PRE could not be reliably measured, as PRE reacted with the 5% blood supplemented FAA (Figure 4.2). Different agars without blood (Brucella agar supplemented with haemin and vitamin K, Brain heart infusion agar), were also used to perform the zone inhibition assay, but insufficient growth was obtained for the selected obligate anaerobe bacteria, *P. gingivalis* W50 and *F. nucleatum* ATCC 49256. The agar well-diffusion method relied on the diffusion ability of inhibitors used in the assay, and both PRE and Zn (II) showed a good diffusion rate throughout the agar. PRE inhibited *S. sanguinis* in a dose-dependent manner (Figure 4.3A). An observed yellow coloured zone could be the result of PRE interaction with the agar. However, this interaction did not affect the inhibitory effect of PRE against *S. sanguinis*, and PRE exhibited a zone of inhibition beyond this yellow region. Similar PRE diffusion patterns were seen with *E. coli*, but PRE did not inhibit *E. coli.* All studied microbes were inhibited with ZnSO4. PRE exhibited antimicrobial activity against Gram-positive bacteria and *C. albicans* but did not show any antimicrobial activity against *E. coli*. Zn (II) showed a clear inhibition around the punched wells. However, PRE did not present a clear inhibition zone compared with Zn (II), probably due to its diffusion rate (Figure 4.2).

	PRE	**Zn (II)**	**0.2% Chlorhexidine digluconate**
S. gordonii NCTC 7865	S	S	S
S. sanguinis NCTC 7863T	S	S	S
P. gingivalis W50	ND	S	S
F. nucleatum ATCC 49256	ND	S	S
E. coli NCTC10418	R	S	S
S. aureus NCTC 8325	S	S	S
S. mutans DSM 20523	S	S	S
C. albicans ATCC 90028	S	S	S
S. salivarius DSM 20560	S	S	S
S. anginosus NCTC 10713	S	S	S

Table 4.3: Zone inhibition assay results for PRE and Zn (II) for indicated microbes. ND, not detected (experiment was not performed); S, susceptible; R, resistant at highest concentration of PRE (50 mg/mL).

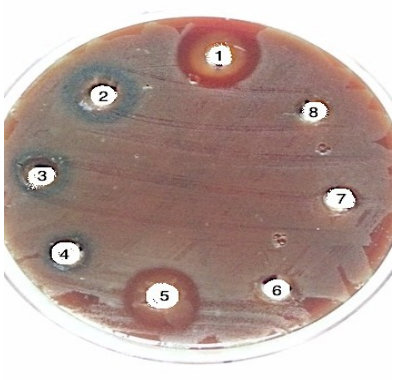
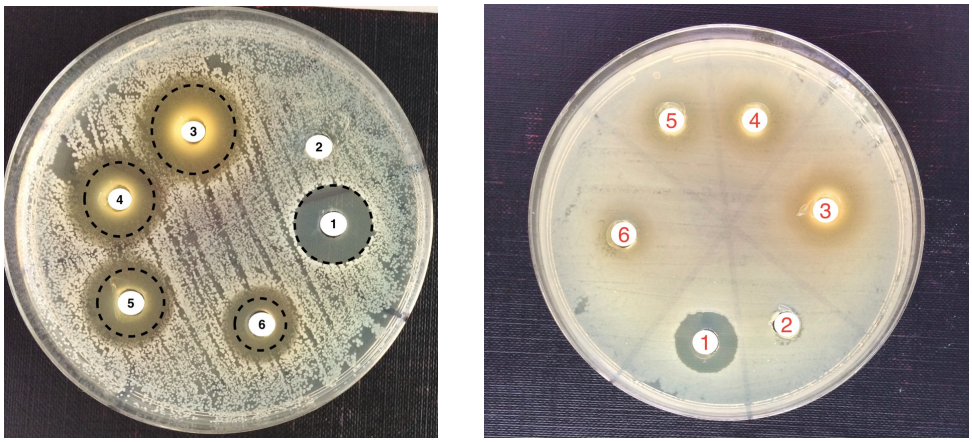


Figure 4.2: Agar diffusion assay showing the reaction between PRE and iron. Wells 1 (0.2% Chlorhexidine digluconate), 2 (25 mg/mL PRE), 3 (12.5 mg/mL PRE), and 4 (6.25 mg/mL PRE), 5 (6.25 mM Zn (II)), 6 (3.125 mM Zn (II)), 7 (1.56 mM Zn (II)) and 8 (0.78 mM Zn (II)) after incubation with bacteria. The black colouration indicates the reaction between PRE and iron.



A **B**

Figure 4.3: PRE diffusion in the agar well diffusion assay. A, PRE effect in agar well diffusion assay against *S. sanguinis*. B, PRE effect in agar well diffusion assay against *E. coli*. Numbers represent different concentrations of PRE, 10 % phthalate buffer in MHB, and chlorhexidine. 1-0.2% chlorhexidine digluconate, 2-10% of phthalate buffer pH 4.5 in MHB, 3-50 mg/mL PRE, 4-25 mg/mL PRE, 5-12.5 mg/mL PRE and 6-6.25 mg/mL PRE) for both images.

4.4.2 Minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC) for studied oral microbes

From the absorbance readings and visual observations, PRE showed antimicrobial activity against all studied Gram-positive bacteria (*S. aureus, S. sanguinis, S. salivarius, S. anginosus*, *S. mutans, S. gordonii*) and *C. albicans,* at indicated concentrations of MIC (Table 4.4). However, as with the agar well diffusion assay, PRE did not show growth inhibition for *E. coli*. Zn (II) showed antimicrobial activity against all bacterial strains at concentrations ranging from 0.39 mM to 6.25 mM.

The MLC values for PRE and Zn (II) are presented in Table 4.4. PRE and Zn (II) exhibited lethal activities at higher concentrations than their inhibitory concentrations against all studied strains. PRE showed lethal activity at ≥4 mg/mL and Zn (II) showed lethal activity at ≥6.25 mM.

Microorganisms	**MIC**		**MLC**	
	PRE (mg/ml)	**Zn (II) mM**	**PRE (mg/ml)**	**Zn (II) mM**
S. gordonii NCTC 7865	2	0.78	4	6.25
S. sanguinis NCTC 7863T	2	0.78	4	6.25
P. gingivalis W50	ND	3.125	ND	6.25
F. nucleatum ATCC 49256	ND	3.125	ND	6.25
E. coli NCTC10418	>4	6.25	>4	>6.25
S. aureus NCTC 8325	2	1.56	>4	6.25
S. mutans DSM 20523	1	1.56	4	>6.25
C. albicans ATCC 90028	4	6.25	>4	6.25
S. salivarius DSM 20560	2	0.39	4	6.25
S. anginosus NCTC 10713	2	0.39	4	>6.25

Table 4.4: Minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC) values for PRE and Zn (II) against studied microbes. ND, not detected.

4.4.3 Checkerboard assay

The data presented in Table 4.5 shows that PRE/Zn (II) exhibited a clear synergistic antimicrobial activity against *S. gordonii* and *C. albicans,* with 0.25 and 0.125 FICindex values, respectively. PRE/Zn (II) showed additive antimicrobial activity with a 0.625 FICindex value for *S. sanguinis*. For *S. aureus* and *S. salivarius,* PRE/Zn (II) FIC_{index} values were found to be indifferent at 1.25 and 1.5, respectively. Again, PRE/Zn (II) showed additive antimicrobial activities against *E. coli, S. anginosus,* and *S. mutans,* with FICindex values of 1, 0.75 and 1, respectively. It is important to note that no antagonistic antimicrobial activity was observed for any studied bacterial strains in terms of antimicrobial activities of PRE/Zn (II).

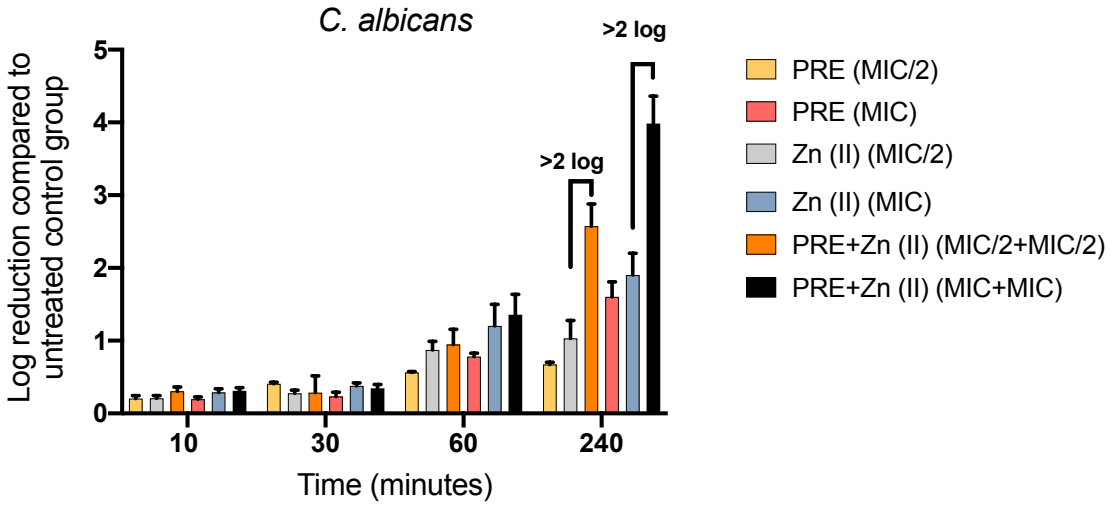
	Fractional Inhibitory Concentration			Remarks
	FIC (PRE)	**FIC (Zn (II))**	**FIC_{index}**	
S. aureus NCTC 8325	0.25	1	1.25	Indifferent
E. coli NCTC10418	0	1	1	Additive
S. gordonii NCTC7865	0.125	0.125	0.250	Synergy
S. sanguinis NCTC 7863T	0.125	0.5	0.625	Additive
C. albicans ATCC 90028	0.0625	0.0625	0.125	Synergy
P. gingivalis W50	ND	ND	ND	ND
F. nucleatum ATCC 49256	ND	ND	ND	ND
S. anginosus NCTC 10713	0.5	0.25	0.75	Additive
S. mutans DSM 20523	0.5	0.5	1	Additive
S. salivarius DSM 20560	1	0.5	1.5	Indifferent

Table 4.5: Fractional inhibitory concentrations (FIC) of PRE and Zn (II) and FICindex values for tested strains. ND, not detected. FICindex was used to classify the combination of antimicrobial agents as synergistic when FICindexes were ≤ 0.5; additive when FIC >0.5 but < 1; indifferent when values were >1 and < 4 and antagonistic when values were ≥ 4.

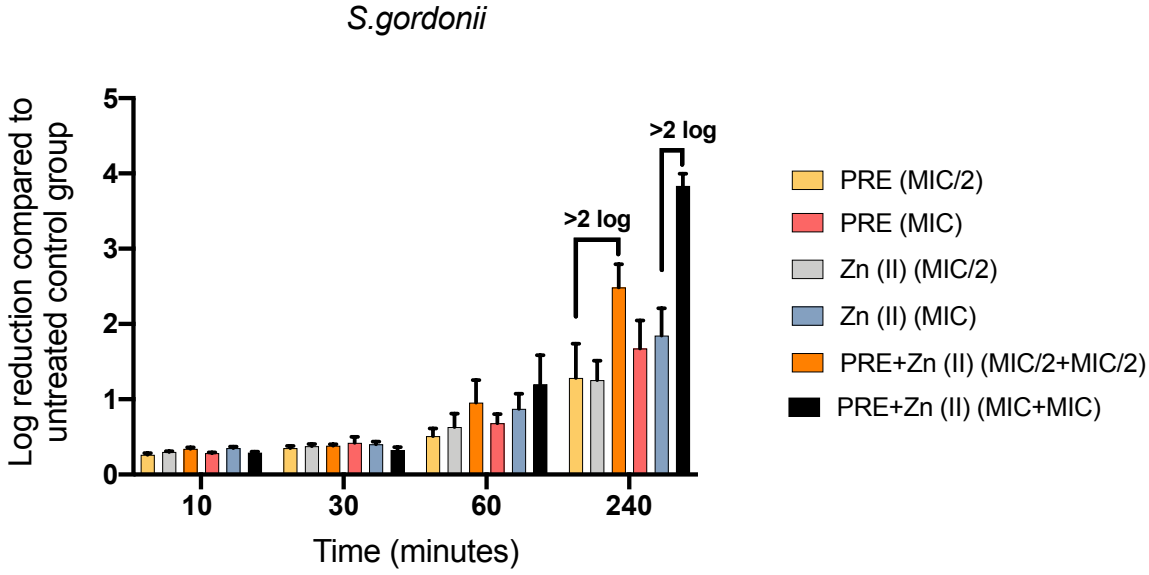
4.4.4 Time kill (log reduction) assay

The *in vitro* time kill kinetic assay was undertaken to assess the bactericidal activity and antimicrobial activity of PRE in combination with Zn (II). In addition to MIC values of compounds alone and their combinations, 0.5x the MIC values were chosen to assess the antimicrobial activity. Using MIC/2 of compounds any synergism between PRE and Zn (II), would be clearer, since, in the checkerboard assay (Figure 4.4). The *in vitro* time kill assay gave comparable results to the *in vitro* checkerboard assay. The log reduction values of compounds and combination were calculated by subtracting the log CFU/mL value of the PRE, Zn (II) and PRE in combination with Zn (II) from the control group. In the time kill assay, when the combination caused ≥2 log reduction in CFU/mL than the compounds in the combination showed the highest log reduction, this could be interpreted as synergistic activity (Weerakkody et al. 2011).

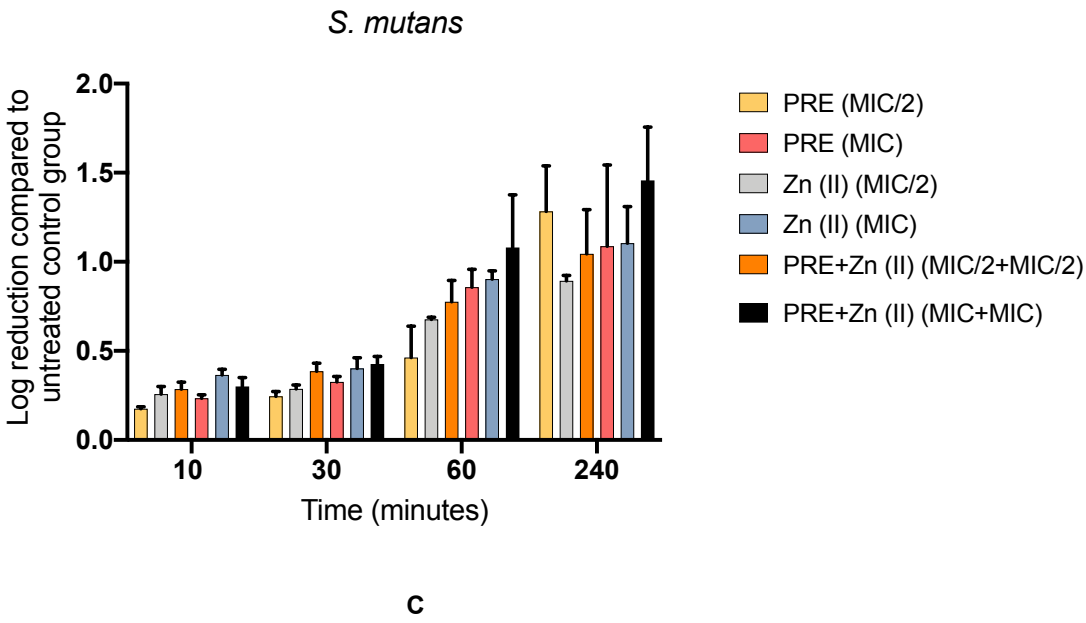
The log reduction assay was performed on *C. albicans, S. gordonii,* and *S. mutans,* where PRE/Zn (II) had shown the synergistic or additive antimicrobial activity in the checkerboard assay. PRE, Zn (II) and PRE/Zn (II)s showed dose- and time-dependent log reductions activity at specified time points. Both studied concentrations of PRE/Zn (II) showed synergistic microbicidal activity with ≥2 log reduction than PRE or Zn (II) combination's log reduction after 240 min. PRE/Zn (II) showed the only additive activity in checkerboard assay for *S. mutans*. Likewise, no synergistic activity was observed at any time points against *S. mutans* in the time kill assay.


C. albicans
Log reduction compared to untreated control group
Time (minutes)
10
30
60
240
>2 log
>2 log
PRE (MIC/2)
PRE (MIC)
Zn (II) (MIC/2)
Zn (II) (MIC)
PRE+Zn (II) (MIC/2+MIC/2)
PRE+Zn (II) (MIC+MIC)

A


S.gordonii
Log reduction compared to untreated control group
Time (minutes)
10
30
60
240
>2 log
>2 log
PRE (MIC/2)
PRE (MIC)
Zn (II) (MIC/2)
Zn (II) (MIC)
PRE+Zn (II) (MIC/2+MIC/2)
PRE+Zn (II) (MIC+MIC)

B

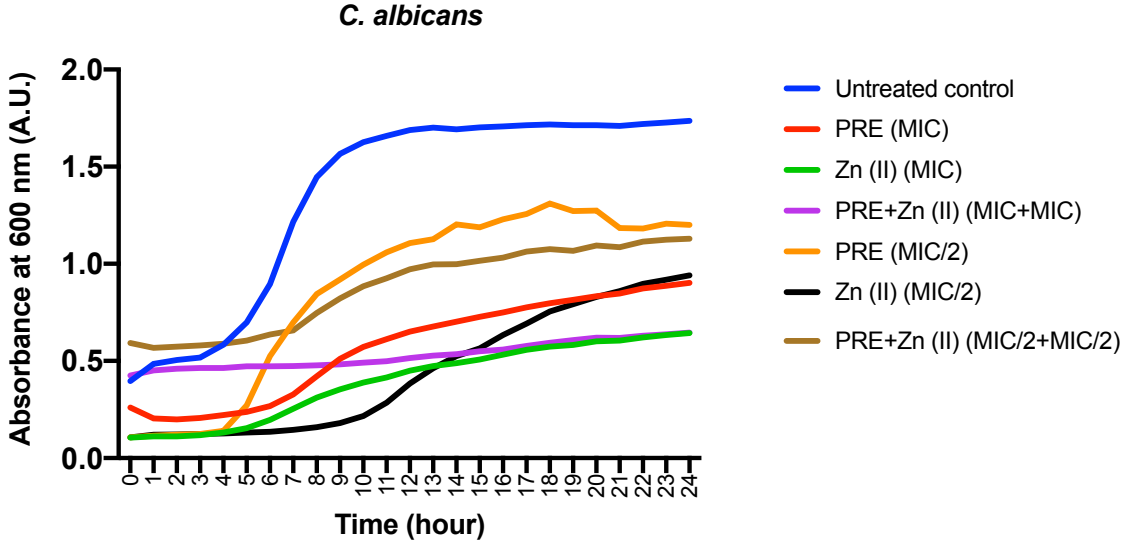


C

Figure 4.4: Time kill kinetic assays (A) *C. albicans*, (B) *S. gordonii* and (C) *S. mutans* at 10 min, 30 min, 60 min and 240 min at the concentrations of MIC and MIC/2 of PRE, MIC and MIC/2 of Zn (II) and the combination of PRE/Zn (II) with concentration MIC+MIC and MIC/2+MIC/2. Presented data are the result of three independent experiments and presented as mean value ± SEM.

4.4.5 Microbial growth curve with PRE, Zn (II) and PRE/Zn (II) treatment

Bacterial growth curves (Figure 4.5) showed an inhibition of microbial growth following treatment with PRE, Zn (II) and PRE/Zn (II)s, at MIC and MIC/2 concentrations, in comparison to untreated controls. Compounds showed a dose-dependent inhibition for all studied microbial growth curves, compared to untreated microbes. PRE/Zn (II) in both MIC and MIC/2 concentrations resulted in prolonged inhibition of *C. albicans* growth. However, the PRE/Zn (II) exerted a changeable growth pattern on *S. mutans* growth when applied at MIC concentrations exhibiting a more biphasic pattern. Combination caused regrowth between 12 h to 20 h, although this pattern changed to growth inhibition after 20 h. In addition, PRE showed *S. gordonii* regrowth after 20 h especially at MIC/2 concentration. Zn (II) inhibited microbial growth in a dose-dependent manner for C. *albicans* and *S. mutans* and caused greater inhibition than the PRE/Zn (II) for *S. mutans* and *S. gordonii*. Overall, all compounds inhibited microbial growth compared to untreated controls after 24 h.



A

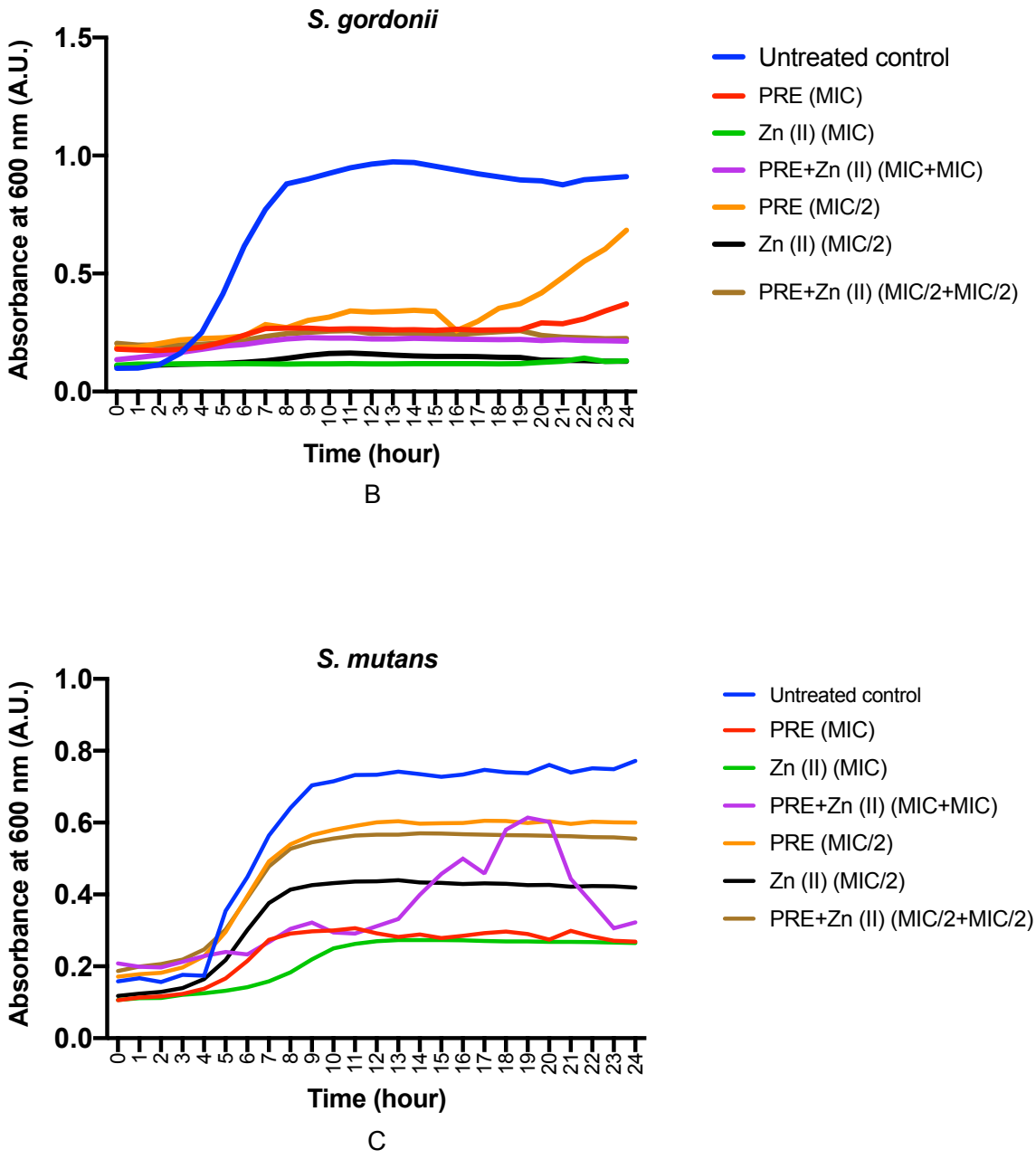
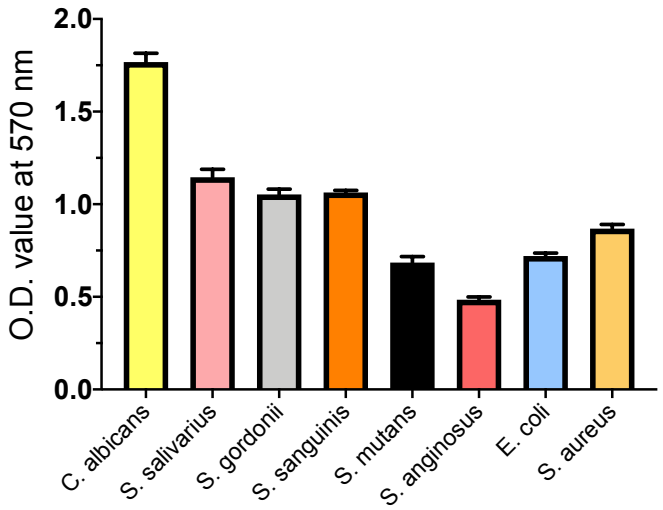


Figure 4.5: Bacterial/fungal growth curves for (A) *C. albicans*, (B) *S. gordonii* and (C) *S. mutans* in the presence of PRE (MIC and MIC/2), Zn (II) (MIC and MIC/2) and PRE in combination with Zn (II) (MIC+MIC and MIC/2+MIC/2) over 24 h (N=3).

4.4.6 Biofilm formation of studied microbes

The potential of studied microbes to adhere and maintain the viability on microtitre plates was evaluated and used to understand the biofilm formation ability of these microbes under *in vitro* conditions. The biofilm mass was quantified for the 24 h biofilms of each bacterial strain and *C. albicans* by staining the attached microbe with crystal violet (Figure 4.6A). All studied microbes showed an ability to form a consistent biofilm. The statistical differences between O.D. values of crystal violet-stained microbes is shown in Figure 4.6B. The biofilm formation ability between studied microbes showed differences probably due to their characteristic features. *C. albicans* exerted the significantly highest O.D. value that mean higher biomass of stained with crystal violet than other microbes ($p<0.0001$). *S. salivarius, S. gordonii* and *S. sanguinis* presented similar biofilm formation abilities according to their O.D. values obtained from crystal violet-stained wells of each microbe ($p>0.5$). *S. mutans* and *S. anginosus* showed statistically lower O.D. values compared to *S. salivarius, S. gordonii* and *S. sanguinis* ($p<0.0001$).

A)



B)

Microbial strains	*S. aureus*	*E. coli*	*S. gordonii*	*S. sanguinis*	*C. albicans*	*S. anginosus*	*S. mutans*	*S. salivarius*
S. aureus			***	****	****	****	***	****
E. coli			**	**	****			***
S. gordonii	***	**			****	****	****	
S. sanguinis	****	**			****	****	****	

Figure 4.6: Comparison of biofilm formation capacity between microbes in MH broth after 24 h in microtitre plates, using the crystal violet staining assay. (A) Crystal violet staining optical density values. Data are expressed as the mean±SEM. N=4. (B) Multiple comparison analysis of significant changes in biofilm formation capacity of studied microbes. Significant differences indicated by *, where **p<0.01, ***p<0.001, and ****p<0.0001 between studied microbes. Blue boxes represent no significant difference between indicated group.

4.4.7 Biofilm formation inhibition and biofilm eradication activity

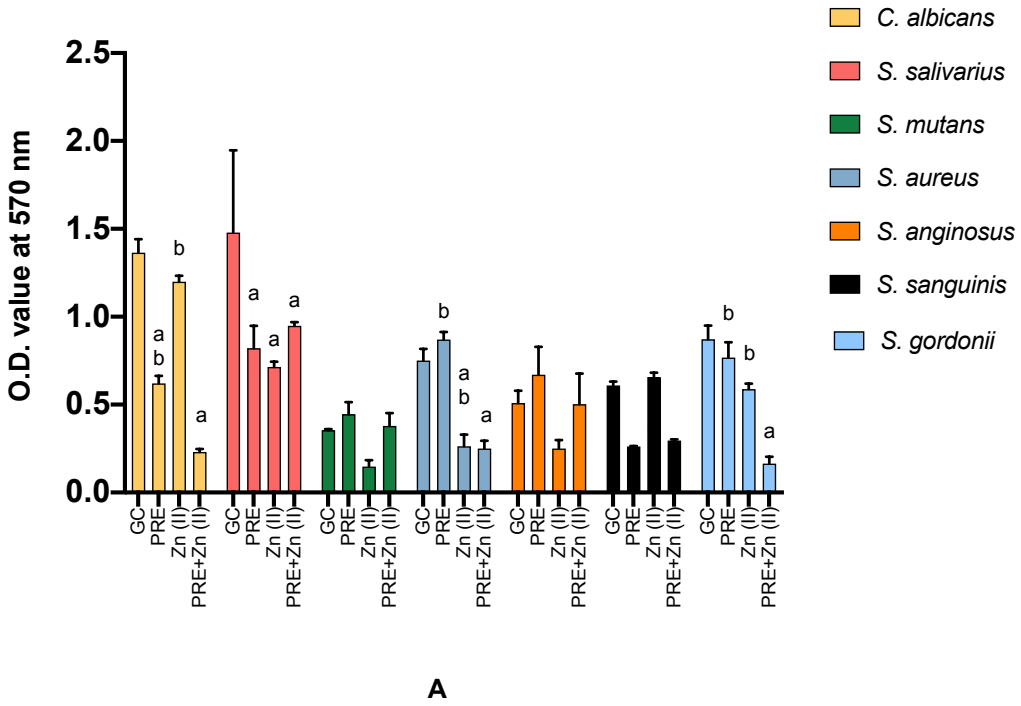
The effects of PRE, Zn (II) and PRE/Zn (II)s on biofilm formation (Figure 4.7) and pre-formed biofilms (Figure 4.8) were studied by using an *in vitro* crystal violet assay in a microtitre plate. In the assay, MICs and half of the MICs of PRE, and Zn (II) were used alone and in combination.

For *S. mutans*, PRE, Zn (II) and PRE/Zn (II) did not show statistically significant inhibition of biofilm formation at both MIC and MIC/2 values ($p>0.05$). Similarly, PRE, Zn (II) and PRE/Zn (II) at MIC/2 concentrations did not affect pre-formed *S. mutans* biofilms ($p>0.05$). However, PRE, Zn (II) and PRE/Zn (II) significantly eradicated pre-formed biofilms of *S. mutans* ($p<0.01$), when applied at MIC concentrations. PRE/Zn (II) did not exert any potentiated effect in terms of the eradication of *S. mutans* biofilms ($p>0.05$).

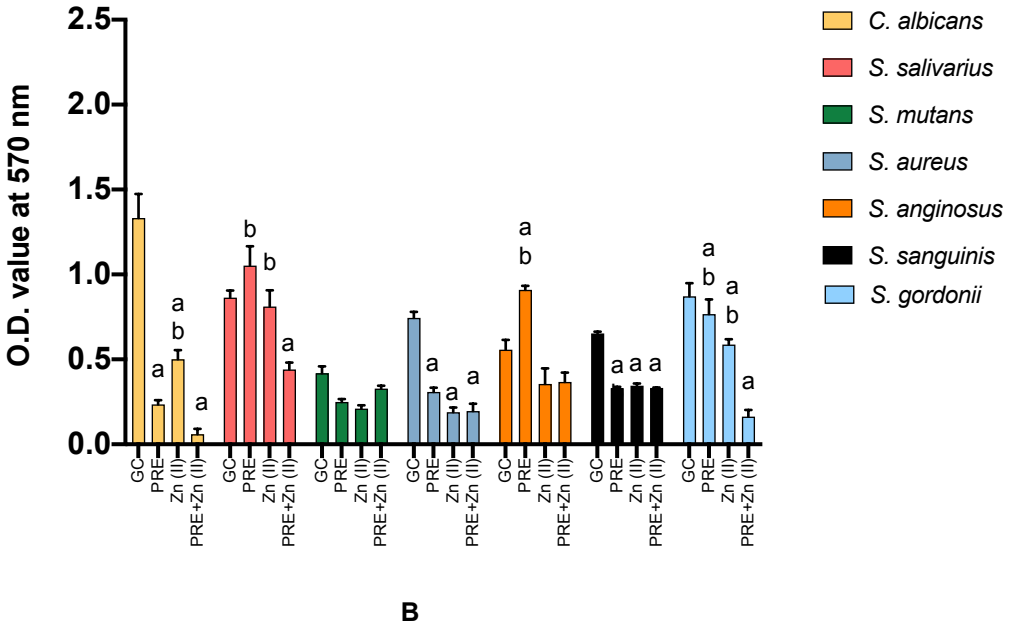
For *C. albicans,* the inhibition of biofilm formation was observed at all studied concentrations of PRE, Zn (II) and PRE/Zn (II). Moreover, PRE/Zn (II) combination (MIC/2+MIC/2) showed a significantly enhanced inhibition of biofilm formation, compared to both the control and PRE or Zn (II) alone ($p<0.01$). PRE/Zn (II) significantly inhibited biofilm formation compared to GC and Zn (II) alone at MIC concentration. Although PRE/Zn (II) exerted a better inhibition in biofilm formation, this effect was not statistically significant compared to PRE alone at MIC concentration. PRE, Zn (II) and PRE/Zn (II) did not show significant eradication on the *C. albicans* biofilm at both MIC and MIC/2 concentrations ($p>0.05$).

PRE and PRE/Zn (II) significantly inhibited *S. sanguinis* biofilm formation, compared to untreated controls ($p<0.01$). Zn (II) alone did not affect either biofilm formation or eradication of *S. sanguinis* at MIC/2 and MIC concentrations ($p>0.05$). PRE and PRE/Zn (II) significantly eradicated pre-formed *S. sanguinis* biofilm ($p<0.01$). However, PRE/Zn (II) did not show any synergistic activity in biofilm formation inhibition and biofilm eradication of *S. sanguinis* in this assay ($p>0.05$).

A potentiated biofilm formation inhibition and pre-formed biofilm eradication activity was observed on *S. gordonii*. Both MIC and MIC/2 values of PRE/ Zn (II) combination significantly decreased biofilm formation of *S. gordonii*, in comparison to both untreated controls and the application of PRE or Zn (II) alone. Similar effects were observed on preformed biofilm of *S. gordonii*. While PRE (MIC/2) and Zn (II) (MIC/2) alone did not show any significant inhibition of biofilm formation and pre-formed biofilm ($p>0.05$), the PRE/Zn (II) (MIC/2+MIC/2) combination showed increased biofilm inhibition and eradication activity ($p<0.01$).



A



B

Figure 4.7: Comparison of biofilm inhibition for (A) MIC/2 and (B) MIC values of PRE, Zn (II) and PRE in combination with Zn (II) on biofilm formation capacity for indicated microbes in MH broth after 24 h in microtitre plates, using the crystal violet staining assay. “a” represents significant difference between treatment compounds and growth control group and “b” represents significant difference between Treatment compounds ($p<0.01$). Data are expressed as mean±SEM. N=3.

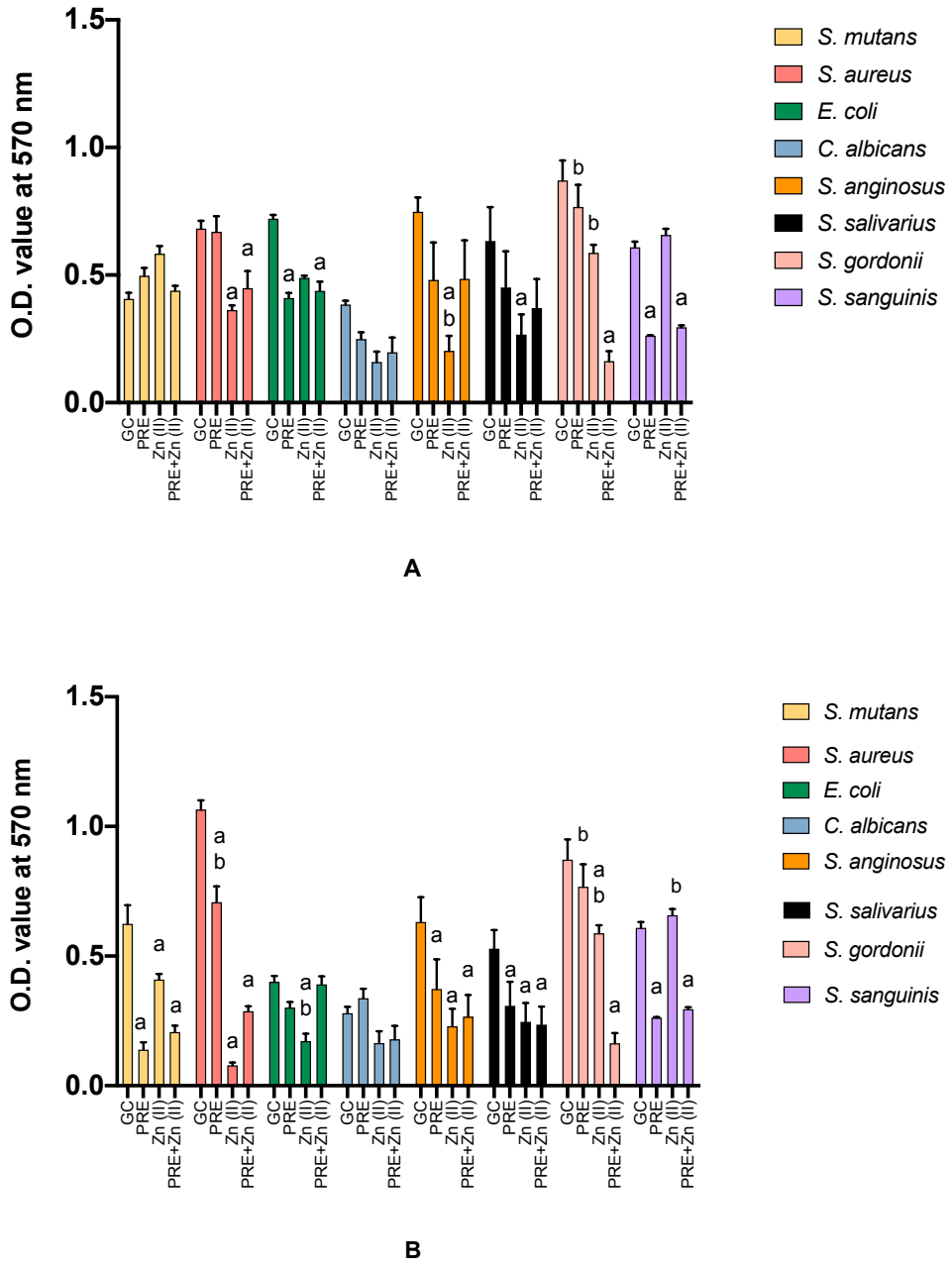


Figure 4.8: Comparison of biofilm eradication for (A) MIC/2 and (B) MIC values of PRE, Zn (II) and PRE in combination with Zn (II) for indicated microbes in MH broth after 24 h in microtiter plates, using the crystal violet staining assay. “a” represents the significant difference between treatment compounds and growth control group and “b” represents significant difference between combination of compounds and their alone usage ($p<0.01$). Data are expressed as mean±SEM. N=3.

4.4.8 Biofilm eradication as assessed by CLSM with live/dead bacterial viability assay

In this assay, the BacLight Live/Dead Bacterial Viability Kit was used to analyse *S. mutans, S. gordonii, S. sanguinis, C. albicans* and *P. gingivalis* biofilm structures under the indicated treatment conditions with PRE (MIC), Zn (II) (MIC) and PRE/Zn (II) (MIC+MIC). Thick and compact biofilms were obtained in the growth controls for each studied bacterial species, and it was observed that biofilms treated with MIC concentrations of the treatment compounds were diminished compared to untreated controls. Quantitative analysis of biofilms using COMSTAT2 with ImageJ allowed different parameters to be analysed to evaluate the biofilm structure from each studied species. The results were shown as CLSM images, using IMARIS software (v4.1.3, Bitplane AG, Zurich, Switzerland) and COMSTAT2 analysis for biofilm mass, mean thickness of entire biofilm, roughness coefficient and dead:live ratio.

MIC values of compounds were used for each studied strain, as previously indicated in Table 4.4. For *P. gingivalis*, MIC values could not be determined because of the precipitation with required broths, BHI and FAB. In this assay, PRE 1 mg/mL was used to evaluate the effect of PRE and PRE in combination with Zn (II) on *P. gingivalis* biofilm. An absorbance-based method was performed to find the minimum biofilm eradication concentration, but according to this assay results suggested that higher concentrations of PRE (>50 mg/mL) and Zn (II) (>50 mM) were needed to eradicate the biofilm (data not shown). However, there were some problems with absorbance-based methods. For example, it was not possible to distinguish between live and dead cells in the absorbance-based assay. For this reason, MIC values of compounds were chosen, as it has been known that microbes are more resistant to antimicrobials in the biofilm condition and subsequently require higher concentration of antimicrobials for inhibition. The MIC value could be considered as a suitable concentration to use in this assay as it is a sufficient concentration to inhibit the microbe in planktonic condition and effect biofilm structure.

PRE and PRE/Zn (II) significantly decreased the biomass of *S. sanguinis* biofilms, compared to untreated controls ($p<0.0001$, Figure 4.9). However, while Zn (II) caused a decrease in the biomass, it did not show a significant reduction in compared with

untreated controls ($p>0.05$). The same effect was also observed for the roughness coefficient parameter. PRE and PRE/Zn (II) caused an increase in roughness coefficient, which has been accepted to reflect heterogeneity in the biofilm structure. However, there was no significant difference between untreated and Zn (II)-treated biofilms of *S. sanguinis* ($p>0.05$). The heterogeneity in the biofilm structure could be observed in images obtained using the IMARIS image program (v4.1.3, Bitplane AG, Zurich, Switzerland). Similarly, all compounds caused a significant decrease in mean biofilm thickness that could be observed from images (Figure 4.9B). PRE and Zn (II) greater dead/live ratio compared to both untreated growth control and PRE/Zn (II). It was interesting that PRE/Zn (II) exerted similar dead/live bacterial ratio compared to untreated growth control and there was not significant difference ($p>0.05$) (Figure 4.9).

The effects of PRE, Zn (II) and PRE/Zn (II) on 24 h *S. gordonii* biofilms was also assessed Figure 4.10. PRE- and PRE/Zn (II)-treated biofilm biomass levels were significantly lower than untreated controls ($p<0.0001$). Only Zn (II) treatment did not cause a significant inhibition in biomass ($p>0.05$). Again, PRE and PRE/Zn (II) treated biofilms showed a greater roughness coefficient in biofilm structure ($p<0.01$ and $p<0.001$ for PRE and PRE/Zn (II), respectively). However, there was no significant difference between untreated control biofilms and Zn (II)- treated biofilms ($p>0.05$). Regarding the mean thickness of the biomass, all treatment groups caused a significant reduction ($p<0.0001$ for PRE and PRE/Zn (II) and $p<0.01$ for Zn (II); and the PRE/Zn (II) showed more inhibition than Zn (II) alone ($p<0.01$). PRE and Zn (II) greater dead/live ratio compared to untreated growth control. Although PRE showed higher dead/live ratio than PRE/Zn (II), there was not a significant difference ($p>0.05$). However, Zn (II) alone showed a greater dead/live ratio compared to PRE/Zn (II) ($p<0.01$) (Figure 4.10).

S. mutans produced an intact biofilm in untreated controls as evident by CLSM stained with Live/Dead stain (Figure 4.11A). All compounds diminished the pre-formed biofilms at their MIC values and combinations by causing disruption in biofilm structure, as shown in Figure 4.11C. PRE and PRE/Zn (II) treated biofilms of *S. mutans* showed a significantly lower biomass, when compared to untreated controls ($p<0.0001$). Zn (II) also significantly reduced the biomass of the biofilm ($p<0.01$), but

this reduction was less than PRE and PRE/Zn (II) treatments. Moreover, the PRE/Zn (II) effect on biomass of biofilm was significantly greater than the Zn (II) alone treatment ($p<0.05$). Similar effects on biofilm were observed for other parameters used to compare the biofilm structure. Again, PRE and PRE/Zn (II) significantly increased the roughness coefficient, compared to untreated controls ($p<0.0001$) and Zn (II) treatment ($p<0.05$). However, Zn (II) alone treatment did not show any statistically significant difference, with untreated controls ($p>0.05$). For the mean thickness of biomass, PRE/Zn (II) showed the greatest reductions, compared to untreated controls ($p<0.001$). PRE alone also showed a decrease in mean thickness of biomass ($p<0.05$), but not as much as PRE/Zn (II) treatment. While Zn (II) treatment reduced the mean thickness of biomass, it was not statistically significant compared to untreated control group ($p>0.05$, Figure 4.11). PRE/Zn (II) exerted a significantly higher dead/live ratio compared to untreated growth control ($p<0.05$) and Zn (II) alone $p<0.01$). PRE, Zn (II) and untreated growth control exerted similar dead/live ratios and there was not a statistically significant difference between them ($p>0.05$) (Figure 4.11).

C. albicans biofilms showed that after 24 h post-treatment, studied compounds changed the *C. albicans* biofilm structure (Figure 4.12). All treatment groups significantly reduced the biofilm biomass, compared with untreated controls (all $p<0.0001$, Figure 4.12C). PRE caused the greatest decrease in mean biofilm thickness ($p<0.001$), but both Zn (II) or the PRE/Zn (II) led to a significant decrease in mean thickness of biomass (Figure 4.12B and C). However, while PRE increased the roughness coefficient, it was not statistically significant ($p>0.05$). Zn (II) showed a higher dead/live ratio, but it was not statistically significant ($p>0.05$) Similarly, other treatment groups and untreated growth control showed similar dead/live ratio without any statistically significant difference ($p>0.05$). (Figure 4.12C).

Application of PRE, Zn (II) and PRE/Zn (II) on *P. gingivalis* biofilms showed that after 48 h incubation under anaerobic conditions, *P. gingivalis* formed a moderate biofilm. *P. gingivalis* biofilms treated with PRE, Zn (II) and PRE/Zn (II) disrupted the biofilms and caused cell death, especially those treated with PRE and the PRE/Zn (II), as shown in Figure 4.13 A and B. PRE and PRE/Zn (II) also significantly decreased the biomass of biofilm ($p<0.001$), while Zn (II) alone did not cause significant reduction in biomass compared to untreated controls. Both the roughness coefficient

and mean thickness of biofilm levels were not affected by studied compounds and their combinations ($p>0.05$). However, all treatments caused significantly higher dead/live ratio, as presented in Figure 4.13C ($p<0.05$).

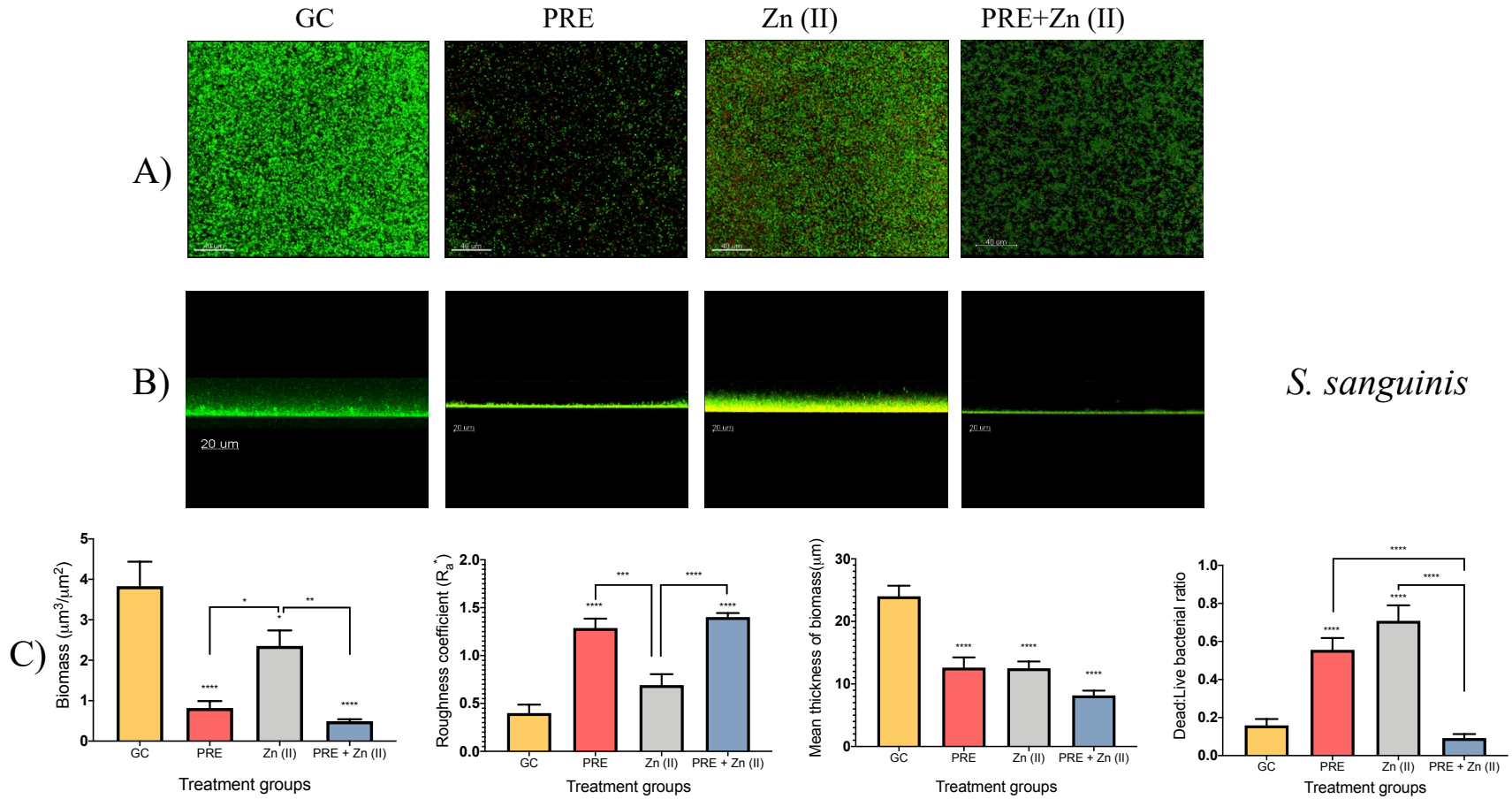


Figure 4.9: Biofilm eradication assay for *S. sanguinis* showing Live/Dead (green/red, respectively) stained confocal laser scanning microscopy (CLSM) images (A and B). PRE, Zn (II) and PRE/Zn (II) were used at their MIC values, both alone and in combination. Corresponding COMSTAT2 analysis (C) of the images shown as mean ± SEM, N=3. Significant indicated by *, where *$p<0.05$, **$p<0.01$, ***$p<0.001$, and ****$p<0.0001$ compared to untreated growth control group (indicated on the bars) and compared to between treatment groups (indicated with lines).

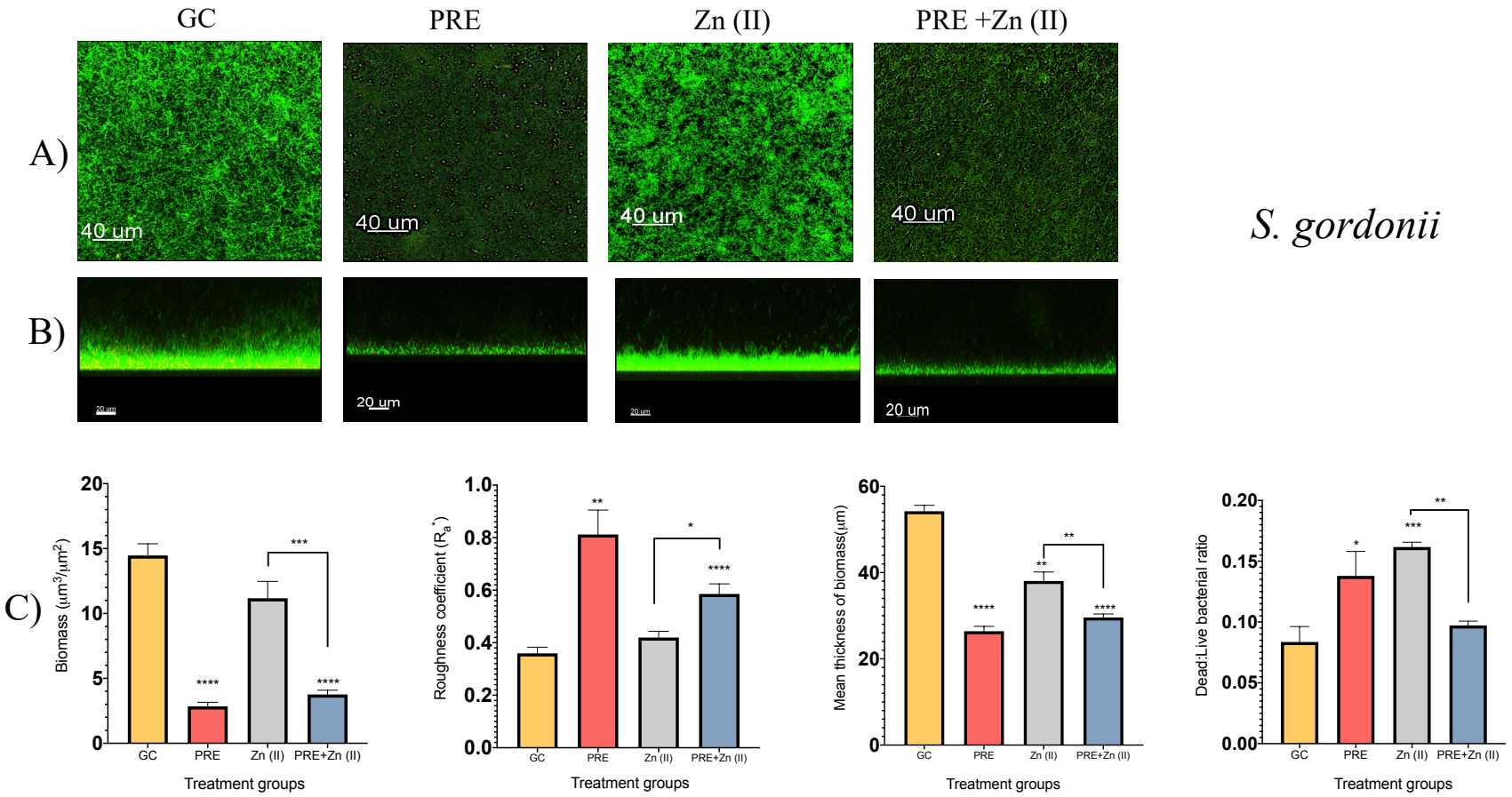


Figure 4.10: Biofilm eradication assay for *S. gordonii* showing live/dead (green/red, respectively) stained confocal laser scanning microscopy (CLSM) images (A and B). PRE, Zn (II) and PRE/Zn (II) were used at their MIC values, both alone and in combination. Corresponding COMSTAT2 analysis (C) of the images is shown mean ± SEM, N=3. Significant indicated by *, where *$p<0.05$, **$p<0.01$, ***$p<0.001$, and ****$p<0.0001$ compared to untreated growth control group (indicated on the bars) and compared to between treatment groups (indicated with lines).

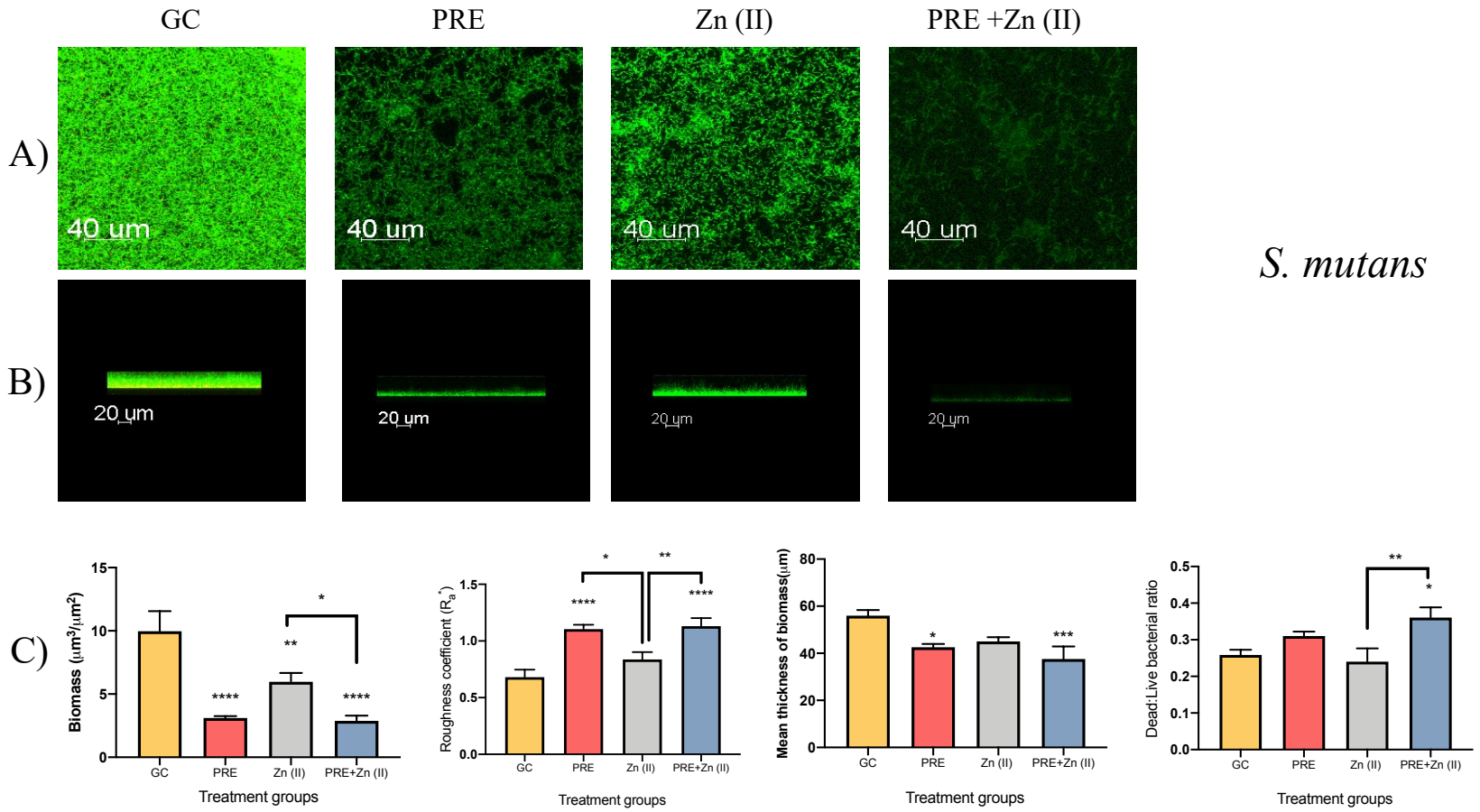


Figure 4.11: Biofilm eradication assay for *S. mutans* showing live/dead (green/red, respectively) stained confocal laser scanning microscopy (CLSM) images (A and B). PRE, Zn (II) and PRE/Zn (II) were used at their MIC values, both alone and in combination. Corresponding COMSTAT2 analysis (C) of the images is shown mean ± SEM, N=3. Significant indicated by *, where *$p<0.05$, **$p<0.01$, ***$p<0.001$, and ****$p<0.0001$ compared to untreated growth control group (indicated on the bars) and compared to between treatment groups (indicated with lines).

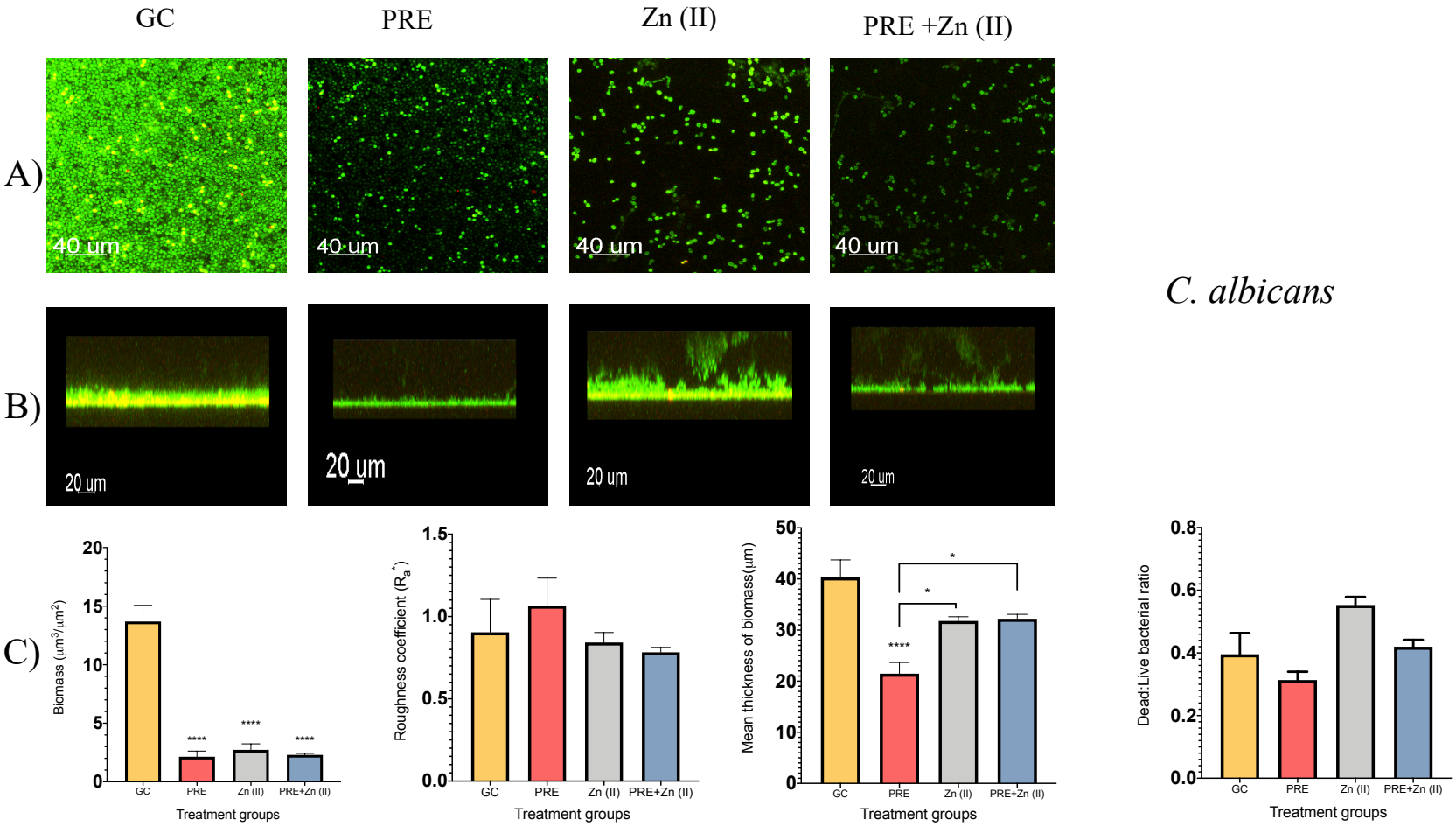


Figure 4.12: Biofilm eradication assay for *C. albicans* showing live/dead (green/red, respectively) stained confocal laser scanning microscopy (CLSM) images (A and B). PRE, Zn (II) and PRE/Zn (II) were used at their MIC values, both alone and in combination. Corresponding COMSTAT2 analysis (C) of the images is shown mean ± SEM, N=3. Significant indicated by *, where *$p<0.05$, **$p<0.01$, ***$p<0.001$, and ****$p<0.0001$ compared to untreated growth control group (indicated on the bars) and compared to between treatment groups (indicated with lines).

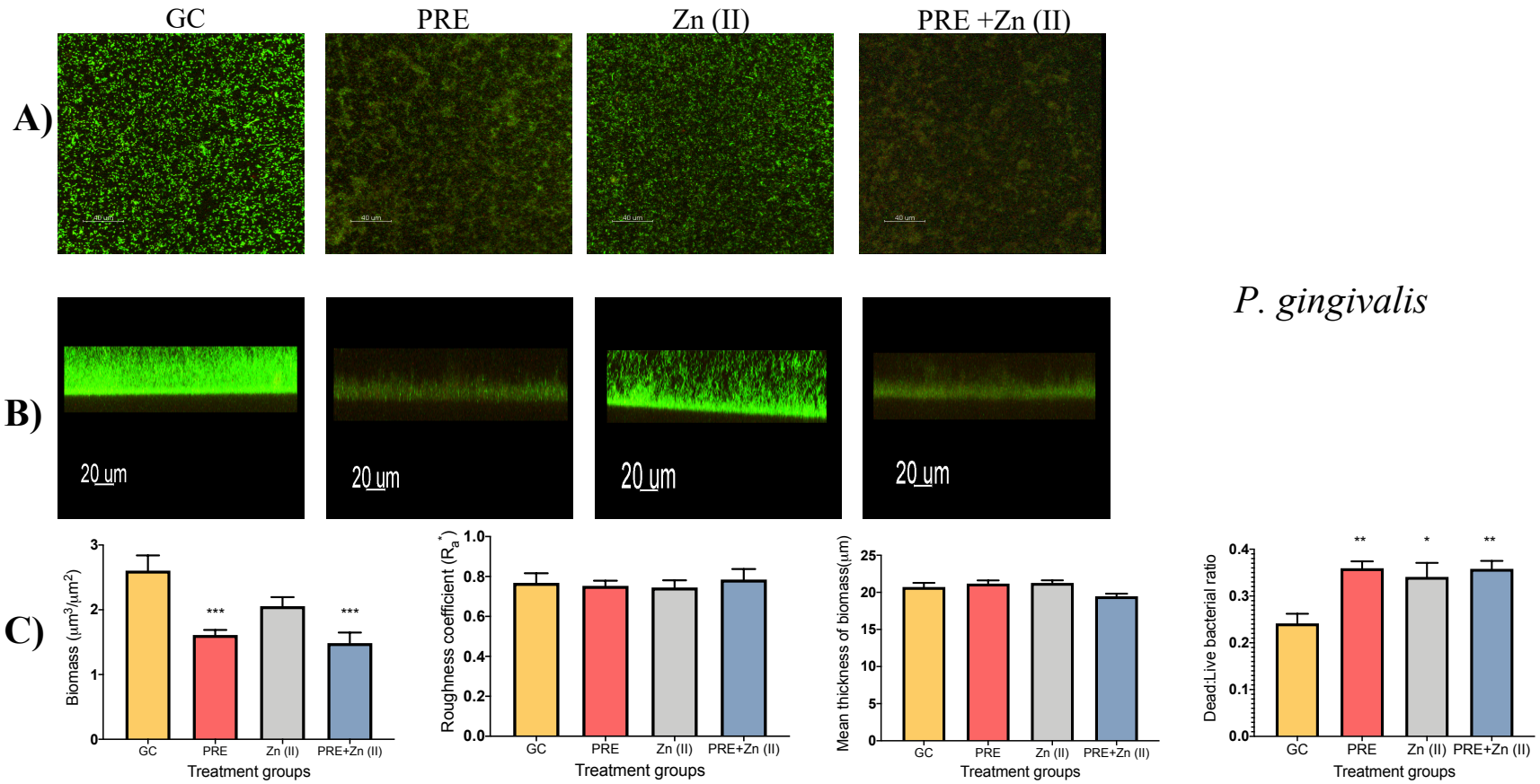


Figure 4.13: Biofilm eradication assay for *P. gingivalis* showing Live/Dead (green/red, respectively) stained confocal laser scanning microscopy (CLSM) images (A and B). PRE, Zn (II) and PRE/Zn (II) were used at their MIC values, both alone and in combination. Corresponding COMSTAT2 analysis (C) of the images is shown mean ± SEM, N=3. Significant indicated by *, where *$p<0.05$, **$p<0.01$, ***$p<0.001$, and ****$p<0.0001$ compared to untreated growth control group (indicated on the bars) and compared to between treatment groups (indicated with lines).

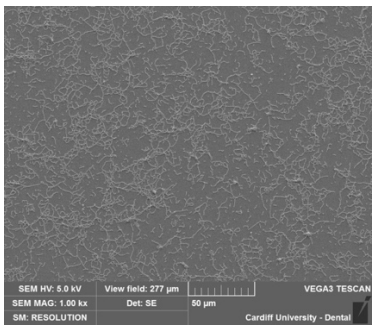
4.4.9 Scanning electron microscopy (SEM)

SEM was used to evaluate the effects of PRE, Zn (II) and PRE/Zn (II) on the phenotypic characteristics of single-species biofilms for selected microorganisms. Biofilms formed on glass cover slips were examined after processing samples by freeze-drying. Figures 4.14, 4.15 and 4.16 are representative scanning electron micrographs for *S. mutans*, *S. gordonii* and *C. albicans*, respectively.

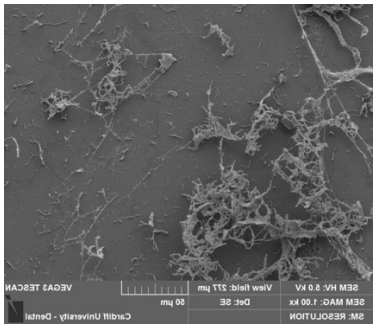
SEM showed that *S. mutans* biofilm under the treatment of PRE exerted an aggregating effect on biofilm, when compared to untreated controls. This effect was not observed with Zn (II)-treated *S. mutans* biofilms, which exhibited a more homogenously dispersed biofilm on the glass coverslip, similar to untreated biofilm, but to a lesser extent. PRE/Zn (II) showed the similar aggregating effect, which could be associated with the presence of PRE in the combination, rather than Zn (II) (Figure 4.14A and B). Similar results were observed for *S. gordonii* biofilms (Figure 4.15). However, *S. gordonii* formed denser biofilms on the glass coverslip than *S. mutans.* PRE-, Zn (II)- and PRE/Zn (II)- treated *S. gordonii* biofilm had an inhibitory effect on the biofilm, compared to untreated controls (Figure 4.15A and B). Chlorhexidine di-gluconate (0.2 %), was used as a positive control and showed similar clumping effects on *S. mutans* and *S. gordonii* biofilms. PRE and PRE/Zn (II) treatments also caused a change in *C. albicans* surface membranes and again showed this clumping effect (Figure 4.16A and B).

S. mutans

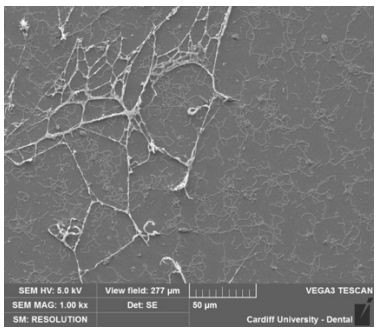
Growth control



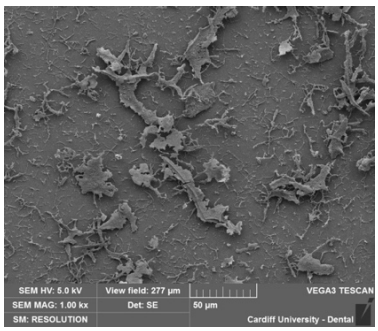
PRE



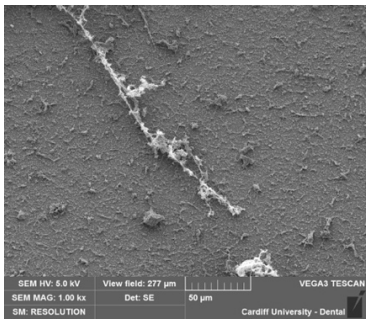
Zn (II)



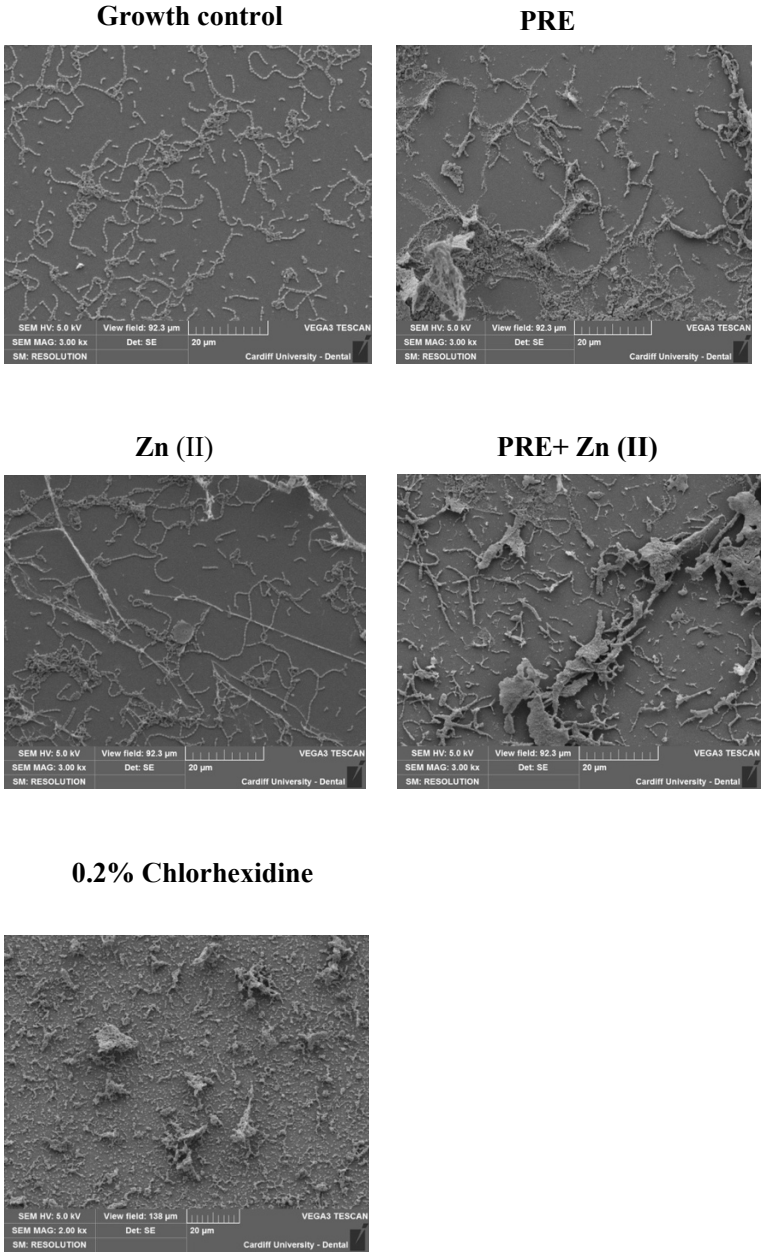
PRE+ Zn (II)



0.2% Chlorhexidine



A

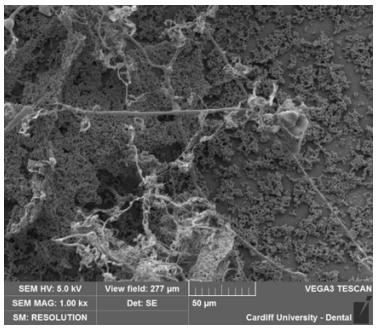


B

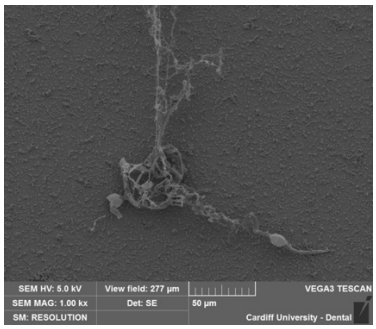
Figure 4.14: Structural characteristics of *S. mutans* biofilms on glass coverslips treated with PRE (MIC), Zn (II) (MIC) and PRE/Zn (II) (MIC+MIC), 0.2% chlorhexidine gluconate (positive control) or without these compounds (untreated, negative control). Representative scanning electron micrographs scale bars were (A) 50 µm and (B) 20 µm for each treatment condition.

S. gordonii

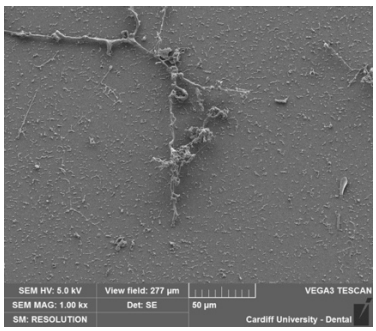
Growth control



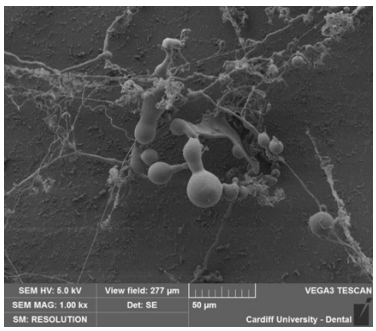
PRE



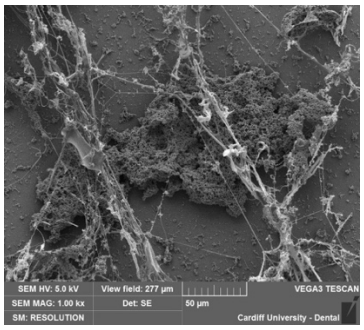
Zn (II)



PRE+ Zn (II)

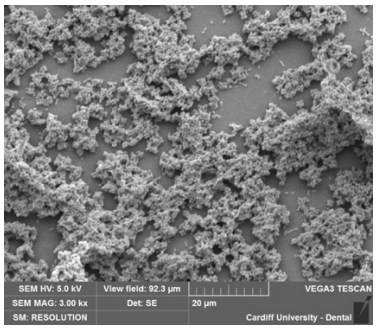


0.2% Chlorhexidine

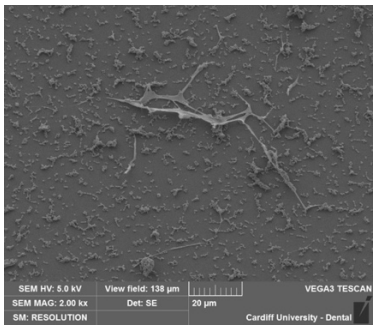


A

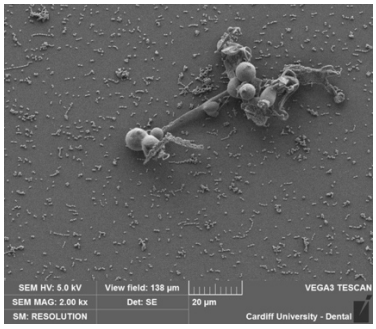
Growth control



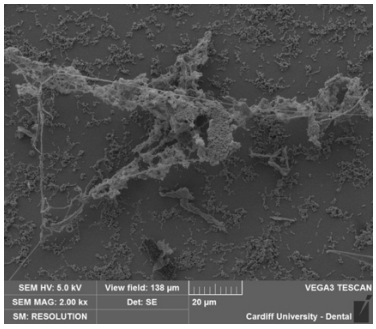
PRE



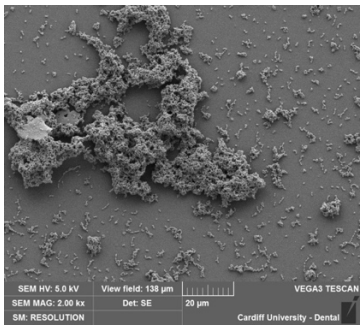
Zn (II)



PRE+ Zn (II)



0.2% Chlorhexidine

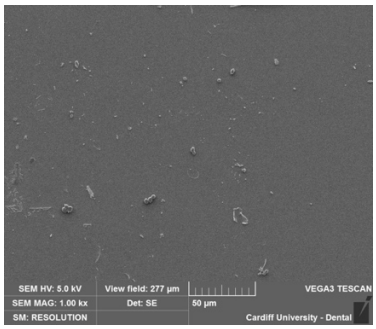


B

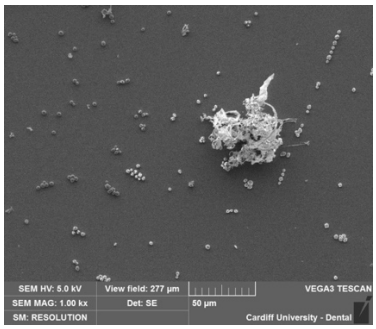
Figure 4.15: Structural characteristics of *S. gordonii* biofilms on glass coverslips treated with PRE (MIC), Zn (II) (MIC) and PRE/Zn (II) (MIC+MIC), 0.2% chlorhexidine gluconate (positive control) or without these compounds (untreated negative control). Representative scanning electron micrographs scale bars were (A) 50 µm and (B) 20 µm for each treatment condition.

C. albicans

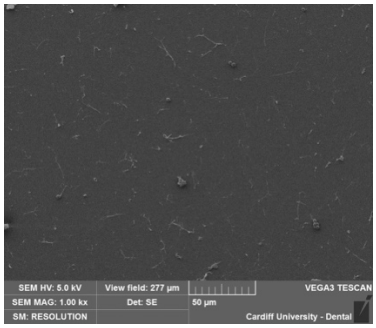
Growth control



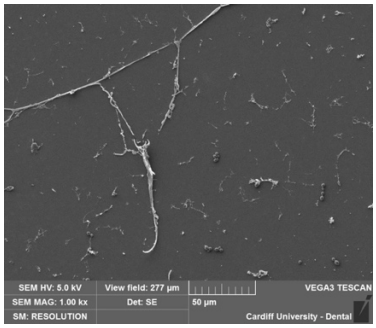
PRE



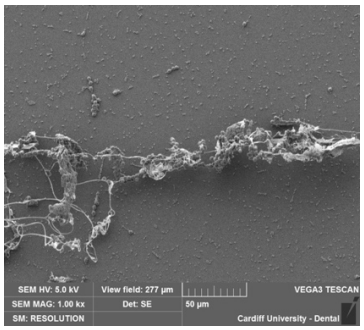
Zn (II)



PRE+ Zn (II)

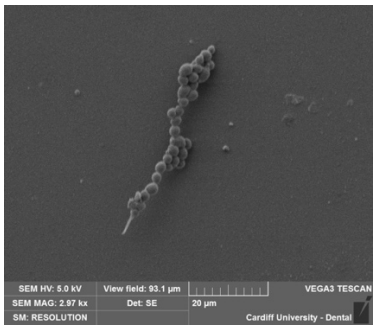


0.2% Chlorhexidine

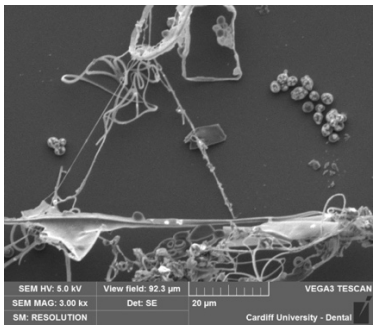


A

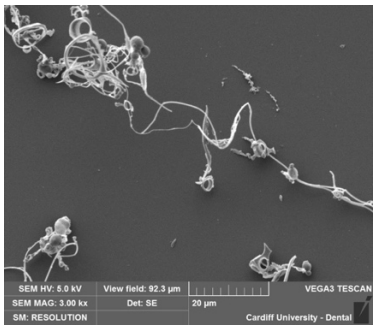
Growth control



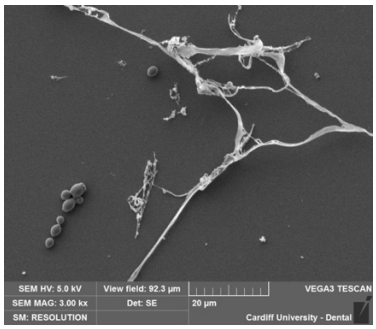
PRE



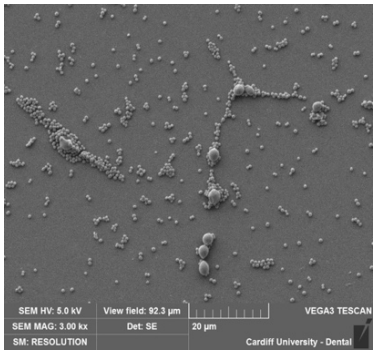
Zn (II)



PRE+ Zn (II)



0.2% Chlorhexidine



B

Figure 4.16: Structural characteristics of *C. albicans* biofilms on glass coverslips treated with PRE (MIC), Zn (II) (MIC) and PRE/Zn (II) (MIC+MIC), 0.2% chlorhexidine gluconate (positive control) or without these compounds (untreated-negative control). Representative scanning electron micrographs scale bars were (A) 50 µm and (B) 20 µm for each treatment condition.

4.5 Discussion

The antimicrobial activity of pomegranate extracts has been studied extensively against a range of Gram-positive, Gram-negative bacteria and yeasts (Negi and Jayaprakasha 2003; Heber et al. 2006; Yehia et al. 2011). Studies showed that pomegranate extracts were abundant with phenolic compounds, especially hydrolysable tannins. Moreover, these phenolic compounds were associated with antimicrobial activity of pomegranate extracts against various microbes (Kanatt et al. 2010; Miguel et al. 2010; Celiksoy and Heard 2021).

The primary aim of this research was to assess the antimicrobial activity of PRE and Zn (II) alone and the potential synergistic antimicrobial activity for PRE/Zn (II) against oral bacteria and *C. albicans*. *E. coli* and *S. aureus* were included in this study to compare our results with previous studies, as the antimicrobial activity of pomegranate peel extract has previously been studied extensively against *E. coli* and *S. aureus* (Braga et al. 2005; McCarrell et al. 2008; Al-Zoreky 2009; Pagliarulo et al. 2016). In this study, PRE showed an antimicrobial activity against *S. aureus,* but was inactive against *E. coli* even at 50 mg/mL. PRE showed a zone of inhibition in the agar well-diffusion assay, and the MIC of PRE against *S. aureus* was determined to be 2 mg/mL. Earlier studies revealed varying results, in terms of the MIC values and zones of inhibition reported for pomegranate extracts. Khan and Hanee (2011) indicated comparatively less antimicrobial activity against *S. aureus* and *E. coli*, than other studied bacteria using a zone of inhibition assay. In the study of Pagliarulo et al. (2016), a clear zone inhibition was observed against both *E. coli* and *S. aureus*, between 15- 30 mm for various hydroalcoholic extracts from pomegranate fruit, including crude and purified peel extract. Meléndez and Capriles (2006) tested the antimicrobial properties of several tropical plants from Puerto Rico, using the disc diffusion against *E. coli* and *S. aureus*. They demonstrated that pomegranate extract produced zone of inhibition sizes of 11 mm and 20 mm for *E. coli* and *S. aureus*, respectively. Mathabe et al. (2006) found that methanol, ethanol, acetone and water extracts obtained from pomegranate exhibited antimicrobial activity against a range of bacteria, including *S. aureus* and *E. coli*. However, Kanatt et al. (2010) found that

pomegranate peel extract was not effective against *E. coli* and a similar extraction method and solvent was used in both the Kanatt et al. (2010) study and the current research.

MIC values against *S. aureus* have been reported to range from 0.62 mg/mL to >250 mg/mL (Prashanth et al. 2001; Machado et al. 2003; McCarrell et al. 2008). The variability in MIC is not surprising, since it is typically observed even with conventional antimicrobials against all clinical isolates (EUCAST data 2013, available at: http://www.eucast.org/), though generally within a more restricted range. These differences in antimicrobial activity and efficacy of pomegranate peel extract or PRE could be associated with different factors. The important factors are the composition of the extract and harvesting time, geographical location of the plant, age of plant, growth stage, drying and extraction technique (Galvão et al. 2012). In addition, the studied bacterial strains could have different sensitivities to plant extracts. Moreover, while standard protocols have been used in laboratories to find MIC values of antimicrobial compounds, there are some potential factors such as duration of measurement and density of inoculum that could cause variation in MIC values. All the above-mentioned reasons may contribute to the variability in obtained concentrations of antimicrobial activity and should be taken into consideration when results are compared from different studies (Holland et al. 2014).

PRE and Zn (II) were assessed for their antimicrobial activity using the agar well diffusion assay against selected oral microbes. All strains were susceptible to Zn (II) by showing clear zones of inhibition and the MIC values of Zn (II) varied between 0.39 and6.25 mM. PRE caused zones of inhibition in all studied streptococcus strains and *C. albicans,* but it did not show any zone of inhibition for *E. coli*. This variation in antimicrobial activity may reflect differences in cell surface structures between Gram-negative and Gram-positive bacteria. Gram-negative bacteria have an outer membrane, and the repulsion between polyphenolic compounds and Gram-negative bacteria surface could causes less efficiency of polyphenolic compounds against Gram-negative bacteria (Ikigai et al. 1993; Fattouch et al. 2007). Even different strains of the same bacterial species can show different sensitivity for one polyphenol (Helander et al. 1998). In this study, the zone of

inhibition assay could not be performed for the anaerobic species *P. gingivalis* and *F. nucleatum*, as PRE reacted with blood in the supplemented agar necessary for the suitable growth of these anaerobic bacteria. Additionally, *P. gingivalis* and *F. nucleatum* did not show enough growth with other agar media, even with Brucella agar supplemented with haemin and vitamin K, which was the suggested agar for anaerobes in CLSI guideline (CLSI documents, M11-A7). Similar problems were observed when performing the MIC assay for these bacteria as MH broth is used in the MIC assay and these anaerobic bacteria did not reach the growth phase with this broth. However, the MIC values for PRE against studied streptococcus strains and *C. albicans* varied between 1-4 mg/mL. The MLC values of both PRE and Zn (II) were found to be at least two-fold than the MIC values. Both PRE and Zn (II) showed bacteriostatic and bactericidal activities against studied microbes dependent on concentrations. Bacteriostatic activity of PRE has been reported with a water extract exerting only weak zone of inhibition activity, with a hazy zone against *B. subtilis ATCC 6633* and *S. cerevisiae Y-139* (Al-Zoreky 2009). McCarrell et al. (2008) found that while pomegranate peel water extract caused a zone of inhibition in first 12 h against *P. aeruginosa*, this activity was not observed at 24 h. Thus, these effects were associated with inhibition of physiological processes that diminished over time. However, bactericidal activity of PRE has been also reported against *S. epidermidis, E. faecalis, P. aeruginosa* and *A. baumannii* (Chebaibi and Filali 2013). Similarly, Zn (II) has been shown to possess bactericidal and bacteriostatic activities. Although many bacteria have a defense mechanism for the detoxification of heavy metal ions, metal compounds have been extensively studied to develop antimicrobial agents. It has been shown that many low-molecular-mass metal compounds demonstrate bacteriostatic and bactericidal activities (Silver 1996). In a previous study, the susceptibilities of *Staphylococcus* strains to metal salts solutions (in the range of 50 μmol to 80mmol) were determined and frequencies of resistance were found to be as follows: CuSO4 and NiCl2 (36.2%), ZnSO4 (13.6%) and CoCl2 (4.5%), (Ug and Ceylan 2003). In another study, ZnSO4 bactericidal activity was demonstrated against enteric bacterial pathogens (Butt and Satti 2011).

The potential synergy between PRE and Zn (II) was evaluated using checkerboard assays and time kill assays. *P. gingivalis* and *F. nucleatum* were not studied, because of

previously discussed limitations in the growth of bacteria and reaction between growth media and PRE. The synergistic activity between PRE and Zn (II) was only evident with for *S. gordonii and C. albicans,* with additive activity observed against *S. sanguinis, S. anginosus, S. mutans* and *E. coli*. While PRE did not show inhibition against *E. coli*, when it was combined with Zn (II) the additive antimicrobial activity was observed by the chequerboard assay. However, for *S. aureus*, no difference was observed in growth inhibition using the chequerboard assay. The results were similar to those reported for *S. aureus* by McCarrell et al. (2008) since detectable growth inhibition was not observed in the combination of PRE and Zn (II) for *S. aureus*. Conversely, in the same study, growth inhibition was not observed for *E. coli*, as well as for PRE combination with Zn (II) (McCarrell et al. 2008).

The time kill assay was performed on selected microbes, *S. mutans, C. albicans* and *S. gordonii*, to measure bactericidal activity depending on time (Jacqueline et al. 2003). *C. albicans* and *S. gordonii* were selected to confirm contact time for synergistic activity. *S. mutans* was used in this study as it is considered the main pathogenic microbe in dental decay (Zhang et al. 2017). In the time kill assay at 240 min, synergistic antimicrobial activity for PRE/Zn (II) was observed for *S. gordonii* and *C. albicans*. PRE/Zn (II) showed more greater (>2 log) reduction than Zn (II) alone, which had the highest reduction in this combination. Similarly, vanillin complex (a phenolic compound) combined with different metal ions in zone of inhibition assays has previously potentiated antimicrobial activity against *S. aureus, E. coli, K. pneumoniae, P. aeruginosa and C. albicans* was observed (Nair and Joseyphus 2008). Potentiated antimicrobial activity against herpes virus was investigated for the PRE/Zn (II) and a 6-log reduction was observed when PRE was combined with different zinc salts (Houston et al. 2017b). To explain the enhanced antimicrobial activity for pomegranate extracts and metal salts, it has been suggested that phenolic compounds form a complex with metal ions and this complex has greater antimicrobial activity. In addition, metallic ions may generate more reactive oxygen species (ROS) generation which causes enhanced PRE activity at the metal centre (Gould et al. 2009; Zhang et al. 2016).

Interactions between pomegranate whole fruit methanolic extract and five antibiotics (chloramphenicol, gentamicin, ampicillin, tetracycline and oxacillin) against 30 clinical isolates of methicillin-resistant and methicillin-sensitive *Staphylococcus aureus* was demonstrated by Braga et al. (2005). In addition, a significant potentiated antimicrobial activity was observed with Zn (II) ion, when used with conventional antibiotics. Zn (II) ions significantly enhanced the antimicrobial activity of tetracycline, macrolides, aminoglycosides, quinolones and vancomycin (Tichy and Novak 1998; Hamdan 2003; Gokhale et al. 2007; Uivarosi 2013; Zarkan et al. 2016). Similarly, fresh pomegranate juice and rifampin in combination showed enhanced antimicrobial activity against multi-drug resistant *M. tuberculosis* (Al Matar et al. 2019). A synergistic activity was also observed between methanolic pomegranate peel extract and ciprofloxacin in a study by Dey et al. (2012). The potentiated antimicrobial activity between pomegranate extracts and antibiotics has been attributed to their efflux blocking actions (Lomovskaya and Watkins 2001; Stavri et al. 2007). Braga et al. (2005) reported efflux inhibition activity for the methanolic extract of the whole pomegranate fruit against *S. aureus RN-7044*, containing the pWBG32 plasmid encoding an ethidium bromide efflux mechanism. Hence, a similar synergistic mechanism may also be apparent with PRE/Zn (II). However, further studies are necessary to confirm this and other possible mechanisms for the potentiated antimicrobial activity of PRE/Zn (II) against certain microbes.

Microorganisms in biofilms are generally more resistant than planktonic equivalents (Carlsson 1967; Gibbons and Houte 1975). Therefore, biofilm-associated infectious diseases can cause prolonged problems by converting acute infections to chronic infection (Hall-Stoodley and Stoodley 2009). It was reported that dental plaque comprises more than 700 microbes and different microbes play a role in the pathogenesis of oral infectious diseases. Complexity of biofilm pathogenesis could cause issues due to antimicrobial resistance problems in the treatment of biofilm-related infectious diseases. Thus, research into unconventional antimicrobial agents, such as plant extracts, could uncover important pathway for antimicrobial drug development. However, antimicrobial studies with plant-derived products are generally restricted to planktonic bacteria, rather than biofilms environments (Costerton et al. 2003). Therefore PRE, Zn (II) and PRE/Zn (II)s were

studied on bacteria in biofilm conditions. The effect of studied compounds activity on biofilm formation and pre-formed biofilm eradication were investigated on 24 h single species biofilms of *S. sanguinis, S. mutans, S. gordonii, C. albicans* and 48 h single species biofilms of *P. gingivalis.*

In this study, PRE and Zn (II) showed antimicrobial activity against *S. mutans*, both in planktonic and biofilm conditions. However synergistic activity was not observed for the PRE/Zn (II). In the biofilm eradication study by CLSM and COMSTAT2 analysis, PRE showed significantly greater biofilm disruption activity than Zn (II) on the biomass of *S. mutans* biofilms and similar results were obtained from the PRE/Zn (II). MIC values against *S. mutans* were 1 mg/mL and 1.56 mM for PRE and Zn (II), respectively, which were also used in the biofilm study. In the study by Sateriale et al. (2020), an MIC of 10 mg/ mL against *S. mutans* for hydroalcoholic extracts of pomegranate peel was reported. Sateriale et al. (2020) studied the same hydroalcoholic pomegranate peel extract's effects on *S. mutans* biofilms, with the pomegranate peel extract demonstrating inhibition of biofilm formation, even at half of the MIC value (5 mg/mL) and disruption to preformed *S. mutans* biofilms at 40 mg/mL. This study indicated a synergistic activity between the pomegranate peel extract and myrtle extract, in terms of antimicrobial activity against *S. mutans* and another oral pathogen, *Rothia dentocariosa*. Ferrazzano et al. (2017) found MIC and MLC values against *S. mutans* of 10 and 15 mg/mL, respectively, for pomegranate peel hydroalcoholic extract, with pomegranate peel exerting better antimicrobial activity than pomegranate juice. In the current study, 1 mg/mL of PRE eradicated pre-formed biofilms of *S. mutans*. PRE significantly decreased the biomass and increased the roughness coefficient of the *S. mutans* biofilms. PRE can inhibit glycosyltransferases in *S. mutans,* and inhibition of glycosyltransferases is important, since it plays a major role in the pathogenesis of *S. mutans* in dental caries (Haslam 1996; Gulube and Patel 2016; Yang et al. 2019).

Dabholkar et al. (2016) compared the antimicrobial activity of a commercial pomegranate mouthwash (Life-extension, USA) and 0.12% chlorhexidine mouthwash using the zone of inhibition assay against *S. mutans, S. salivarius* and *Aggregatibacter*

actinomycetemcomintans. Pomegranate mouthwash exerted zones of inhibition against all three bacteria, although this inhibition was lower than chlorhexidine. Similarly, in this study, *S. salivarius* was found susceptible to PRE with an MIC of 2 mg/mL and PRE 2 mg/mL diminished the biofilm. However, while Zn (II) also inhibited the growth and biofilm with an MIC of 0.39 mM, the PRE/Zn (II) did not exert synergistic or antagonistic activity. Aldhaher et al. (2015) found pomegranate peel aqueous extract had an MLC of 25 mg/mL against *S. mutans*, using agar diffusion assay. In addition, 25 mg/mL of pomegranate peel aqueous extract inhibited adherence of *S. mutans* to teeth and stainless-steel wire. In another study, pomegranate peel methanolic extract inhibited the growth of *S. mutans, S. sanguinis, S. salivarius, Lactobacillus acidophilus* and *S. aureus*, but was ineffective against *C. albicans* and *Actinomyces viscous*, in the form of 8 and 12 mg/mL extract-loaded discs assessed using the agar diffusion assay. All observed inhibition was lower than ciprofloxacin and nystatin positive controls (Abdollahzadeh et al. 2011). Pomegranate extract was found effective against *C. tropicalis* and *C. glabrata* in the study by Oliveira et al. (2013). In the present study, PRE and Zn (II) inhibited *C. albicans* growth, with MIC values of 4 mg/mL and 6.25 mM, respectively. In addition, the PRE/Zn (II) showed synergistic activity against planktonic cells using checkerboard and time kill assays starting at 240 min (4 h). Anti-biofilm activities of the compounds was assessed for 24 h *C. albicans* biofilms and all treatments showed significant anti-biofilm activity. The *C. albicans* biofilm biomass was significantly reduced. However, PRE had the greatest effect on the mean biofilm thickness whilst PRE/Zn (II) did not exhibit synergistic antibiofilm effects. Mehta et al. (2014) performed *in vitro* zone of inhibition studies to evaluate antimicrobial activity against a range of oral microbes. Aqueous pomegranate peel extract showed a dose-dependent antimicrobial response against *S. mutans, S. mitis* and *C. albicans*. However, *P. gingivalis* and *P. intermedia* were resistant. While Mehta et al. (2014) showed the inefficiency against *P. gingivalis* and *P. intermedia*, blood supplemented agar was used in their study and that could be the reason for no antimicrobial activity against these two obligate anaerobes, since tannins in pomegranate peel extract are able to bind iron in the blood, resulting in a black compound. This reaction could prevent the antimicrobial action of pomegranate peel extract. In this Chapter, the antimicrobial activity of PRE against *P. gingivalis* and *F. nucleatum* could not be

determined due to precipitation and turbidity problems with the growth media required by these obligate anaerobic bacteria in planktonic form. The agar well diffusion assay was not performed due to precipitation of tannin with iron in blood. While *P. gingivalis* and *F. nucleatum* showed growth in FAB and BHI broth, PRE precipitated in BHI broth, and this precipitation prevented turbidity-based assessments. Resazurin was considered to measure growth, but reproducible results were not obtained possibly due to the PRE redox effect on colour change of resazurin (Cordier and Steenkamp 2015). Therefore, PRE antimicrobial effect was studied on *P. gingivalis* biofilms. After forming *P. gingivalis* biofilms in FAB or BHI, PRE, Zn (II) and PRE/Zn (II) antimicrobial effect was observed via live/dead fluorescence staining. This concept helped to overcome turbidity, precipitation or redox problems. PRE and PRE/Zn (II) significantly reduced the biofilm biomass, while Zn (II) alone did not cause any significant changes. Hence, the effect of the PRE/Zn (II) on *P. gingivalis* biofilms could be primarily attributed to PRE. Widyarman et al. (2018) reported the antimicrobial activity of pomegranate juice against *P. gingivalis*, *Treponema denticola* and *A. actinomycetemcomitans* biofilms; undiluted pomegranate juice gave similar inhibition results to 0.2% chlorhexidine. It was indicated that pomegranate juice showed greater antimicrobial activity against multispecies biofilms comprising these three microbes.

In this study, the effect of PRE, Zn (II) and PRE/Zn (II) effect were studied on biofilms of *C. albicans, S. mutans* and *S. gordonii*. Morphological damage was also observed in PRE-treated biofilms of *C. albicans*. Furthermore, similarities in phenotypic characteristics were observed between PRE-, PRE/Zn (II)- and 0.2% chlorhexidine-treated biofilm of studied microbes.

In one clinical study, after rinsing the mouth with 30 mL of pomegranate juice, 23% and 46% reductions of streptococci and lactobacilli, respectively (Kote et al. 2011). However, the material and methodological information related to pomegranate variety and juice preparation was not provided for this study. In another clinical study, 60 mg/mL of hydroalcoholic extract from whole pomegranate fruit, distilled water and 0.12% chlorhexidine were compared in 60 healthy patients. The hydroalcoholic extract of

pomegranate mouthwash was found to be effective against dental microorganisms with 84% reduction in CFU/mL. Similar values were obtained for chlorhexidine, with 79% inhibition and only 11% inhibition was observed with distilled water (Menezes et al. 2006). Umar et al. (2016) performed a study to compare the antimicrobial activities of different parts of pomegranate (peel, aril and juice), using an *in vitro* zone of inhibition assay against *S. mutans*. It was found that pomegranate peel exerted higher activity, whilst pomegranate juice did not show any inhibitory activity. A mouthwash formulation was then clinically evaluated to determine its effects on salivary pH and *S. mutans* levels. It was found that salivary pH increased for both groups after 10 min and 60 min. Furthermore, *S. mutans* numbers significantly decreased for both groups, while chlorhexidine resulted in an increase. A recent systematic review of the literature compared natural antimicrobial phenolic compounds and synthetic antimicrobials using 16 clinical studies for qualitative synthesis and 12 studies for meta-analysis (Martins et al. 2020). The result showed that natural antimicrobial phenolic compounds were less effective than chlorhexidine in biofilm control. However phenolic compounds, including pomegranate phenolics, showed similar reductions in oral microbe counts of *S. mutans* and streptococcus species.

The antimicrobial mechanism of polyphenols is not clear, but there are some suggested mechanisms. In general, the mode of action for polyphenols is associated with their ability to damage the cytoplasmic membrane, inhibition of nucleic acids and energy metabolism that could be a result of reaction between sulfhydryl groups or by non-specific interactions with the proteins (Cowan 1999). In addition, polyphenols can interfere the synthesis of cell membrane and cell wall (Cushnie and Lamb 2011). The phenolic acids-cell membrane interface can cause hyper-acidification and induce permeability of cell membranes. This could partly explain the varied antimicrobial efficiency of pomegranate against different microbes (Miguel et al. 2010; Lou et al. 2011). Polyphenols also inhibit the respiratory chain of microbes and decrease the oxygen consumption, resulting in limited oxidation of NADPH (Haraguchi et al. 1998).

The antimicrobial activity of PRE could be attributed to its high content of hydrolysable polyphenols, mainly gallotannins and ellagitannins, such as punicalagins, punicalin and ellagic acid (Cowan 1999; Patel et al. 2008; Kasimsetty et al. 2009; Glazer et al. 2012). It has been suggested that the chemical structure of tannins play a role in their antimicrobial activities. For instance, hydrolysable tannins exerted antimicrobial activity in lower concentrations than condensed tannins (Ekambaram et al. 2016). The degree of galloylation, configuration of the digalloyl or trigalloyl groups that to the glucose core and free galloyl groups have an effect on antimicrobial activity of tannins which are the most abundant compounds in pomegranate extracts (Chung et al. 1993; Tian et al. 2009; Engels et al. 2010). For example, while punicalagin is effective against a range of bacteria, another ellagitannin, granatin A, was found to be ineffective against microbes, probably due to the absence of free galloyl groups (Machado et al. 2002; Shimozu et al. 2017). A higher degree of galloylation means higher protein binding capacity (Ekambaram et al. 2016). Moreover, Reddy et al. (2007) studied the antimicrobial activity of ellagic acid, gallagic acid, punicalin and punicalagin and found that punicalagin and gallagic acid exhibited the highest antimicrobial activity. Therefore, it was suggested that the gallagyl and hexahydroxydiphenol (HHDP) moieties could be more important than the ellagic acid moiety in terms of antimicrobial activity of these phenolic secondary compounds (Reddy et al. 2007). Hydroxyl groups can react with both the cell membrane and active site of microbial enzymes, and as a result damage microbial metabolism (Silva-Beltrán et al. 2015).

Anti-biofilm activity mechanism of polyphenols is not clear, but it could be attributable to multiple factors. The antibiofilm activity of pomegranate polyphenolic compounds comprises the precipitation of vital proteins, which play role in biofilm formation, such as adhesins. In addition, the pomegranate polyphenolics ellagic acid and ellagitannins, could change the cell surface charge and interfere with cell-substratum interactions and biofilm development (Liu et al. 2008). The adhesion of microbes is mainly related to physicochemical characteristics of the surface. However, bacterial characteristics and other environmental factors should not be ignored in the adhesion process during biofilm development. A change in bacterial surface could affect and interfere with the attachment

of cell-substratum and distract the normal phase of biofilm development (Simões et al. 2007). In addition, it should be noted that polyphenols can also cause aggregation of microbes, and this situation changes the bacterial attachment preference and affects surface attachment (Cushnie et al. 2011). Another factor is Quorum Sensing (QS), QS is described as the control of gene expression in response to changes in cell population density. Moreover, QS assists microbes in escaping from the host immune system (Rudrappa and Bais 2008; Ni et al. 2009). Several studies have suggested that QS inhibition plays a role in the anti-biofilm activity of pomegranate (O'May and Tufenkji 2011; Sarabhai et al. 2013).

4.6 Conclusion

PRE and Zn (II) exhibited antimicrobial activity against all studied *Streptococcus* strains and *C. albicans*. Synergistic antimicrobial activity of PRE/Zn (II) was found only against planktonic *S. gordonii* and *C. albicans.* Generally, PRE showed better anti-biofilm activity than Zn (II) and PRE/Zn (II). As such, biofilm eradication activity could be more associated with PRE, rather than Zn (II). Further studies are necessary to see the effect of PRE, Zn (II) and PRE/Zn (II) in more complex biofilms, rather than single species biofilms. In addition, more research is required to elucidate the antimicrobial and anti-biofilm mechanism of PRE and PRE/Zn (II) and the main compound in PRE responsible for the antimicrobial activity. While punicalagin is known to be one of the most abundant compounds in PRE and it has been highly associated with the antimicrobial activity of PRE, structure-activity studies are necessary to find the compound or compounds responsible for the antimicrobial activity of PRE.

Chapter 5: Anti-adhesive Activity of PRE, Zn (II) and PRE/Zn (II)

5.1 Introduction

The oral cavity including tooth surfaces comprise a highly complex environment for biofilm development, involving specific and highly interrelated microorganisms. The first step of bacterial biofilm infections is the adhesion of the bacteria to host cells and/or surface, is considered an important target to prevent/treat biofilm-related infections (Stewart 2001; Davies 2003). Microbes in biofilms are much more resistant to antimicrobial agents, compared to their planktonic counterparts (Stewart and Franklin 2008). Even microbes which are normally susceptible to antimicrobial agents can develop multidrug resistance when they are present in a biofilm, due to acquired mobile resistance genes (Levin and Rozen 2006). Thus, prevention of adhesion is important for treatment and prevention of biofilm infections (Krachler and Orth 2013).

Bacteria have multiple mechanisms for attachment to the host cell or tissue. Initial adherence is weak and reversible. Irreversible adherence occurs subsequently through covalent bonds. Adherence can initiate through non-specific binding with hydrophobic molecules and other non-specific interactions (Quintero- Villegas et al. 2013). In addition, bacterial adhesins can interact with specific receptors of host surfaces. It is possible that these different adhesion mechanisms can act together and/or play a role in the different phases of adhesion (Ofek and Doyle 1994). After initial adhesion occurs between bacteria and host cell and/or surface, bacteria produce a stronger bond which comprises protein and polysaccharides compared to initial adherence (Miorner et al. 1983). Protein-protein binding, a type of specific adhesion, occurs between protein adhesives and EPS components (Sharon 1987). EPS includes extracellular polysaccharides, nucleic acids (DNA and RNA) and proteins. In addition to these compounds, peptidoglycans, lipids, phospholipids and other bacterial compounds can also be found in the EPS (Sutherland 2001; Branda et al. 2005).

Periodontal diseases have been strongly associated with the formation and accumulation of sub-gingival plaque, which is a bacterial biofilm enveloped by and tightly adhered to the tooth surface via EPS (Yang et al. 2011). Bacterial biofilms of

S. mutans, F. nucleatum, P. gingivalis and *C. albicans* can be found in dental unit water lines, on tooth surfaces and dental prosthetic appliances, and on oral mucous membranes. Biofilms in the form of supra-gingival and sub-gingival plaque are considered the main aetiologic factors in dental caries and periodontal diseases (Van Houte 1994; Socransky et al. 1998; Haffajee and Socransky 2000; Stenudd et al. 2001). The aim of anti-adhesion therapy is to interfere with the adhesion of microbes to the host cell/tissue and/or substrate surface, as adhesion is the first step of biofilm related infections (Ofek et al. 2003).

In this Chapter, the anti-adhesive properties of PRE, Zn (II) and PRE/Zn (II) were assessed against *Streptococcus mutans, Streptococcus gordonii* and *Candida albicans.* Many reports suggest that *Streptococcus mutans* is the main pathologic microbe in caries, due to its acidogenic biofilm formation on tooth surfaces (Hamada and Slade 1980; Hamada et al. 1984; Carlsson 1997; Ccahuana-Vásquez 2010). *S. mutans* cells adhere to acquired enamel pellicle via sucrose- dependent and sucrose-independent mechanisms (Ahn et al. 2008; Wen et al. 2010; Zijnge et al. 2010). *S. gordonii* is an early coloniser, which initiates dental plaque formation and is found in high numbers in both supra-gingival and sub- gingival plaque (Socransky et al. 1998; Quirynen et al. 2005). Moreover, as an early coloniser, *S. gordonii* helps to drive the temporal and spatial development of dental plaque. After *S. gordonii* colonisation on tooth surfaces, it provides an environment for subsequent colonisers and more pathogenic microbes, such as *P. gingivalis*, by providing an attachment substrate. Many reports have shown co-aggregation of *P. gingivalis* with oral streptococci (Stinson et al. 1991; Lamont et al. 1992; 1994; 2002; Amano et al. 1997; Maeda et al. 2004a,b). *C. albicans* has specialised adhesins, which allow *C. albicans* attachment to microbes, biotic and abiotic surfaces such as mucosal or prosthetic surfaces in the oral cavity (Verstrepen and Klis 2006; Campos et al. 2008; Garcia et al. 2011).

The protective effect of several food components against infectious bacterial diseases has been demonstrated in *in vitro* and *in vivo* studies (Mahady 2005; Krachler and Orth 2013). Plants have been to aid for oral hygiene for centuries and there is a link between improved oral health and the regular consumption of certain food and beverages which include bioactive components (Safiaghdam et al. 2018). In a clinical study, it was shown that regular and moderate consumption of red wine and coffee can

tilt the microbial community of dental plaque towards less pathogenic bacteria (Signoretto et al. 2010). The bioactivity of polyphenols has been demonstrated to inhibit odontopathogenic and periodontopathogenic bacteria. For instance, plant secondary compounds, especially polyphenols and proanthocyanins, have been found promising for anti-adhesive treatment. It has been reported that a non-dialysable material from cranberry juice concentrate inhibits co-aggregation of many oral bacteria and inhibited adherence and biofilm formation of *P. gingivalis* (Steinberg et al. 2004; Yamanaka et al. 2004; Steinberg et al. 2005; Labrecque et al. 2006). Moreover, it was reported that polyphenols and proanthocyanins inhibit bacterial surface attachment by binding to flagella and pili of bacteria, causing decreased aggregation and swarming mobility in biofilms (Burger et al. 2002; Signoretto et al. 2012).

In Chapter 4, the antimicrobial activity of PRE, Zn (II) and PRE/Zn (II) combination was shown against planktonic and biofilm cells, with different methods aimed to investigate the inhibitory activities of these compounds. Here, the anti-adhesion potential of these treatments was investigated.

5.2 Aims and objectives

The aim of this Chapter was to investigate initial attachment of *S. mutans, S. gordonii* and *C. albicans* on PRE, Zn (II) and PRE/Zn (II) combination to coated glass surfaces. This aim was evaluated by studying the:

- PRE, Zn (II) and PRE/Zn (II) combination anti-adhesive effect on pre-coated glass surfaces of *C. albicans, S. mutans* and *S. gordonii*, using live/dead bacterial staining with confocal laser scanning microscopy (CLSM).

- PRE, Zn (II) and PRE/Zn (II) combination anti-adhesive effect on pre-coated glass surfaces of *C. albicans, S. mutans* and *S. gordonii*, by enumerating colony forming units.

5.3 Materials and methods

Agar plates, broth, and bacterial cultures were prepared as described in Chapter 2 (Section 2.2.3). All bacterial strains and their origin were described in Table 2.4. Equipment, glass and plastics, chemical consumables and assay kits used in this chapter were presented in Section 2.1 in Table 2.1, Table 2.2, and Table 2.3, unless otherwise stated.

5.3.1 Anti-adhesive activity

5.3.1.1 Coating glass slide with PRE, Zn (II) and PRE/Zn (II) combination

Eight-well glass chamber slides (8-Well Falcon Culture Slide, Corning Incorporated Life Sciences, New York, USA) were used in this assay. Each well of the 8-well chamber slides was coated with either PRE, Zn (II) or PRE/Zn (II). Desired concentrations of compounds were prepared in phthalate buffer pH 4.5, with phthalate buffer pH 4.5 alone used as an untreated control. The concentrations of compounds and the volumes are shown in Table 5.1 for each studied microorganism. Slides were wrapped in parafilm to prevent evaporation and then in aluminium foil to protect from light, and then incubated at 37 ℃ for 24 h.

Microorganism	**PRE (200 μL)**	**Zn (II) (200 μL)**	**PRE +Zn (II) (100 μL +100 μL)**
S. mutans	1 mg/mL	1.56 mM	2 mg/mL +3.12 mM
S. gordonii	2 mg/mL	0.78 mM	4 mg/mL +1.56 mM
C. albicans	4 mg/mL	6.25 mM	8 mg/mL +12.5 mM

Table 5.1: The concentrations of PRE, Zn (II) and PRE/Zn (II) combinations for coating wells of glass slide for studied microbes.

5.3.1.2 Microscopy evaluation of anti-adhesive effect

The anti-adhesive activity of PRE, Zn (II) and PRE/ Zn (II) was determined by evaluating *S. mutans, S. gordonii* and *C. albicans* attachment to glass surfaces coated with PRE, Zn (II) and PRE/ Zn (II). Eight-well chamber slides (8- Well Falcon Culture Slide, Corning Incorporated Life Sciences, New York, USA) were coated, as described in Section 5.3.1.1. Different 8-well chamber slides were used for each microorganism and wells were coated with compounds as described in Table 5.1.

After incubation, coating solution were aspirated from wells and 200 µL of overnight cultures of *S. mutans, S. gordonii* or *C. albicans* with 10^8 CFU/mL were added. Then, slides were incubated for 2 h at 4 ℃ to assess initial attachment of studied microorganisms. After 2 h incubation, the supernatant from each well was discarded and washed with phosphate buffered saline (PBS), to remove unattached bacteria to the glass surface. Baclight Live/Dead staining was prepared and 5 µL of Live/Dead stain in PBS applied as outlined in Chapter 4 (Section 4.3.6.3). Slides were examined by CLSM (Leica SP5, AOBS Spectral Confocal Microscope, Heidelberg, Germany) using a 40x objective lens. Each experiment was performed on three separate occasions with replicates for each group, and four images were taken from each well. COMSTAT2 analysis was performed as described in Chapter 4 (Section 4.3.6.3) (Heydom et al. 2000). Results were shown as obtained images and biomass ($\mu m^3/ \mu m^2$) (mean± SEM) from COMSTAT2 analysis.

5.3.1.3 Evaluation of antiadhesive effect via viable cell enumeration

In addition to images and biomass ($\mu m^3/ \mu m^2$) from COMSTAT2 analysis, viable microbes from coated and uncoated wells were counted. Briefly, the procedure was repeated as in Section 5.3.1.2. However, instead of staining wells for CLSM microscopy imaging, the chamber slides were removed, and cells were collected, using a 25 cm cell scraper to harvest cells in a sterile centrifuge tube containing 1 mL PBS. The bacterial suspensions were vortexed for approximately 15 s. Then, 20 µL sample was taken from the tubes and diluted 10-fold in 96-well plates containing 180 µL PBS in each well. 20 µL of the diluted samples were plated on MH agar and incubated for 24 h. Using the Miles and Misra method (Miles et al. 1938), colonies

were subsequently counted, and CFU/mL values calculated. Each experiment was performed on three separate occasions with triplicate for each group, and data was expressed as CFU/ mL mean ± SEM.

5.3.2 Statistical analysis

All experiments were performed on in least three independent occasions, including two internal replicates using independent microbial cultures for all assays. Results were statistically analysed using GraphPad Prism software (GraphPad Software, Version 8.2.1, San Diego, CA, USA), by performing one-way ANOVA, with post-test Tukey analysis and $p<0.05$ was considered statistically significant. All data were presented as the mean value with SEM.

5.4 Results

5.4.1 Anti-adhesive activity

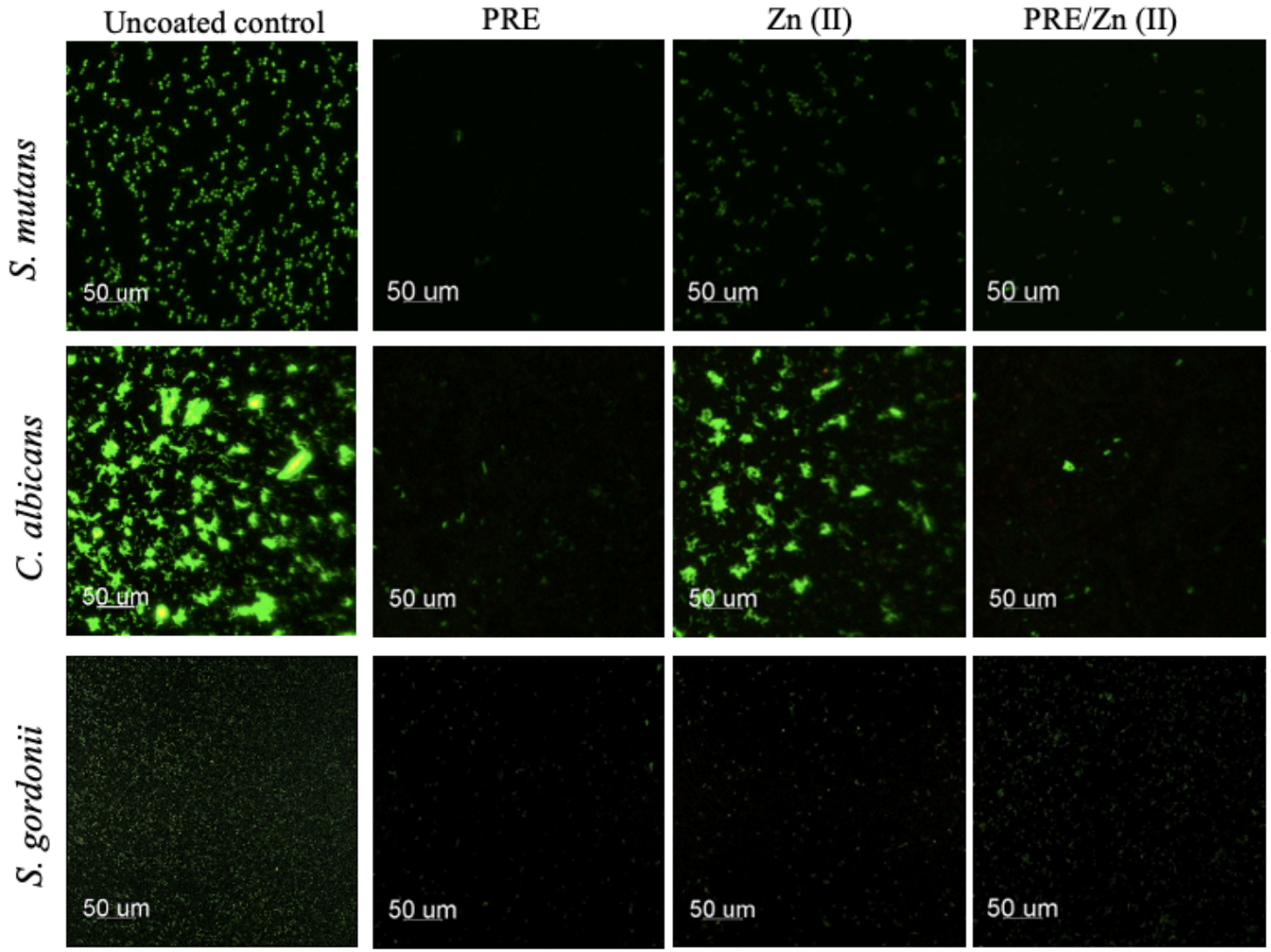
5.4.1.1 Confocal laser scanning microscopy (CLSM) and COMSTAT2 analysis

Figure 5.1A represents the live (green) and dead (red) stained confocal images from 8-well chamber slides pre-coated with PRE, Zn (II) and PRE/Zn (II), in addition to uncoated controls, and co-cultured with *S. mutans, S. gordonii* and *C. albicans*. The biomass ($\mu m^3/ \mu m^2$) of attached bacteria for each species was determined via COMSTAT2 analysis. Figure 5.1B represents biomass ($\mu m^3/ \mu m^2$) for each studied microorganism. From the images, it was evident that all coated wells had fewer live bacteria attached to PRE, Zn (II) and PRE/Zn (II) coated surfaces, when compared to uncoated negative controls (Figure 5.1A). However, interesting to note that non-viable bacteria (red fluorescence stain in Figure 5.1A) was not observed according to staining result after 2 h initial attachment of tested microbes. PRE- and PRE/Zn (II)-coated wells resulted in fewer live bacteria/fungal attachment than Zn (II). Statistical analysis for biomass ($\mu m^3/ \mu m^2$) showed that uncoated slides exhibited significantly higher biomass ($\mu m^3/ \mu m^2$) than coated wells for each microorganism (Figure 5.1B). There was a significant difference between the uncoated control group and PRE-coated wells ($p<0.001$, Figure 5.1B) for *S. mutans.* Similar results were obtained for *C. albicans*, the biomass ($\mu m^3/ \mu m^2$) of attached *C. albicans* to PRE and PRE/Zn (II) coated surfaces was significantly less than the un-coated surface ($p<0.01$, Figure 5.1B). Furthermore, there was a significant difference between PRE/Zn (II) and Zn (II) coated surfaces for *C. albicans* ($p<0.05$). *S. gordonii* showed significantly less live cell attachment in PRE, Zn (II) and PRE/Zn (II) coated surfaces compared to uncoated surface. However, there was not any significant difference between PRE, Zn (II) and PRE/Zn (II) ($p>0.05$) (Figure 5.2).

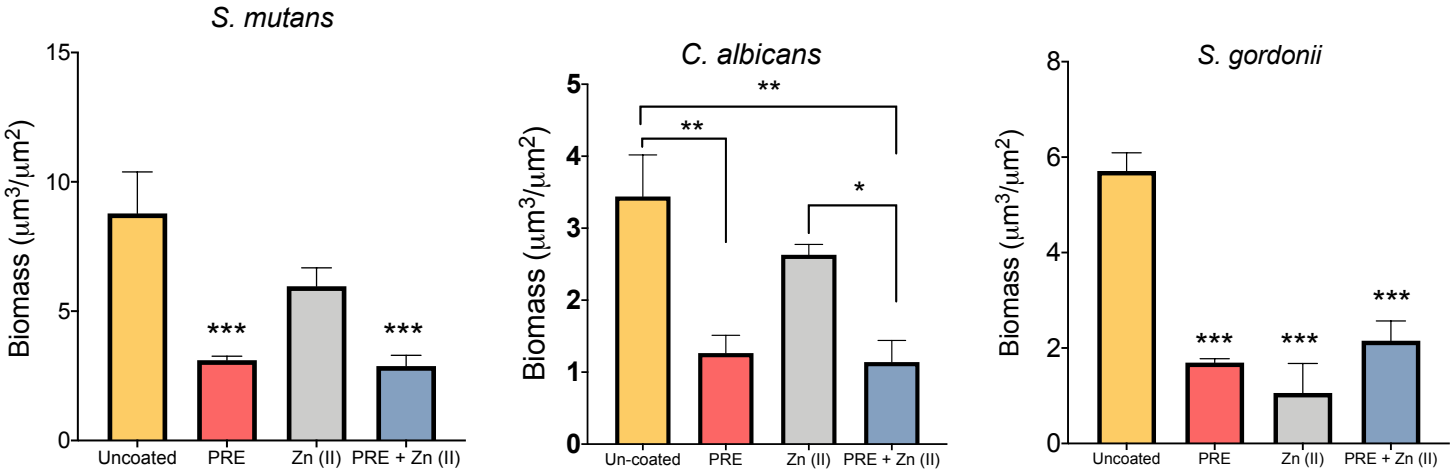
5.4.2 Recovery of viable bacterial and fungal cells

The anti-adhesive activity of PRE, Zn (II) and the PRE/Zn (II) combination were also analysed by enumerating the microbial cells after 2 h attachment to coated and uncoated surfaces of 8-well chamber slides (Figure 5.2). *S. mutans* attachment was significantly reduced in wells coated with PRE and PRE/Zn (II) combination ($p<0.05$ and $p<0.01$ for PRE vs uncoated controls and PRE/Zn (II) vs uncoated controls, respectively).

For *C. albicans*, both PRE and the PRE/Zn (II) combination caused less attachment and PRE/Zn (II) combination exerted significantly reduced ($p<0.01$) attachment, compared to the uncoated controls. Furthermore, the PRE/Zn (II) combination was significantly effective ($p<0.05$) in inhibiting the attachment of *S. gordonii,* in comparison to uncoated surfaces.


Uncoated control
PRE
Zn (II)
PRE/Zn (II)
S. mutans
C. albicans
S. gordonii
50 um

(A)



(B)

Figure 5.1: Adherence of *S. mutans, C. albicans* and *S. gordonii* to PRE (MIC), Zn (II) (MIC), PRE/Zn (II) (MIC+MIC) coated and uncoated 8-well chamber slides; A) Surfaces visualised by CLSM after staining with BacLight Dead/Live bacterial staining kit. (B) Biofilm biomass via COMSTAT2 analysis of. Significance indicated by *, where *p<0.05, **p<0.01, ***p<0.001 between each group. Bars represent mean±SEM, N=3.

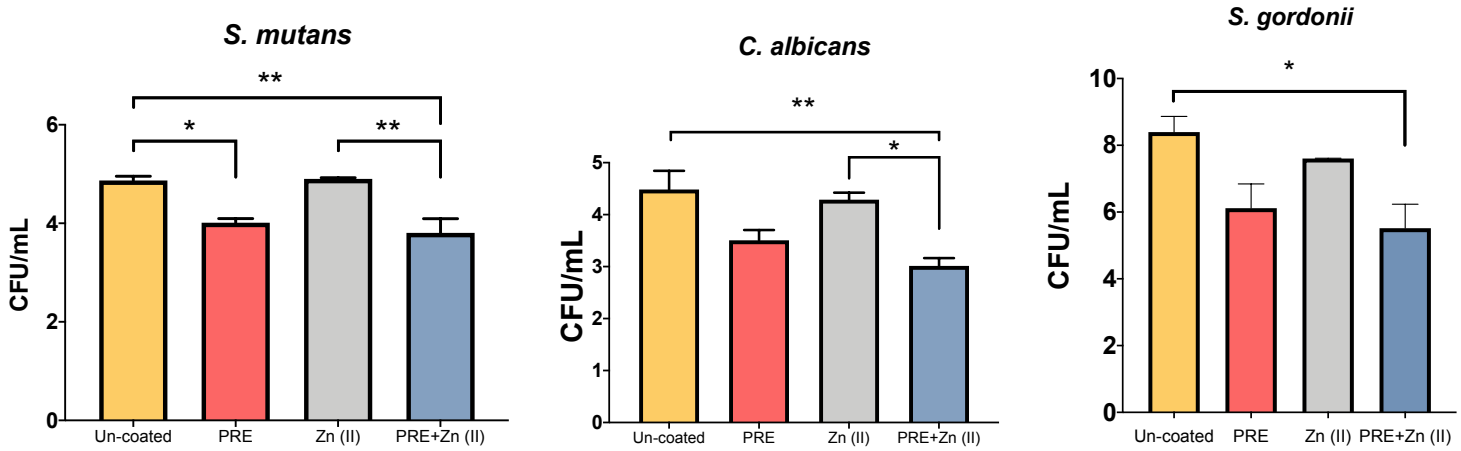


Figure 5.2: Viable cell numbers of *S. mutans, C. albicans* and *S. gordonii* from 8-well chamber slides uncoated or coated with PRE (MIC), Zn (II) (MIC), PRE/Zn (II) (MIC+MIC). Bars represent mean±SEM, N=3. Significance indicated by *, where *$p<0.05$, **$p<0.01$ between each group.

5.5 Discussion

A biofilm is commonly described as a multicellular (homogenic or heterogenic) microbial community, which attaches to a surface/interface through a self- made organic material matrix (Costerton et al. 1999, Wimpenny et al. 2000; Donlan and Costerton 2002; O'Toole and Stewart 2005; Flemming and Wingender 2010). The development of biofilms starts with adhesion of a microorganism to an immersed surface, which then proliferates to create the biofilm. This ability to adhere provides numerous advantages to microbes to form a biofilm. Surface attachment provides growth stability and retain cells in close proximity to each other, which results in increased catalytic activity of microorganism. In addition, it facilitates microbial survival to overcome other environmental issues, such as extreme pH levels, UV exposure, metals, antimicrobial agents, phagocytosis and dehydration (Espeland and Wetel 2001; Mah and O'Toole 2001; Leid et al. 2002; McNeill and Hamilton 2003; Almatroudi et al. 2018). Therefore, adherence of microorganisms to the tooth surface plays an important initial role in the development of dental caries and periodontal diseases, and thus, interference of bacterial adhesion is a promising strategy in combating these diseases (Selwitz et al. 2007; Slimestad et al. 2007, Pitts et al. 2017).

Numerous studies have shown inhibition of cariogenic bacteria by polyphenolic compounds. In the previous Chapter, the antimicrobial effect of PRE, Zn (II) and PRE/Zn (II) was evaluated against these microbes both in planktonic and biofilm conditions. In this Chapter, PRE was evaluated for its potential anti-adhesive activity with or without Zn (II) combination against *S. mutans, S. gordonii* and *C. albicans*. *S. mutans* is considered the main cause of dental caries and *C. albicans* is often found with *S. mutans* in dental plaque biofilms, especially from children with early childhood caries. *Candida* species have been found in enamel, root and dentinal caries (Falsetta et al. 2014). *S. gordonii*, a primary coloniser of the human oral cavity and an opportunistic pathogen, has several adhesins on its surface. Moreover, *S. gordonii* and *P gingivalis* display co-adhesion, which could play an important role in invasion of *P. gingivalis* (Love et al. 2000).

In the present study, the anti-adhesive activities of PRE, Zn (II) and PRE/Zn (II) were investigated on coated glass surfaces, which has been widely used as representative of

the hard surface of the tooth in the literature (Hamada and Torii 1978; Hamada and Slade 1980; Mattos-Graner et al. 2000; Carter et al. 2001; Tao and Tanzer 2002; Sasaki et al. 2004; Furiga et al. 2008).However, in further studies enamel blocks from animal or human could be a better choice to stimulate the dental surface and assess tested compounds' anti-adhesive activities (Kato et al. 2010; Taha et al. 2017; Braga 2019).

Results showed that PRE and the PRE/Zn (II) significantly inhibited adherence of *S. mutans* onto glass surfaces. This adherence was expressed as biomass ($\mu m^3/ \mu m^2$) on the coated surface versus un-coated surfaces ($p<0.001$). Viable microbial number was also enumerated, and results compared between coated and uncoated wells. The *S. mutans* CFU was significantly lower in PRE and PRE/Zn (II) coated wells than with uncoated wells. Zn (II) only coated wells reduced *S. mutans* biomass ($\mu m^3/ \mu m^2$), but this was not significant ($p>0.05$). Thus, decreased biomass ($\mu m^3/ \mu m^2$) and viable attached *S. mutans* in PRE/Zn (II) coated wells are most likely associated with PRE rather than Zn (II). Similar results were obtained for *C. albicans*. PRE and PRE/Zn (II) coated wells significantly decreased biomass ($\mu m^3/ \mu m^2$) of *C. albicans* and the viable CFU of *C. albicans* was significantly lower with PRE/Zn (II), compared to uncoated controls ($p<0.01$). In addition, in wells coated with the PRE/Zn (II), the number of viable CFU of *C. albicans* was significantly lower than other experimental groups, with exception of PRE-coated wells. This result can be interpreted as PRE playing the key role in preventing the adhesion of *C. albicans*, rather than Zn (II). However, PRE/Zn (II) resulted in a significant decrease in the attached viable CFU of *C. albicans*. This may be due to the previously confirmed synergistic fungistatic and fungicidal activity of the PRE/Zn (II) combination against *C. albicans*, as confirmed by checkerboard assays and time-kill assays in Chapter 4. Regarding to *S. gordonii*, all coated wells significantly reduced *S. gordonii* biomass ($\mu m^3/ \mu m^2$), but only the PRE/Zn (II) combination caused a significant reduction in viable CFU, (>2 log reduction) of attached microbes onto the surfaces.
This again could be associated with the synergistic activity between PRE and Zn (II) against *S. gordonii*.

The anti-adhesive activity of polyphenol-rich extracts has been studied by many researchers with various compounds and extracts from different plant sources. Smullen et al. (2007) showed that unfermented cocoa, green tea and red grape seed

extracts exerted antimicrobial activity against *S. mutans* and reduced its adherence to glass surfaces. The anti-adhesive activity of PRE has been attributed to its phenolic compounds, including tannins, anthocyanins and flavonoids. Since it is well known that tannins can interact with macromolecules, including carbohydrates and proteins, this makes these compounds promising anti-adhesive and anti-biofilm agents (Janecki et al. 2010). In addition, PRE can inhibit pathogen attachment to host cells. Thus, pre-treatment of host surfaces with PRE could provide an unfavourable environment that prevents adhesion of pathogenic microbes (Sandasi et al. 2010). In one study, promising anti-adhesive activity of different pomegranate rind extract (alcohol, acetone and distilled water) was found to reduce the *S. aureus* and *E. coli* biofilm formation ability and adhesion ability on epithelial cells (Al-Wazni and Hadi 2016). In another study, pomegranate extract showed reductions in the adherence of *C. albicans* and *E. coli* on pre-treated glass surfaces (Sharma and Sabnis 2010). It has also been reported that polyphenols play an important role in protein precipitation and enzyme inhibition in microorganisms (Fan et al. 2007). Al-Zoreky (2009) showed that pomegranate extracts interfere with the secretion of proteins from bacteria cells. This result was confirmed in a study by Suresh et al. (2013), showing a dose-dependent decrease in the protein content of bacteria which have been inoculated with different concentrations of PRE. In addition to proteins, pomegranate peel compounds can also react with other macromolecules, including carbohydrates (Janecki and Kolodziej 2010).

Vasconcelos et al. (2006) studied the anti-adherence activity of pomegranate-based gel formulation, including 540 mg pomegranate peel powder against S. mutans (1:16), S, sanguis (1:16), S. mitis (1:128) and C. albicans (1:64). The MICs of pomegranate gel increased when different bacteria were mixed, or bacteria were associated with C. albicans. Miconazole gel was used as a positive control in this study, and it was claimed that pomegranate peel gel exerted greater activity than the miconazole gel in terms of anti-adhesive activity against the studied microbes. Voravuthikunchai et al. (2006) confirmed the anti-adhesive activity of PRE against *H. pylori* to the gastric mucosa. It was reported that PRE exerted these effects by changing bacterial cell surface hydrophobicity, which helps in the elimination of microbes from the human body. Furthermore, in an *in vivo* study, the alcoholic extract of pomegranate peel

showed a modest reduction in *H. pylori* colonisation and infection in Wistar rats, following eight days of treatment with a dose of 50 mg/kg (Mayyas et al. 2021).

Surface chemistries have an important role in the adhesion of microbial cells and in the present study a glass surface was chosen as the most suitable. Glass surface pre-treatment with PRE, Zn (II) and the PRE/Zn (II) combination provided an unfavourable environment to all studied microbes, *S. mutans, S. gordonii* and *C. albicans*, by reducing both the biomass ($\mu m^3/ \mu m^2$) of attached microbes and the CFU/mL on PRE and PRE/Zn (II) pre-coated glass surfaces. The result of reduced adhesiveness of the microorganisms to the treated glass surfaces could be the result of the change in the physicochemical properties of the surface, or the bacterial adhesive properties. Moreover, the decreased number of CFU could be the result of no microbial attachment or growth of microbes onto these surfaces.

5.6 Conclusion

The anti-adhesive activity of PRE, Zn (II) and the PRE/Zn (II) combination were investigated in this Chapter. Initial adhesion to a solid surface is considered the first step in biofilm formation. Therefore, prevention of adherence by coating surfaces could be an antimicrobial method to fight biofilm- related infectious diseases. This study has shown that PRE- and PRE/Zn (II)- coated glass surfaces caused significantly reduced biomass ($\mu m^3/ \mu m^2$) of attached cells for: *S. mutans, S. gordonii* and *C. albicans.* However, Zn (II) coated wells did not show significant reductions against the studied microbes, except for *S. gordonii*. PRE/Zn (II) coated surfaces caused significant reductions in CFUs (≥1 log reduction) of collected cells for all studied microorganisms, compared to their uncoated control counterparts.

Chapter 6: The Pro-oxidant Activity of PRE/Zn (II) Against *C. albicans*

6.1 Introduction

As mentioned in Chapter 4 and Chapter 5, *Candida albicans* is one of the most prevalent fungal pathogens of humans and particularly important in immunosuppressed patients such as those with acquired immunodeficiency syndrome (AIDS), implanted medical devices and patients who are receiving immunosuppressive treatments (Kullberg and Oude Lashof 2002; Nobile and Johnson 2015). While *C. albicans* is considered a commensal microorganism, the overgrowth of *C. albicans* on the oral mucosa can cause oral candidosis, which is an important oral dermatological disease. Predisposition to oral candidosis occurs with the alteration of both systemic and local factors (Zegarelli et al. 1993). *C. albicans* is the most isolated species amongst *Candida* species from the oral cavity (Scully et al. 1994; Millsop and Fazel 2016). In addition, *C. albicans* has been considered the one of main aetiologic factors of oral candidosis, denture stomatitis, early childhood caries, periodontal diseases, and endodontic lesions, especially in immunocompetent hosts (Baumgartner et al. 2000; de Carvalho et al. 2006; Campisi et al. 2008; Canabarro et al. 2013). Furthermore, *C. albicans* biofilms are extremely resistant to conventional antifungal agents. The inherent resistance of *C. albicans* to antifungal agents is mainly due to the extracellular matrix it produces, and the upregulation of efflux pumps, among other factors. The ATP-binding cassette transporter superfamily (including *CDR1* and *CDR2*) and the major facilitator class (including *MDR1*) are the main classes of efflux pumps which regulate antifungal drug exportation (Ramage et al. 2002; LaFleur et al. 2006).

Reactive oxygen species (ROS), which include the superoxide anion radical ($O2^{\cdot-}$), hydroxyl radical ($OH^{\cdot}$), alkyl peroxyl free radicals ($ROO^{\cdot}$) and non-radical molecules, such as hydrogen peroxide (H_2O_2), are generated continuously because of aerobic metabolism (Krumova and Cosa 2016). In eukaryotic cells, mitochondria are an important source of ROS. As ROS balance is a critically important consideration in regulating cell functions and survival, cells are equipped with intracellular antioxidant

defence systems. However, when this system is insufficient to maintain the ROS balance, ROS homeostasis is altered. Hence, ROS accumulation starts, which causes damage to biomolecules, such as proteins, lipids, and DNA, leading to cell death (Baronetti et al. 2011; Chang 2011; Dickinson 2011).

Oxidative stress is an important challenge for pathogens to grow and invade the host. *C. albicans* possesses a robust antioxidants defence mechanism to escape increased oxidative stress. ROS balance in *C. albicans* is regulated by superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), glutathione or glutaredoxin and thioredoxin (Kaloriti et al. 2014; Poopedi et al. 2020). The existence of SOD in *C. albicans* is associated with the need for a cytoprotective mechanism for *C. albicans* against not only exogenous ROS, but also endogenous fungal-derived ROS (Kelly et al. 1997; Hwang et al. 1999; Rhie et al. 1999). Antifungal agents currently used clinically in the treatment of fungal infections, include azoles (miconazole), polyenes (amphotericin B, clotrimazole) and echinocandins, which have also demonstrated activity against fungal biofilms. Furthermore, these drugs lead to increased ROS levels in fungal planktonic and biofilm cells (Delattin et al. 2014).

PRE has been widely investigated for its antioxidant activity and found to be a promising antioxidant that prevents injury caused by ROS through different mechanisms, including direct scavenging of ROS and the activation of antioxidant enzymes (Procházková et al. 2011). The radical scavenging activity of PRE has been associated with its highly polyphenolic content (Gil et al. 2000). While polyphenols are reported to provide high antioxidant activities to the host, they can also act as a pro-oxidant agent against microorganisms through the induction of elevated ROS and oxidative stress (Fathima and Rao 2016). The production of a phenoxyl radical or a redox complex with a transition metal ion is the basis for phenolic compounds' direct prooxidant actions. $O_2^{\cdot-}$, H_2O_2, and a complicated mixture of semiquinones and quinones can be produced when phenoxyl radicals react with oxygen (Hodnick et al. 1988). Numerous phenolic compounds' prooxidant activity is thought to cause lipid peroxidation, DNA damage, and apoptosis in both normal and malignant cells (Zheng et al. 2008).

In previous chapters (Chapter 4 and Chapter 5), the antimicrobial activity of PRE, Zn (II) and PRE/Zn (II) combination was studied against planktonic and biofilms of *C. albicans*. PRE/Zn (II) demonstrated synergistic activity in *in vitro* time-kill and checkerboard assays resulting in >2 log reductions in *C. albicans* cells, compared with PRE or Zn (II) alone. In addition, the anti-adhesive activities of PRE/Zn (II) were evaluated, and it was found to potentiate anti-adhesive activity by reducing viable cells on coated glass surfaces. However, the mechanism behind this synergistic antimicrobial activity remains to be identified.

In this chapter, the effect of PRE, Zn (II) and PRE/Zn (II) combination on ROS production and oxidative stress in *C. albicans* and whether these treatments influenced their redox status and antifungal activities overall was studied.

6.2 Aims and objectives

To determine whether ROS production is associated in synergistic antifungal activity of PRE/Zn (II) against *C. albicans*.

- ROS levels were evaluated using a of 2',7'-dichlorofluorescein diacetate (DCFH-DA) probe for planktonic cells of *C. albicans* (after 24 h treatment) and *C. albicans* biofilm (after 1 h and 24 h treatment) by confocal laser scanning microscopy (CLSM).
- Assess the effect of PRE, Zn (II) and PRE/Zn (II) on hyphal formation by *C. albicans*, using Calcofluor White staining and CLSM.
- To determine whether these effects can explain the synergistic antifungal activity of PRE/Zn (II) against *C. albicans.*

6.3 Materials and methods

Culture media, and *C. albicans* cultures was prepared as described in Chapter 2 (Section 2.2.3). Equipment, glass and plastics, chemical consumables and assay kits used in this chapter were presented in Section 2.1 in Table 2.1, Table 2.2, and Table 2.3, unless otherwise stated.

6.3.1 Effect of PRE, Zn (II) and PRE/Zn (II) on ROS generated by *C. albicans*

6.3.1.1 Preparation of dichloro-dihydro-fluorescein diacetate (DCFH-DA) solution

Dichloro-dihydro-fluorescein diacetate (DCFH-DA) (1 g) was dissolved in 40 mL of DMSO (99.9%) in a sterile tube to obtain 25 mg/mL (0.0515 M) solution and this stock solution was stored at –20 ℃. Then, 194 µL of DCFH-DA stock solution was added to 806 µL of DMSO to obtain 10 mM of DCFH-DA solution in a sterile tube. Then 10 µL of DCFH-DA stock solution was added to 9990 µL DMSO in a sterile tube (x1000 dilution) to obtain 10 µM final concentration and this was protected from light with foil.

6.3.1.2 Oxidative metabolite assay for biofilms

ROS generation was assessed on planktonic and biofilm states of *C. albicans* using a DCFH-DA probe (Peralta et al. 2015). For planktonic state, *C. albicans* cells (10^8 CFU/mL) were treated with PRE (MIC), Zn (II) (MIC), and PRE/Zn (II) (MIC+MIC) for 24 h to investigate the ROS generation during biofilm formation on glass bottomed 96-well plates in an aerobic incubator at 37 ℃. After 24 h, the supernatant was discarded, and the plates were first stained with 5 µL of Calcofluor-White (with 0.05 % v/v) for 1 min, this stain was excited at 355 nm. Then, 15 µL of 10 µM of DCFH-DA was added and incubated for 15 min in the dark at room temperature. This stain was excited at 488 nm excitation and used to measure ROS production.

For *C. albicans* biofilms, a similar method was performed. Briefly, 24 h *C. albicans* biofilms were generated and treated with PRE (MIC), Zn (II) (MIC), and PRE/Zn (II) (MIC+MIC) in glass bottomed 96-well plates as described in Chapter 4 (Section 4.3.6.3). Two different treatment times (1 h and 24 h) were used to see the effect on ROS generation. After treatment, the supernatant was discarded, and the same staining and imaging procedure was applied.

Stained wells were then visualised with a Leica TCS SP5 Confocal Microscope (Leica Microsystems Ltd, Milton Keynes, UK). Images were obtained with 60x 1.8 oil objective with a z-step size of 1 μm. Z-stack images were analysed by using COMSTAT2 with ImageJ analysis.

The randomly selected fields of view were analysed using COMSTAT2 software with the NIH-ImageJ analysis software for biomass ($\mu m^3/\mu m^2$) of two channels, blue for *C. albicans* cell wall, and green for ROS. The ROS level was normalised to total biomass of Calcofluor-White *C. albicans* and presented in a graph as mean±SEM.

6.3.1.3 Effects on hyphal growth

The effect of PRE (MIC), Zn (II) (MIC), and PRE/Zn (II) (MIC+MIC) on *C. albicans* hyphal growth was ascertained by Calcofluor White staining of *C. albicans* biofilms. *C. albicans* biofilms were developed in glass bottomed 24- well plates by adding 500 μL of overnight experimental culture of *C. albicans* (10^8 CFU/mL) and incubating at 37 ℃ for 24 h in an aerobic environment. Supernatants were discarded and the biofilms treated with 500 μL of PRE (MIC), Zn (II) (MIC), or PRE/Zn (II) (MIC+MIC) for 24 h in an aerobic incubator at 37 ℃. Supernatants were carefully removed and gently washed once with phosphate buffered saline (PBS) (pH 7.2), to remove unattached cells. Biofilms were stained with Calcofluor-White (0.05% v/v) for 1 min imaged using CLSM. Three random images were taken from each well and three replicate wells were used for each untreated control and treatment groups. Images were analysed using ImageJ analysis software and the results presented as mean±SEM of fluorescence intensity.

6.3.2 Statistical analysis

All experiments were performed on at least three independent occasions, with two internal replicates. Results were analysed using GraphPad Prism software (GraphPad Software, Version 8.2.1, San Diego, CA, USA), by performing one-way ANOVA with post- test Tukey analysis and $p<0.05$ was considered statistically significant. All data were presented as the mean value with SEM.

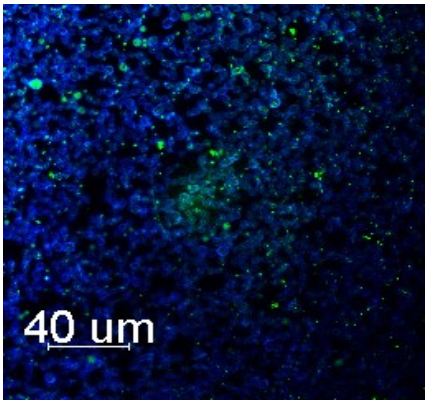
6.4 Results

6.4.1 Confocal laser scanning microscopy and COMSTAT2 analysis

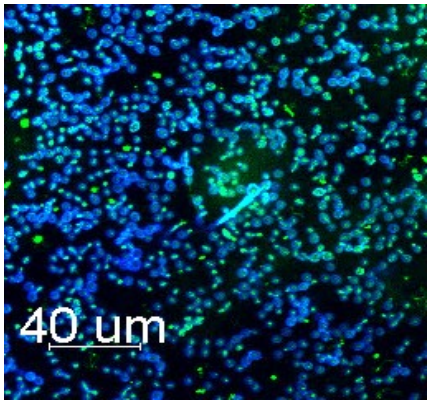
The images obtained for *C. albicans* planktonic cells after 24 h treatment with PRE (MIC), Zn (II) (MIC), and PRE/Zn (II) (MIC+MIC) are presented in Figure 6.1A. ROS production was observed in each group, with Zn (II) and PRE/ Zn (II) showing higher green fluorescence than growth control (GC) and the PRE- treated group. COMSTAT2 analysis showed the untreated GC group and the PRE-treated group exhibited similar levels of ROS production (Figure 6.2B). While *C. albicans* cells treated with Zn (II) produced more ROS, there was no significant difference compared to untreated GC group ($p>0.05$). However, PRE/Zn (II) showed enhancement in ROS production, which was significantly higher than the GC, PRE, and Zn (II) treated groups ($p<0.0001$).

ROS levels were visualised in *C. albicans* biofilms treated for 1 h and 24 h with PRE (MIC), Zn (II) (MIC), and PRE/Zn (II) (MIC+MIC). After 1 h, similar images were obtained for all treatment groups and the GC group, with no noticeable effect on ROS levels in *C. albicans* preformed biofilms (Figure 6.2A). In addition, there was no statistically significant differences between normalised ROS levels between all groups (Figure 6.2A and B). However, after 24 h treatments, the PRE/Zn (II) combination exhibited higher intracellular ROS production, as evident in Figure 6.3A. In addition, Figure 6.3B showed that the normalised levels of ROS for the PRE/Zn (II) treatment were significantly higher compared with the GC group ($p<0.001$) and PRE and Zn (II) treatments ($p<0.01$).

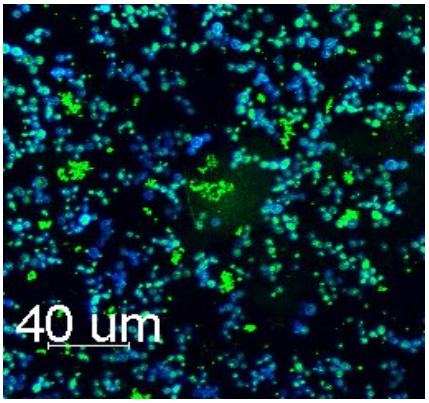
GC



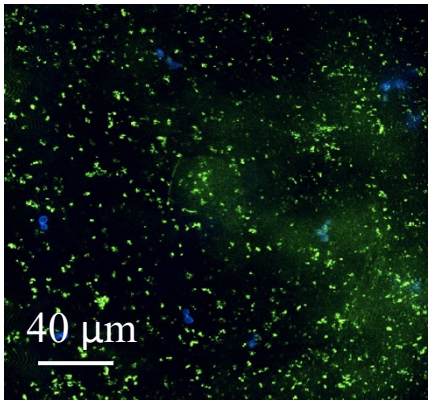
PRE



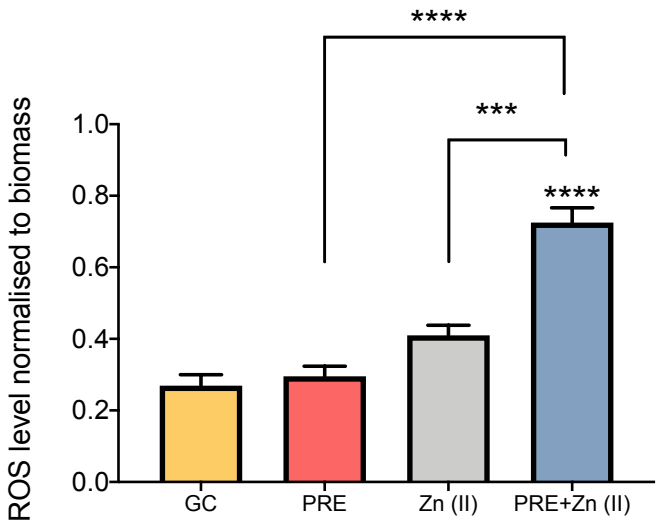
Zn (II)



PRE +Zn (II)



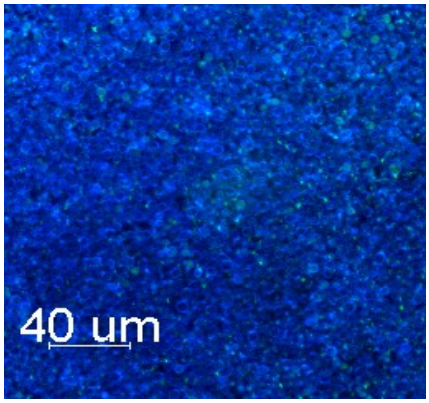
(A)



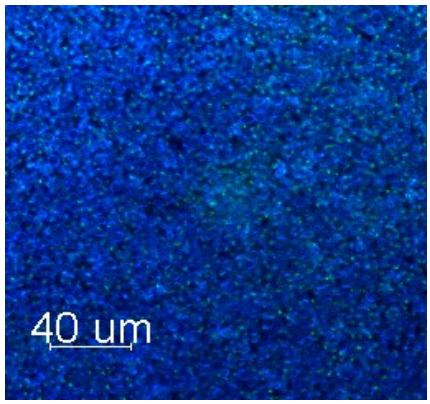
(B)

Figure 6.1: (A) CLSM of *C. albicans* incubated with PRE (MIC), Zn (II) (MIC), and PRE/Zn (II) (MIC+MIC) for 24 h. Blue channel shows calcofluor *in C. albicans* cell walls, and the green channel shows the oxidation of the DCFH probe as an indicator of ROS production. (B) The graph shows ROS levels for treated and untreated C. albicans cells normalised to total biomass, as quantified using COMSTAT2 and Image J software. Mean ± SEM, N=3. Significant differences indicated by *, where ***$p<0.001$, and ****$p<0.0001$, compared to the untreated growth control group (indicated on bars) and compared between treatment groups (indicated by lines).

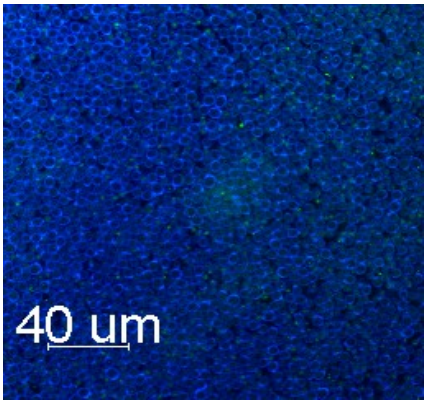
GC



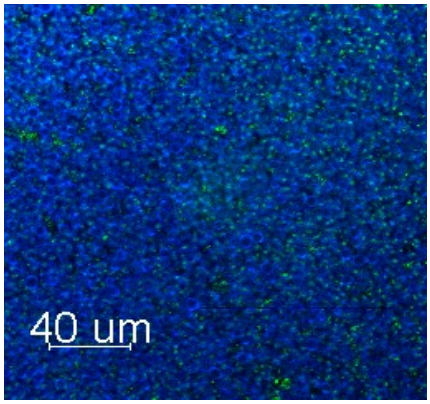
PRE



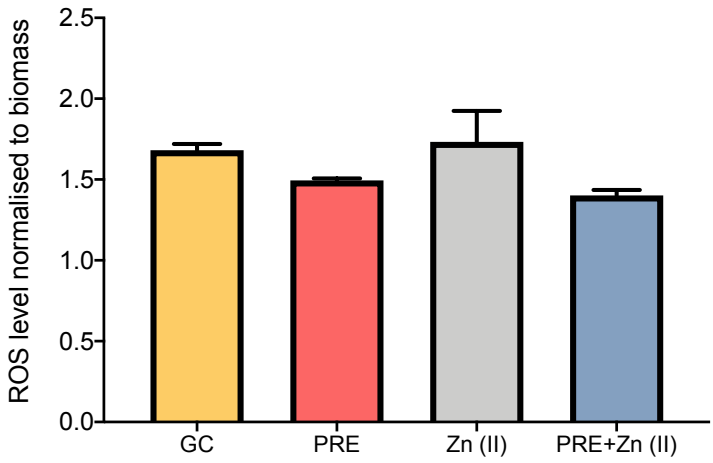
Zn (II)



PRE +Zn (II)



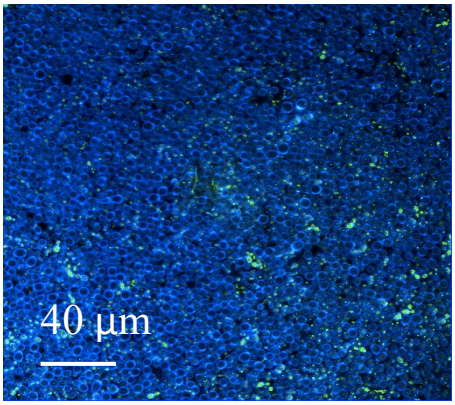
(A)



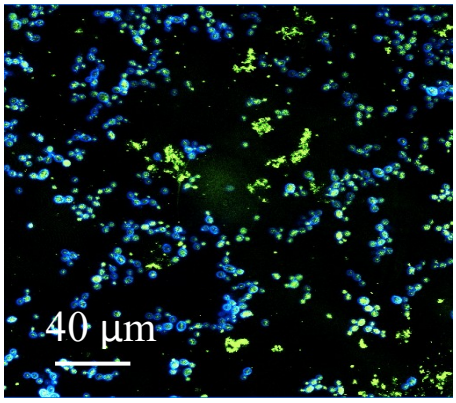
(B)

Figure 6.2: (A) CLSM of *C. albicans* biofilms incubated with PRE (MIC), Zn (II) (MIC), and PRE/Zn (II) (MIC+MIC) for 1 h. Blue channel shows calcofluor in *C. albicans* cells wall in biofilm, and the green channel shows the oxidation of the DCFH probe as an indicator of ROS production. (B) The graph shows ROS levels for treated and untreated *C. albicans* biofilms normalised to total biomass, as quantified using COMSTAT2 and Image J software. Mean ± SEM, N=3.

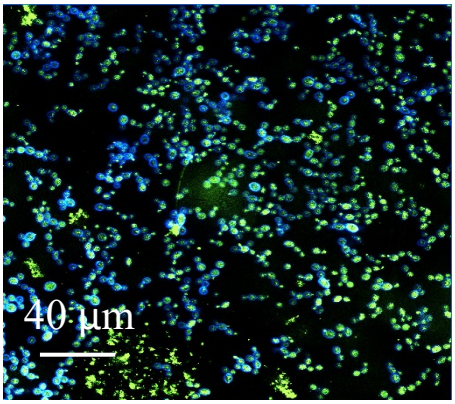
GC



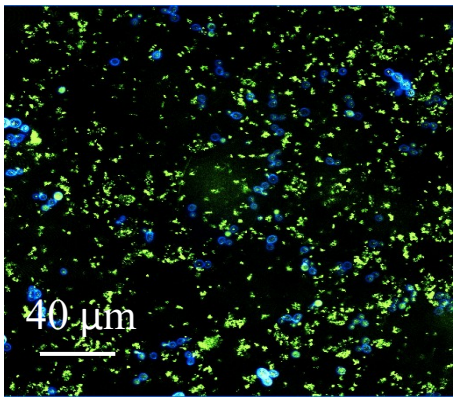
PRE



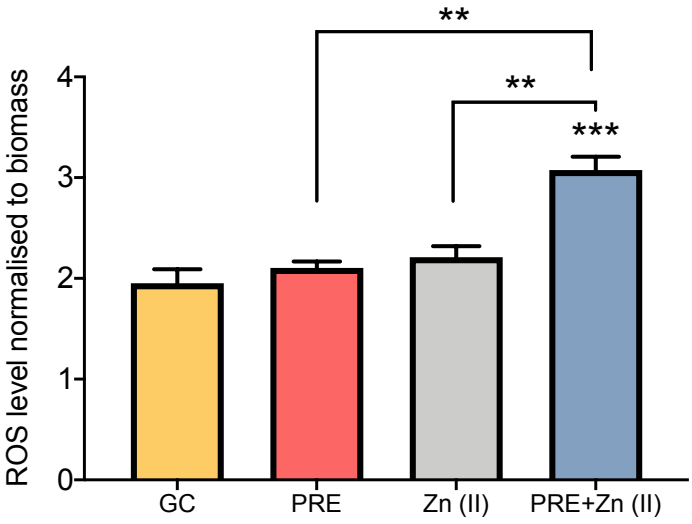
Zn (II)



PRE +Zn (II)



(A)



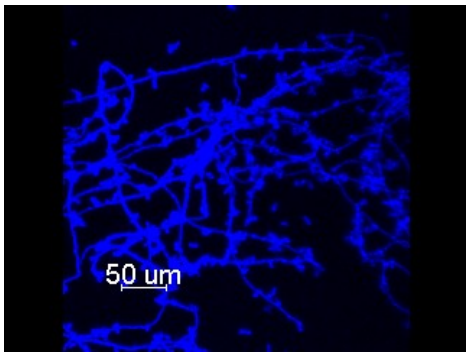
(B)

Figure 6.3: (A) CLSM of *C. albicans* biofilms incubated with PRE (MIC), Zn (II) (MIC), and PRE/Zn (II) (MIC+MIC) for 24 h. Blue channel shows calcofluor in *C. albicans* cell walls in biofilms, and the green channel shows the oxidation of the DCFH probe as an indicator of ROS production. (B) The graph shows ROS levels for treated and untreated *C. albicans* biofilms normalised to total biomass, as quantified using COMSTAT2 with Image J software. Mean ± SEM, N=3. Significant differences indicated by *, where, $^{**}p<0.01$, and $^{***}p<0.001$ compared to the untreated growth control group (indicated on bars) and compared between treatment groups (indicated by lines).

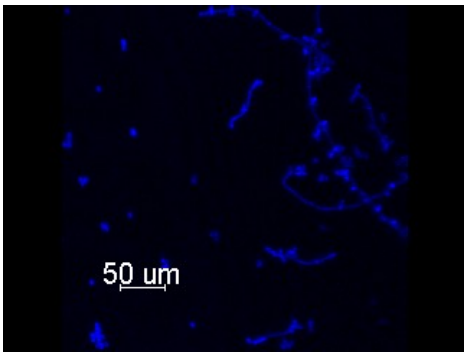
6.4.2 Effects on hyphal growth of *C. albicans* in biofilm

PRE (MIC) showed the greatest inhibition of hyphal growth in biofilm (Figure 6.4A). PRE/Zn (II) also inhibited hyphal growth, compared to the GC group, but this inhibition was lower than with PRE-only treated group. PRE exhibited the lowest fluorescence intensity and there was a significant difference between PRE treated group and both the GC group and PRE/Zn (II) group ($p<0.0001$). Furthermore, Zn (II) and PRE/Zn (II) also showed statistically lower fluorescence intensities, compared to the GC group ($p<0.001$ and $p<0.0001$ respectively).

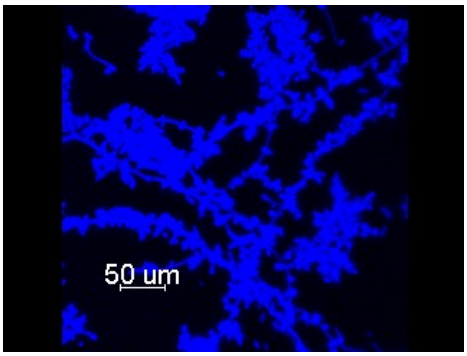
GC



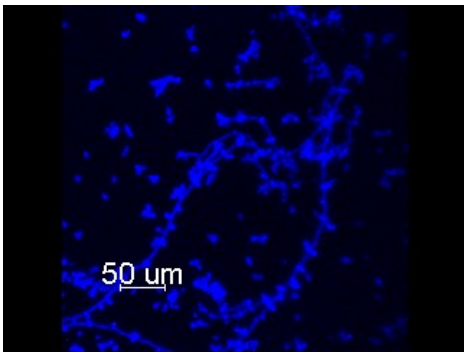
PRE



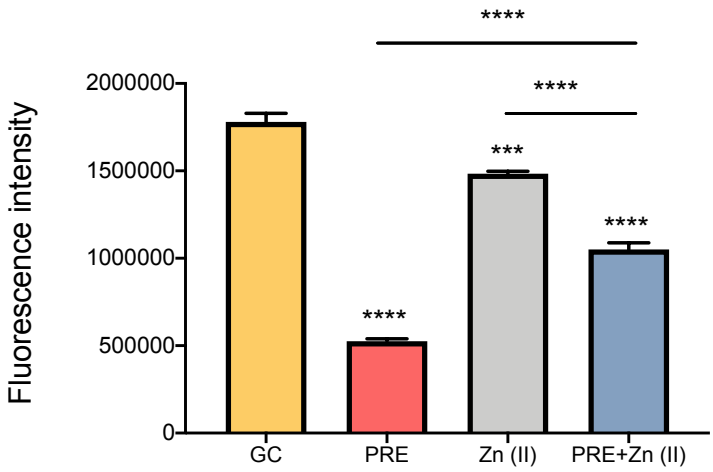
Zn (II)



PRE +Zn (II)



(A)



(B)

Figure 6.4: Effect of PRE, Zn (II) and PRE/Zn (II) combination on pre-formed biofilms of *C. albicans.* (A) CLSM imaging of treated and untreated *C. albicans* biofilms, stained with Calcofluor-White (for *C. albicans* cell walls in blue, scale bar = 50 µm). (B) The fluorescence intensities of CLSM images. Significance indicated by *, where ****p<0.001,* and *****p<0.0001* compared to untreated growth control group (indicated on the bars) and compared to between treatment groups (indicated by lines). Data represented as mean±SEM (N=3).

6.5 Discussion

Treatment of *C. albicans* infection can be difficult, due to the biofilm formation capabilities of *Candida* species and their increased resistance to antifungal agents (Paraje et al. 2008; Mah 2012). The increased rates of antifungal drug resistance are a global concern, making treatment of fungal infections more difficult (Delattin et al. 2014). There are several mechanisms suggested for increased resistance against antifungal agents, including two types of efflux pump genes, the ATP binding cassette transporters, CDRs (CDR1 and CDR2) and the major facilitator, MDR1 (White et al. 2002; Mah 2012). For example, azoles are commonly used in clinical management of fungal infections and the resistance against azole antifungal agents is considered a major challenge in the treatment of fungal infections (Whaley et al. 2017).

In Chapter 4, synergistic activity of the PRE/Zn (II) against *C. albicans* was demonstrated. PRE/Zn (II) demonstrated synergistic activity in an *in vitro* checkerboard assay (FICI <0.5) and time-kill assay by causing >2 log reductions in *C. albicans*, compared to when PRE or Zn (II) were applied alone. In addition, in Chapter 5, the anti-adhesive activities of the PRE/Zn (II) combination exerted a potentiated anti-adhesive activity, by reducing viable cells on coated glass surfaces. Although several suggested mechanisms were discussed in previous chapters, the mechanism behind this activity is not clear. In this chapter, however, the focus was on whether addition of Zn (II) to PRE influenced the anti-/pro-oxidant activity, and if it had any role in this synergistic antifungal activity against *C. albicans*.

In general, antifungal compounds with ROS inductive activities have been considered a promising fungicidal strategy against both planktonic and biofilm forms of *C. albicans* (Delattin et al. 2013). For instance, it was reported that miconazole induces the accumulation of endogenous ROS in *C. albicans* biofilms (Vandenbosch et al. 2010). Moreover, a high level of ROS inactivating activity has been found in miconazole-tolerant cells (Mah 2012).

ROS has been considered during development of effective antifungal agents, due to the apoptotic effects on different cell types. In eukaryotic cells, mitochondria have

fundamental roles, including energy production, synthesis of key metabolites, apoptosis regulation, calcium buffering and production of endogenous ROS (Ott et al. 2007). Therefore, cells have developed various enzymatic and non-enzymatic antioxidant defence mechanisms. SOD, catalase (CAT), and glutathione peroxidases (GPX) are examples of enzymatic antioxidant systems in cells, including *C. albicans*. It has been reported that SOD has an important role in the virulence of *C. albicans* (Ott et al. 2007; Bink et al. 2011). Several studies induced *C. albicans* apoptosis, due to increased ROS levels with acetic acid, resveratrol, farnesol, and antimicrobial peptides (Phillips et al. 2003; Cho and Lee 2011; Zhu et al. 2011; Lee and Lee 2015). Moreover, the fungicidal activity of commonly used antifungal agents, such as azoles, have been partly associated with their ROS elevated effects, in addition to their target specific activities (Delattin et al. 2013).

PRE has been widely considered as an antioxidant, due to its high polyphenolic content, particularly hydrolysable tannins which include a central polyol, such as glucose surrounded by several gallic acid units, and more complex ellagitannins from pentagalloylglucose derivatives by oxidative reactions (Gil et al. 2000; Mueller-Harvey 2001). The characterization of PRE in Chapter 3, identified polyphenolic compounds in PRE including hydrolysable tannins and anthocyanins. The *in vitro* antioxidant activity of polyphenols has been well described. Generally, this *in vitro* antioxidant activity has been associated with polyphenols' redox behaviour and electron transfer reactions (Chiorcea-Paquim et al. 2020). For instance, punicalagin, which is one of the most abundant polyphenolic compounds in PRE, has been found as α- and β-punicalagin anomers. These compounds rapidly interconvert under acidic pH conditions, and this chemical shift change occurs upon deprotonation, which generally enhances antioxidant activity (Kraszni et al. 2013; Oudane et al. 2017).

However, antioxidants can also act as pro-oxidants, depending on concentration or presence of metal cations resulting in free radicals (Yamasaki et al. 1998). The dual antioxidant and pro-oxidant actions of plant phenolic compounds has been reported (Parejo et al. 2003; Murzakhmetova et al. 2008; Šamec et al. 2015). Similar dual action in oxidative mechanisms has been demonstrated for Zn (II). Zn (II) is an essential element with critical functions for structural development and catalysis of enzymes

and in signal mediation in biological systems. Zn (II) plays an important role in redox regulation by being a key component of the active site of Cu/Zn SODs and antioxidant systems overall (Mocchegiani et al. 2011). Similarly, defining the role of Zn (II) as an antioxidant or pro-oxidant is not straightforward, due to the variability and complexity of Zn (II) activity (Lee 2018). There are specific circumstances that influence Zn (II) activity towards being either antioxidant or pro-oxidant. While Zn (II) does not have redox activities itself, it has been referred to as a pro-antioxidant (Hao and Maret 2005). As treatment with extracellular Zn (II) increases the free Zn (II) level, the imbalance of this increased free Zn (II) level plays an important role in its effect on oxidative stress. For instance, high concentrations of Zn (II) can trigger oxidative stress through intracellular suppression of metabolism and mitochondrial functions, because elevated free Zn (II) concentrations can cause protein mis-folding (Butler and Loh 2007; Lu et al. 2016).

Pro-oxidative agents typically cause oxidative stress by generating ROS. However, a similar effect can arise due to inhibition of endogenous antioxidant systems. In this study, *C. albicans* was treated with PRE (MIC), Zn (II) (MIC) and PRE/Zn (II) (MIC+MIC) and effects on ROS levels in *C. albicans,* observed via a commonly used DCFH-DA fluorescence-based method (Bonini et al. 2006). DCFH-DA is a non-polar and non-fluorescent molecular probe and can diffuse across membranes. DCFH-DA then hydrolyses intracellularly to the polar, non-fluorescence, membrane impermeable form, 2′,7′- dichlorodihydrofluorescein (DCFH). DCFH is rapidly oxidised to the highly fluorescence derivative, 2′,7′-dichlorofluorescein (DCF) by ROS (Weissman et al. 2005). ROS impacts on the viability of cells through enzyme inactivation, membrane disruption and induction of cell death (Kobayashi et al. 2002). Furthermore, excessive accumulation of ROS is a fundamental biomarker of apoptosis that supports morphological changes, nuclear fragmentation, chromatin condensation, cellular swelling, and phosphatidylserine externalisation (Pozniakovsky et al. 2005). Results showed that PRE and Zn (II) did not cause induced intracellular ROS production when applied alone. However, the PRE/Zn (II) (MIC+MIC) combination produced increased intracellular ROS levels, which could be the reason of synergistic antifungal activity of this combination against *C. albicans*. Moreover, this increased

ROS level was observed for planktonic and biofilm forms of *C. albicans* after 24 h treatment.

Elevated oxidative stress is harmful for cells and tissues (Rafieian- Kopaie and Baradaran 2013). The pro-oxidant properties of phenolic compounds under specific conditions, such as the presence of redox active transition metals, have been shown (Prochazkova et al. 2011). It has been proposed that this dual action of polyphenols on oxidative stress depends on the physiological conditions and on the polyphenol chemical properties (Chiorcea-Paquim et al. 2020). Phenolic compounds can act as pro-oxidants by generating ROS, such as H_2O_2 and phenoxy radicals, via polyphenol autooxidation. This autooxidation happens via a Fenton reaction and the presence of oxygen sources, such as O_2 or H_2O_2, and metal ions, such as Fe (II) and Cu (II) (Cao et al. 1997; Fukumoto and Mazza 2000; Sakihama et al. 2002). In addition, it has been reported the redox inactive metals, including Al (III), Zn (II), Cd (II), Mg (II), and Ca (II), can also induce the pro-oxidant activity of polyphenols, due to their stabilising role on phenoxyl radicals (Yamasaki et al. 1998; Rout and Das 2003; Kalinowska et al. 2018). Although phenoxyl radicals are generally unstable and can be converted to non-radical molecules through polymerisation, enzymatic and non-enzymatic reductions, the presence of metal ions can enhance the stability of phenoxyl radicals by prolonging their lifetime.

This could be the reason for the elevated ROS levels observed in this study for PRE/Zn (II) combination in both planktonic and biofilm forms of *C. albicans*, as this pro-oxidant activity may cause critical damage to mitochondrial transmembrane potential (Galati and O'Brien 2004). The pro-oxidative properties of the PRE/Zn (II) combination may inhibit mitochondrial respiration enzymes, such as NADH oxidase and succino-oxidase (Bohmont et al. 1987). However, penetration of cell by PRE and PRE/Zn (II) to cells is necessary and further studies should be conducted to assess whether this occurs in *C. albicans* cells. Hence, it has been suggested that pro-oxidant activity could be the reason for polyphenols antimicrobial and anti-pathogenic bioactivities (Chiorcea-Paquim et al. 2020). In this present study, the ROS-mediated antifungal activity of the PRE/Zn (II) combination was demonstrated by oxidation of the fluorescence dye DCF in *C. albicans,* following PRE/Zn (II) treatment.

ROS can be harmful causing DNA damage and mutagenesis for a wide variety of cell molecules, including nucleic acids, proteins, and lipids (Hwang et al. 2011). However, it is difficult to determine the specific events which damage *C. albicans,* and more in-depth studies are necessary to have a fuller understanding of the role of ROS in this synergistic antifungal activity of the PRE/Zn(II).

In this chapter, PRE/Zn (II) effects on induced ROS levels within *C. albicans* were confirmed. Several studies have demonstrated oxidative stress induction from many plant polyphenols in the presence of transition metal ions, such as copper (Ahmad et al. 1992; Azmi et al. 2005; Arif et al. 2017). *C. albicans* has an ability to grow in three morphological forms, namely budding yeast, pseudo hyphal and hyphal forms. This morphological adaptivity provides an advantage to *C. albicans* in the development of biofilms on surfaces of medical devices, resulting in biofilm-associated infections (Sudbery 2011; Zhao et al. 2013). The disruptive effects of PRE, Zn (II) and PRE/Zn (II) on *C. albicans* biofilms were shown via CLSM images and quantitative COMSTAT2 analysis. In the absence of test compounds, *C. albicans* developed high-density biofilms. All treatments led to significant reduction in biofilm biomass, but only PRE-treated *C. albicans* biofilms showed significant reductions in mean thickness. In this chapter, the effect of the compounds on the morphological form of *C. albicans* biofilms was assessed with Calcofluor White staining. The fluorescence intensity was measured since Calcofluor White is a fluorochrome that binds to chitin in the fungal cell wall, which is synthesised by enzymes in the plasma membrane. Thus, any change in plasma cell membrane can show an effect on cell wall chitin, and damage in the cell wall results in lower fluorescence intensities compared to undamaged cell walls (Chaffin et al. 1998). PRE was the most effective compound at inhibiting hyphal growth and this result is similar to PRE's effect on *C. albicans* biofilm mean thickness. It was an expected outcome since the hyphal form of *C. albicans* contribute to the 3D architecture of *C. albicans* biofilms.

There are limited numbers of antifungal agents and there is thus an urgent need to discover new drugs to treat fungal infections. Polyphenols are increasingly attractive as alternative agent in the treatment of infectious diseases due to their broad-spectrum

antibacterial and antifungal properties (Viuda-Martos et al. 2010). Pomegranate rind is abundant with phenolic compounds and the PRE/Zn (II) combination is known for its synergistic antibacterial, anti-viral and anti- inflammation activities (Houston et al. 2017b; Celiksoy and Heard 2021; Celiksoy et al. 2021).

6.6 Conclusion

The possible synergistic activity of the PRE/Zn (II) combination against *C. albicans* was investigated, evaluating the effect on intracellular ROS production of *C. albicans*. PRE/Zn (II) induced intracellular ROS production, for both planktonic and biofilm cells for *C. albicans*. Therefore, this could be the mechanism behind the synergistic activities previously observed. Synergistic activity was also observed in the assays based on the viable number of bacteria, rather than other parameters. For example, it was also found that PRE had the highest inhibition activity on hyphal growth of *C. albicans,* and the fluorescence intensity result of Calcofluor-White staining demonstrated that the fluorescence intensity of PRE was significantly lower than for the PRE/Zn (II) combination ($p<0.0001$). However, from the current results, it could not be determined whether the effects of the PRE/Zn (II) combination on *C. albicans* were caused by elevated ROS production or by inhibition of the antioxidant mechanisms in *C. albicans*. However, further research is required into the impact of such an increase in ROS levels on *C. albicans* behaviour following PRE/Zn (II) application, in order to better understand its mechanisms of action.

Chapter 7: Antioxidant and Wound-Healing Activity of PRE, Punicalagin and in Combination with Zn (II)

7.1 Introduction

Wound healing is a complex process, involving a chain of well-orchestrated biochemical and cellular events that affect the growth and regeneration of wounded tissue. This is mainly achieved through four precise and programmed phases: homeostasis, inflammation, proliferation, and remodelling. These phases must occur in an orderly and suitable timeframe essential for proper healing, which can be inhibited by many factors causing delayed or non-healing wounds to occur (Guo and Dipietro 2010).

Oral wounds can be caused from a trauma, periodontal diseases, tooth extraction and oral surgery. However, the oral cavity has a distinct environment bathed in a continuous warm fluid and millions of microorganisms. There are some differences between dermal and oral mucosal tissues in terms of wound healing. It has been shown that the oral mucosa heals more rapidly and without scar formation, compared to dermal tissue (Szpaderska et al. 2003). Fibroblasts, keratinocytes, saliva, and some other intrinsic factors have a role in this clinical difference between dermal and oral mucosal wound healing (Mandel 1987; Zelles et al. 1995; Schor et al. 1996; Turabelidze et al. 2014). However, oral wounds cannot be protected with a bandage as can dermal wounds, whilst the oral cavity also faces potential trauma due to events such as mastication (Politis et al. 2016). Moreover, the oral cavity has a relatively large commensal microflora. For the treatment of oral wounds, antibiotics, corticosteroids, non-steroidal anti-inflammatory drugs (NSAIDs) and disinfectants such as chlorhexidine have been used to accelerate the healing process (Cleland et al. 2001). However, these drugs have many side effects, such as failure bacterial resistance, gastrointestinal damage, tooth discoloration, dysgeusia and excessive sensitivity of the oral mucosa (Gjermo et al. 1989). Therefore, new alternative compounds are needed for oral wound healing.

The wound healing capabilities of PRE have been reported, in addition to its anti-inflammatory, antimicrobial and antioxidative activities, that have been attributed its phenolic content (Murthy et al. 2004). However, no studies have been performed to evaluate such activities from an oral wound perspective. Moreover, punicalagin, which is the main ellagitannin in PRE, has not yet been studied for a deeper evaluation of the compositional-functional relationship for this bioactivity. The possibility of potentiated wound healing activity for PRE and punicalagin in combination with Zn (II) was chosen for investigation in this Chapter, as Zn (II) has a demonstrated activity in wound healing. The potentiated antimicrobial and anti- inflammatory activity of the PRE/Zn (II) has also previously been reported (McCarell et al. 2008; Houston et al. 2017a; Houston et al. 2017b; Houston et al. 2017c). Therefore, in this study, it was hypothesised that potentiated activity could be a promising combination for oral wound healing activity.

7.1.1 The role of zinc in wound healing

Zinc is a fundamental trace element in the human body, and it plays an important role for skin and body health (Schwartz et al. 2005; Lansdown et al. 2007; Kogan et al. 2017). Zinc is the second most abundant element after iron in the human body and has many crucial roles in diseases, including wound healing (Prasad 1995). Zinc and zinc-containing compounds have an active role in every step of wound repair (Jones and Williams 2004). Zinc has crucial roles in the modification of extracellular matrix (ECM), cell migration, protein synthesis and in the reduction of inflammation (O'Dell 1992; Vallee and Falchuk 1992; O'Dell 2000).

Zinc plays a key role as a cofactor in many transcription factors and enzyme systems (Lansdown et al. 1999). These zinc finger transcription factors modify the gene expression and thereby regulate cellular behaviour (Zhu et al. 2004). Zinc finger transcription factors act with DNA and RNA polymerases, which are zinc- dependent enzymes, to induce transcription of key genes for wound healing, such as in cellular replication and genes encoding extracellular matrix (ECM) proteins (Berg and Shi 1996; Bao et al. 2003; Sum et al. 2005). Matrix metalloproteinases (MMPs) are zinc-dependent enzymes, that are actively involved in wound repair. MMPs augment auto-

debridement and keratinocyte migration during the wound healing process. Moreover, MMPs activity can be inhibited by removing zinc using chelating agents (Springman et al. 1995; Gomis 2003; Nagase et al. 2006).

7.1.2 ROS activity in periodontal diseases

Periodontal diseases are inflammatory diseases. While the pathogenesis mechanism of periodontal diseases is not fully understood, it has been suggested that bacterial colonisation, predominantly Gram-negative, aerophilic or microaerophilic bacteria, in the sub-gingival area release toxic products (Bartold et al. 2005). These toxic products cause tissue destruction directly. Additionally, they induce the host immune defence response and start the inflammation process (Page and Kornman 2000; Nair 2006). The host defence systems, especially polymorphonuclear leucocytes (PMNs), produce reactive oxygen species (ROS) to the inflammation site (Page and Kornman, 1997). However, the accumulation of ROS at the inflammatory site could further increase and worsen the inflammation process (Battino et al. 1999; Waddington et al. 2000). Excessive ROS production can cause indiscriminate biomolecular and cellular damage contributing to periodontal disease progression and impeding repair processes.

7.1.2.1 The antioxidant effects of PRE, Zn (II) and PRE in combination with Zn (II)

Pomegranate has been used since ancient times by many cultures, due to its various health effects as pomegranate extracts are a rich source of phenolic compounds. This beneficial health activity could relate to different phenolic components, such as punicalagin isomers, ellagic acid derivatives and anthocyanins (delphinidin, cyanidin and pelargonidin 3-glucosides, and 3,5-diglucosides) (Singh et al. 2009; Bekir et al. 2013a; Bekir et al. 2013b).

Recently, the antioxidant ability of pomegranate extracts has been studied in *in vitro* and *in vivo* assays. Free radical scavenging and lipid oxidation capacity of these phenolic compounds have been reported with *in vitro* assays (Gil et al. 2000; Noda et al. 2002). Moreover, it has been shown that pomegranate peel extracts have more

antioxidant activity and phenolic compounds than the pomegranate pulp, seed, and arils (Tomas-Barberan et al. 2001; Guo et al. 2003; Li et al. 2015). Pomegranate peel extract protects against highly reactive, hydroxyl radicals (·OH), which can damage most biological molecules, and this is through its metal-chelating property (Ou et al. 2002; Huang et al. 2005). Murthy et al. (2002) undertook an *in vivo* study to show the protective effect of pomegranate peel extract against CCl4- induced hepatoxicity, which involves highly destructive damage by ROS, and it was shown that pomegranate peel extract protected Wistar rats from ROS damage.

Cells have enzymatic and non-enzymatic antioxidant systems to remove excessive ROS. Superoxide dismutase, glutathione peroxidase, glutathione reductase, catalase and heme oxygenase are some of the major factors in enzyme-dependent antioxidant cellular mechanisms (Castro and Freeman 2001; Rahman 2007; Bhattacharyya et al. 2014). Superoxide dismutase has several isoforms, and zinc is a cofactor of cytosolic and extracellular Zn/Cu superoxide dismutase. This enzyme is a ROS scavenger and catalyses the O^{2-} radical into less harmful O_2 and H_2O_2 (Mariani et al. 2008). Zinc also induces metallothionein production, which is effective scavenger of OH. Iron and copper play a role in the production of OH from H_2O_2. However, zinc competes with iron and copper for cell membrane binding and causes reduced production of OH (Prasad 1993). Zinc also exerts antioxidant activity via inducing heme oxygenase and inhibiting the NADPH oxidase, which is a plasma membrane-associated enzyme that catalyses production of O^{2-} using NADPH as the electron donor (Tapiero and Tew 2003; Prasad 2014). It was reported that critical transcription factors are involved in regulation of gene expression of antioxidant and antioxidant molecules are up regulated by zinc (Zhao et al. 2010). Zinc has antioxidant activity in the oral cavity by reducing production of ROS in above mentioned mechanisms. Furthermore, it disturbs the respiration system in *Fusobacterium nucleatum* and other microbes, and with this antimicrobial activity it prevents ROS production in the oral cavity (Nayak et al. 2010; John et al. 2011; Salari et al. 2017).

7.2 Aims and objectives

The bioactivity of pomegranate extracts manifests through as antimicrobial, antioxidant and anti-inflammatory properties, as described above. The aims of this Chapter were to evaluate:

- PRE, Zn (II), and PRE/Zn (II) effects on the viability, proliferation, and migration effect on keratinocytes (HaCaTs) and human primary gingival fibroblasts (HPGFs).
- Punicalagin has been considered the main phenolic compound in PRE and it was included this study to obtain a deeper understanding about the compositional-functional relationship of PRE on wound healing activities. Therefore, punicalagin, Zn (II) and punicalagin/Zn (II) effects on viability, proliferation, and migration effect on HaCaTs and HPGFs were also investigated.
- The antioxidant activities of PRE, punicalagin and their combination with Zn (II) were further assessed using *in vitro* free radical scavenging assays.

7.3 Materials and methods

Equipment, glass and plastics, chemical consumables and assay kits used in this chapter were presented in Section 2.1 in Table 2.1, Table 2.2, and Table 2.3, unless otherwise stated.

7.3.1 Assessment of the antioxidant activities of PRE, punicalagin and Zn (II) using *in vitro* assays

7.3.1.1 DPPH (2,2-Diphenyl-1-picrylhydrazyl) radical scavenging assay

The DPPH assay was used to evaluate scavenging of stable radicals by PRE, punicalagin and their combination with Zn (II). In this assay, the antioxidant reacts with stable free radicals, by transferring electrons or hydrogen atoms to DPPH and neutralising the free radical. In this colorimetric-based assay, the dark violet colour of DPHH changes from dark violet to yellow. The assay was performed as previously described (Okonogi et al. 2007). Briefly, 0.2 mM DPPH solution was prepared by dissolving 39.4 mg DPPH in 1 mL methanol and adjusted to 500 mL methanol using a volumetric flask. The DPPH solution was wrapped with aluminium foil to prevent light degradation and stored at 4°C. The 96-well plate was separated into columns and columns were labelled DPPH only, samples only, and DPPH and samples. Two-fold serial dilutions were made for each sample, then the plate was wrapped with foil to protect from light degradation and incubated for 30 min at room temperature. At the end of the incubation, the plate was read at 515 nm using a plate reader to measure absorbance (Abs), as described above, including a blank sample containing only DPPH as the negative control and ascorbic acid as the positive control.

The percentage of the radicals scavenging activity of compounds were calculated using the formula:

% DPPH scavenging = 100 x [(Abs Sample+ DPPH) - (Abs Sample Blank)]/ [(Abs DPPH) - (Abs Solvent)].

The concentration of the sample where scavenging was 50 % of the initial DPPH radical was calculated by interpolating the [(Abs of the sample) - (Abs Sample Blank)] into a calibration line generated by the Abs of DPPH at different concentrations. Each experiment was repeated on at least three independent occasions, and each repeat contained at least three replicate wells. Data was presented as mean±SEM.

7.3.1.2 ABTS (2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic-acid) radical scavenging assay/TEAC (trolox equivalent antioxidant activity)

The ABTS/TEAC assay was performed, as described by Re et al. (1999) with minor changes for determination of trolox equivalent antioxidant capacity (TEAC) values for PRE, punicalagin and their combinations with Zn (II). The assay was based on the ability of compounds to scavenge the ABTS radical (ABTS·). The ABTS· radical was produced by reaction between 7 mM ABTS solution and 2.45 mM potassium persulphate solution. These components were mixed and stored in the dark for 12-16 h at 30˚C. Both solutions were prepared in distilled water in a total volume of 100 mL. The prepared ABTS· solution was diluted with 5 mM PBS solution, pH 7.4, until a final absorbance of 0.7±0.2 at 734 nm was obtained. Concentrations of 0.5 mg/mL PRE, 0.5 mg/mL punicalagin and 0.125 mM Zn (II) were used in this assay. Trolox 0-400 µg/mL was used as a positive control to prepare a standard curve and to enable results to be expressed as TEAC. Each experiment was repeated at least three times independently, and each repeat contained at least three replicate wells. Data was presented as mean±SEM.

7.3.2 Effects of PRE, punicalagin and PRE/Zn (II) combination on fibroblast and keratinocyte wound healing responses

The methodology for general cell culture methods and the preparation of extracts and buffers are as described in Chapter 2 (Section 2.2.4)

7.3.2.1 MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) Colorimetric Assay

Human primary gingival fibroblasts (HPGFs) and human adult, spontaneously immortalised, epidermal keratinocytes (HaCaT cells), were seeded in 96 well-plates. HPGF cells were used between passages 2-7. The experiment protocol was performed based on the method of Moses et al. (2020).

PBS (100 µL) was placed in all outer wells of the 96 well-plates to maintain the cells' environmental conditions during the experiment. HaCaTs were seeded at a cell density of $5x10^3$ cells per well in 10 % serum-containing medium (DMEM, L- glutamine, antibiotics, FCS). In contrast, HPGFs were seeded in the same culture medium, but at $2.5x10^3$ cells per well. At 24 h post-seeding, culture media was replaced with serum-free media, and the cells re-incubated again for another 24 h. Following serum starvation, the serum-free medium was replaced with medium containing the test agent for a further 72 h. Test agent solutions were prepared fresh on the day of use. Freeze-dried PRE, Zn (II) and punicalagin were dissolved in phthalate buffer pH 4.5 to make stock solutions and were then filtered with a 0.2 µm syringe filter. A range of concentrations of the compound were prepared in 97 % DMEM/1 % FCS/1 % L-glutamine/1 % antibiotics. The respective treatment media (100 µL) were added to each well. An untreated control, comprised of 1 % phthalate buffer pH 4.5, was also included in each experiment.

Concentration of compounds and combinations were PRE (0.1µg/mL, 1 µg/mL, 10 µg/mL, 100 µg/mL), punicalagin (0.1µg/mL, 1 µg/mL, 10 µg/mL), Zn (II) (0.001 mM, 0.1 mM, 1 mM), PRE/Zn (II) (0.1µg/mL+0.1 mM, 1 µg/mL+0.1 mM, 10 µg/mL+0.1 mM), and punicalagin/Zn (II) (0.1µg/mL+0.1 mM, 1 µg/mL+0.1 mM, 10 µg/mL+0.1 mM). All test concentrations, and the control had six replicate wells. Each experiment was performed on three independent occasions. MTT solution (5 mg/mL of MTT in phosphate buffered saline, [PBS]) was prepared and filter-sterilised using a 0.2 µm syringe filter. After 24 h, 25 µL MTT solution was added to each well at 24 h and incubated for 4 h at 37˚C/5% CO_2/95% in an air environment. After 4 h of incubation, the medium was aspirated, avoiding disruption of the precipitated formazan crystals.

DMSO (100 µL) was added to solubilise hydrophobic formazan crystals, then the plate was wrapped with cling film and incubated at 37˚C/5% CO_2/95% in an air environment for 30 min. The plate was read spectrophotometrically at 540 nm using a microtitre plate reader. HPGF and HaCaT cell viability and proliferation were assessed at 24 h, 48 h and 72 h. The percentage of cell viability was calculated as follows:

% cell viability = (absorbance of the compound/ mean absorbance of the control group) x 100

Each experiment was performed on at least three separate occasions, and six replicates per condition. Data was presented as mean±SEM.

7.3.2.2 Scratch-wound assay

The experimental protocol was followed as described by Moses et al. (2020). 70-90% confluent HPGFs and HaCaTs were trypsinised, counted with a haemocytometer and seeded at densities of $2.5x10^4$ cells/mL and $7.5x10^4$ cells/mL, respectively, in culture medium (DMEM, L-glutamine, antibiotics, 10% FCS) per well of the 24-well plate, and incubated for 48 h. After incubation, the culture media was replaced with serum-free media for 24 h. After 24 h serum-starvation, the serum-free media was aspirated from the cells and an artificial scratch was made in each well using a sterile 200 µL pipette tip. The wells were washed (x2) with PBS. to remove cell debris. Each respective concentration of PRE, Zn (II) and punicalagin was added to the wells, with three wells per condition. Cells were also exposed only to phthalate buffer pH 4.5 (1%) in serum-free media for all experiments to observe the effect of carried phthalate buffer pH 4.5 with the treatment solutions effect on cells. The concentration of compounds and combinations were PRE (0.1µg/mL, 1 µg/mL, 10 µg/mL, 100 µg/mL), punicalagin (0.1µg/mL, 1 µg/mL, 10 µg/mL), Zn (II) (0.001 mM, 0.1 mM, 1 mM), PRE/Zn (II) (0.1µg/mL+0.1 mM, 1 µg/mL+0.1 mM, 10 µg/mL+0.1 mM) and punicalagin/Zn (II) (0.1µg/mL+0.1 mM, 1 µg/mL+0.1 mM, 10 µg/mL+0.1 mM).

For HPGFs, the plates were examined by Time Lapse Microscopy using the Cell IQ Time-Lapse Microscope to monitor wound repopulation of the cells at 37°C, in a 5% CO_2/95% air environment. Fibroblast cell movement was tracked using a tracking plugin with Image J and 6 cells/video and 3 independent videos were analysed per condition, resulting in 18 data points/culture condition. These data points were chosen to monitor HPGF migration in each well, and the software was arranged to take digital images every 20 min, over a 48 h period. These images were converted to a video using Imagen program with Cell IQ Time-Lapse Microscope. Each experiment was performed on three independent occasions. The images were analysed using ImageJ Software (NIH Software, Version 1.49). Scratch assay analysis was made using a cell tracking method to determine the distance (Tt), cell displacement (Td), speed (Tt/min) and overall velocity (Td/min), instead of measuring the percentage of wound area for HPGF cells.

For HaCaTs, the plate was placed in Time Lapse Microscopy using the Leica TCS SP5 Confocal Microscope (Leica Microsystems (UK) Ltd., Buckinghamshire, UK) to monitor the wound repopulation of the cells at 37°C, in a 5% CO_2/95% air environment. Nine different images, resulting in 18 data points/culture condition, were chosen for each well to monitor the HaCaT wound repopulation in each well, and the software was arranged to take digital images every 20 min, over a 48 h period. These images were converted to video using LAS AF Lite (Leica Software, Version 4.0.11706, Leica Microsystems (UK) Ltd). Each experiment was performed on three independent occasions. For HaCaTs, images were analysed using ImageJ Software (NIH Software, Version 1.49). The percentage wound closure at 24 h and 48 h were calculated for each condition and expressed as mean±SEM.

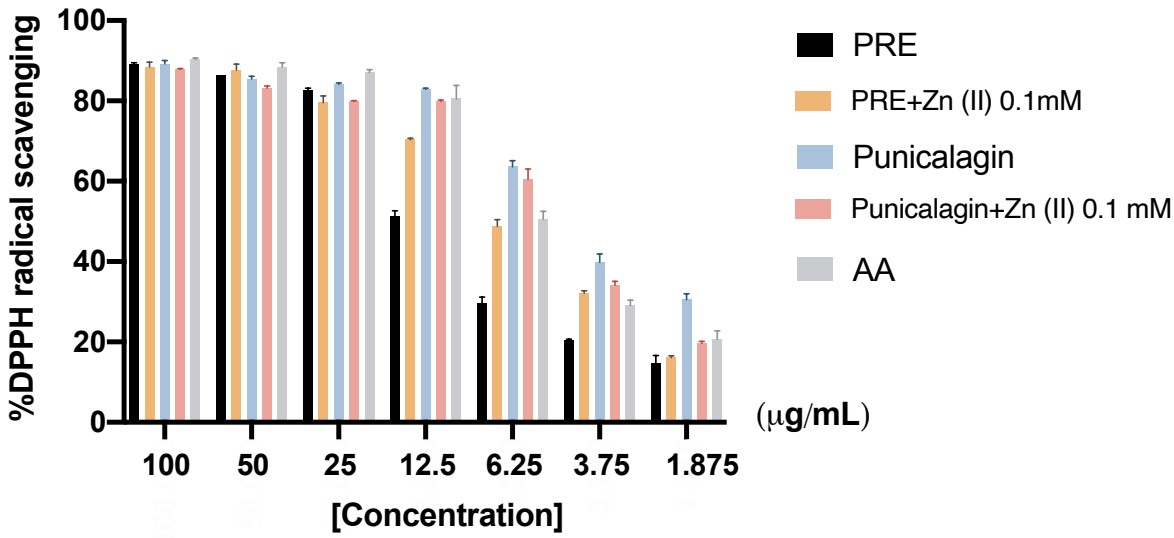
7.3.3 Statistical analysis

All experiments were repeated on three independent occasions, with data expressed as mean±SEM. Antioxidant, cell proliferation/viability and wound repopulation data were analysed using one-way analysis of variance (ANOVA) with post-test Tukey analysis, with p values of ≤ 0.05 considered significant (*, $p < 0.05$), very significant (**, $p < 0.01$) or highly significant (***, $p < 0.001$).

7.4 Results

7.4.1 Antioxidant activity

Radical scavenging capacities of PRE, punicalagin and their combination with Zn (II) 0.1 mM were assessed using DPPH and ABTS/TEAC assays. The results for DPPH assay were reported as percentage of DPPH inhibition (Figure 7.1A) and IC50 values (the concentration of compound needed to inhibit 50 % of the initial DPPH free radical) (Figure 7.1B). All studied compounds showed a dose-dependent response in the percentage of free DPPH inhibition. While PRE and PRE/Zn (II) exerted similar percentage in DPPH radical scavenging at 100 µg/mL, 50 µg/mL and 25 µg/mL, PRE/Zn (II) exerted percentage of DPPH radical scavenging at 12.5 µg/mL, 6.25 µg/mL, and 3.75 µg/mL. In these concentrations, punicalagin also showed better percentage inhibition in DPPH radical scavenging compared to positive control, ascorbic acid (AA) (Figure 7.1A). Punicalagin and punicalagin/Zn (II) exerted similar percentage values in DPPH radical scavenging in all concentrations except at 1.875 µg/mL where punicalagin caused higher inhibition in DPPH radical scavenging. PRE (10.69±0.44 µg/mL) and PRE in combination with Zn (II) 0.1 mM (8.1±0.27 µg/mL) were required at a higher concentration than the positive control ascorbic acid (8.31±0.64 µg/mL) to inhibit free DPPH. However, punicalagin (6.04±0.29 µg/mL) and its combination with Zn (II) 0.1 mM (6.99±0.2 µg/mL) required a lower concentration of compound than ascorbic acid to inhibit free DPPH. While there was a slight difference between the compounds and their Zn (II) combinations, no statistical difference was observed ($p>0.05$; Figure 7.1B). The ABTS/TEAC assay showed a similar pattern of results. Punicalagin and punicalagin with Zn (II) had significantly higher TEAC values than PRE and PRE/Zn (II) ($p<0.001$). Similarly, Zn (II) 0.1 mM addition did not result in any significant change in free radical scavenging activity for PRE and punicalagin with ABTS/TEAC assay ($p>0.05$; Figure 7.1B).



A

Compounds	ABTS·+ /TEAC	DPPH· IC50
	(mM/mg)	(µg/mL)
PRE	3.27±0.08^{A}	10.69±0.44^{A}
PRE + Zn (II) 0.1mM	3.47 ±0.2^{A}	8.1±0.27^{A}
Punicalagin	6.3±0.02^{B}	6.04±0.29^{B}
Punicalagin + Zn (II) 0.1mM	6.15±0.01^{B}	6.99±0.2^{B}
AA	*ND	8.31±0.64^{A}

B

Figure 7.1: ROS antioxidant scavenging activities. (A) Percentage of DPPH· radical scavenging capacity of Zn (II) 0.1 mM and different concentrations of PRE (1.875-100 µg/mL), and punicalagin (1.875-100 µg/mL); (B) ABTS/TEAC column shows trolox equivalent antioxidant capacity of PRE (0.5 mg/mL), punicalagin (0.5 mg/mL), and their combinations with Zn (II) 0.1 mM. DPPH IC50 column shows the concentrations of PRE, punicalagin and their combinations with Zn (II) 0.1 mM which scavenge 50 % of the initial DPPH radical. Values with different uppercase superscript letters in the same column indicate the significant difference ($p<0.05$) between the compounds analysed by post-test Tukey analysis. each value presented as the mean±SEM (N=3). *ND; not detected. AA: Ascorbic acid.

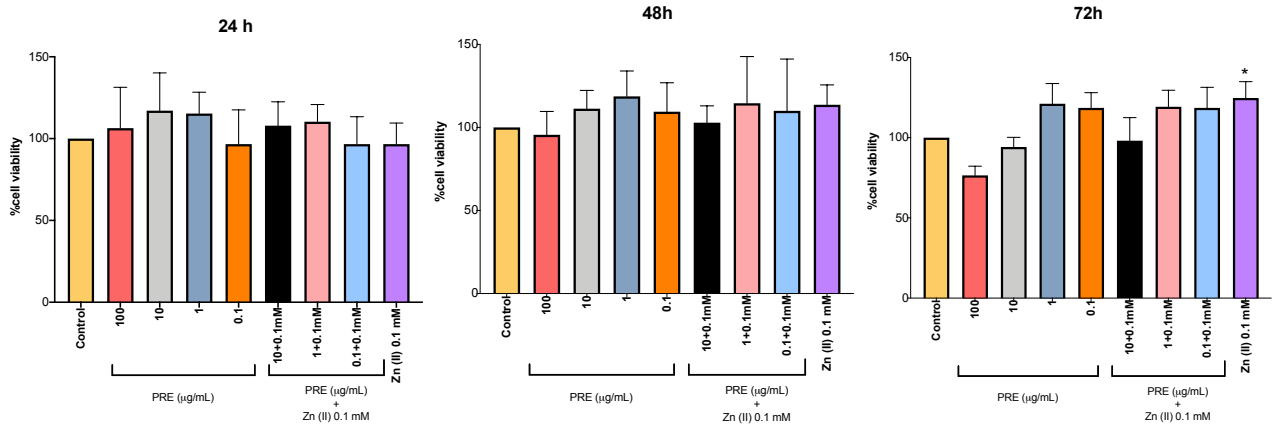
7.4.2 Effects of PRE, Punicalagin, Zn (II) and PRE and punicalagin in combination with Zn (II) on HaCaT and HPGF viability and proliferation (MTT Assay)

7.4.2.1 HaCaT cells

The cell viability experiments were performed on HaCaTs cultured in different concentrations of PRE, punicalagin, Zn(II), and PRE/Zn (II) and punicalagin/Zn (II).

PRE and PRE in combination with Zn (II) 0.1 mM did not show any significant decrease and increase in cell viability at all time points (Figure 7.2A, 7.2B and 7.2C). PRE caused an increase in cell proliferation at lower concentrations (10, 1, and 0.1 µg/mL). Zn (II) 0.1 mM exerted a significant cell proliferation at 72 h, but PRE/Zn (II) did not show same proliferation effect on HaCaTs at all time points.

Both PRE and punicalagin showed a dose-dependent response in the MTT cell viability/proliferation assay (Figure 7.2). Punicalagin at 10 µg/mL led to a significant decrease in cell viability from 24 h onwards ($p<0.001$; Figure 7.2D, Figure 7.2E, and Figure 7.2F). Similarly, punicalagin at 10 µg/mL in combination with Zn (II) 0.1 mM was cytotoxic to HaCaT cells by 72 h ($p<0.001$) (Figure 7.2D, Figure 7.2E, and Figure 7.2F). While punicalagin 1 µg/mL and 0.1 µg/mL showed a significant increase in cell proliferation at 24 h, their combination with Zn (II) did not ($p<0.05$) (Figure 7.2D). In contrast, Zn (II) 0.1 mM increased cell viability at 48 h and 72 h (Figure 7.2B, 7.2C, 7.2D, and 7.2E).


24 h
48h
72h
%cell viability
150
100
50
0
Control
100
10
1
0.1
10+0.1mM
1+0.1mM
0.1+0.1mM
Zn (II) 0.1 mM
PRE (µg/mL)
PRE (µg/mL)
+
Zn (II) 0.1 mM
*
(A)
(B)
(C)

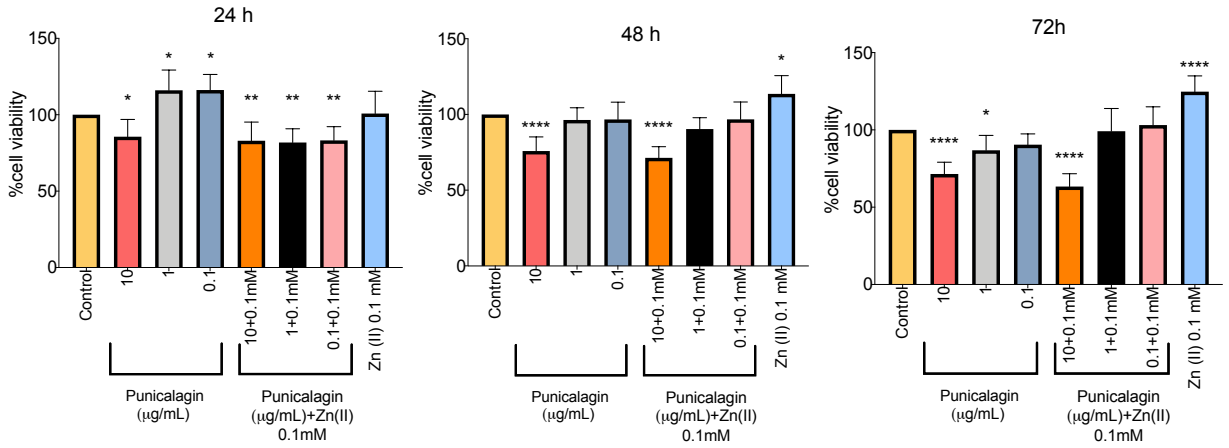
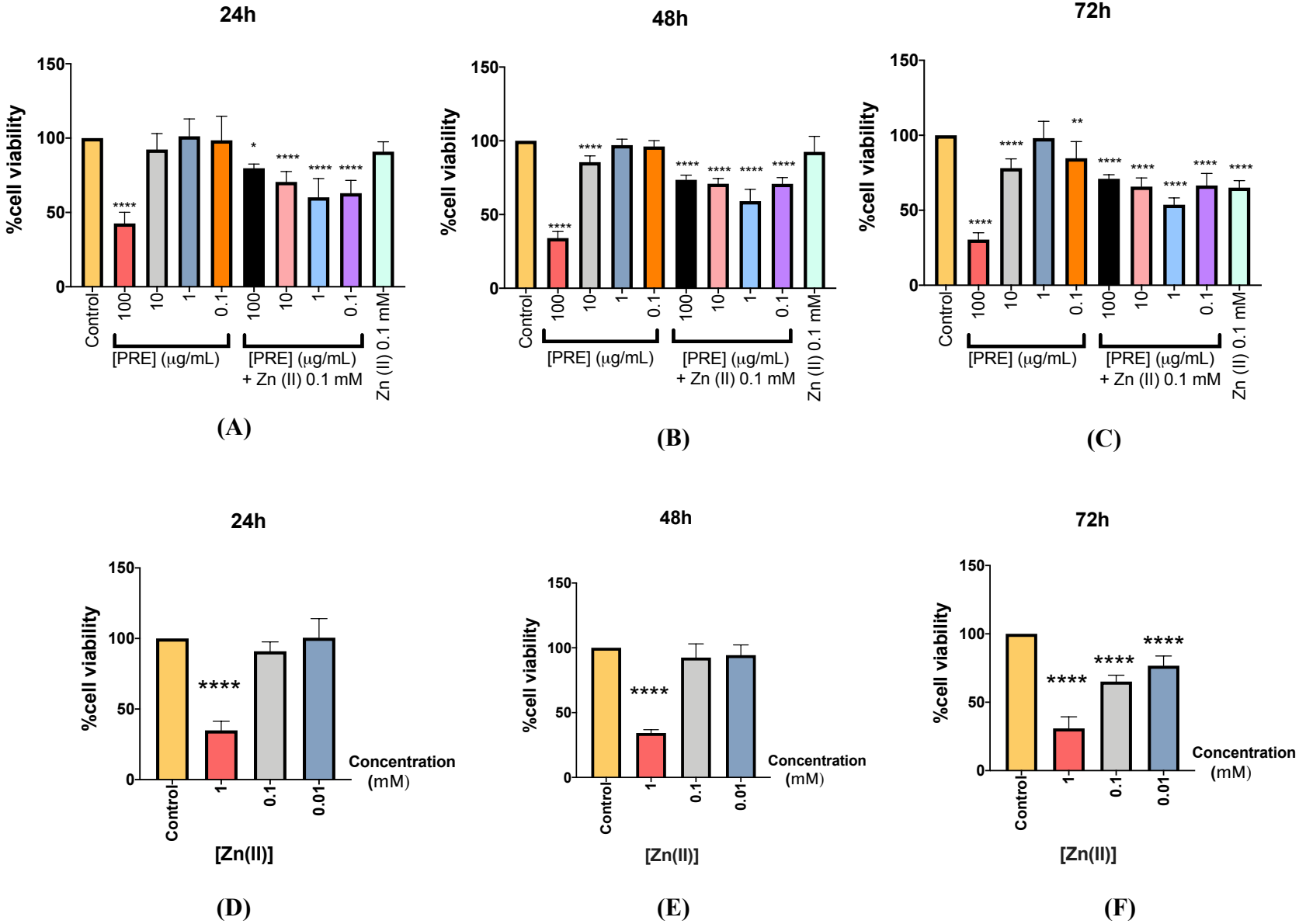


Figure 7.2: Cell viability was evaluated using the MTT assay. Effects of PRE (0.1-100 µg/mL), Zn (II) 0.1 mM and PRE (0.1-100 µg/mL) in combination with Zn (II) 0.1 mM on HaCaTs viability for 24 h (A), 48 h (B) and 72 h (C). Effects of punicalagin (0.1-10 µg/mL), Zn (II) 0.1 mM and punicalagin (0.1-10 µg/mL) in combination with Zn (II) 0.1 mM on HaCaTs viability for 24 h (D), 48 h (E) and 72 h (). Values were expressed as a mean percentage with standard error of the mean values. (N=3, average±SEM, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, p>0.05).

7.4.2.2 HPGFs

The effects of PRE, punicalagin, Zn (II) and Zn (II) combinations with PRE and punicalagin on the viability of HPGFs, were also determined by MTT assay. Zn (II) 1 mM significantly inhibited HPGF proliferation ($p<0.001$; Figure 7.3D, 7.3E, and 7.3F), while lower concentrations of Zn (II) did not show a significant decrease in proliferation at 24 h and 48 h and showed a delayed cytotoxicity at 72 h ($p>0.05$; Figure 7.3D, 7.3E, and $p<0.0001$; Figure 7.3F). When HPGFs were treated with PRE or punicalagin alone, both showed dose-dependent viability responses. The highest concentrations of PRE (100 µg/mL) and punicalagin (10 µg/mL) significantly reduced HPGF viability ($p<0.001$), but lower concentrations (1 and 0.1 µg/mL) did not affect cell viability ($p>0.05$). However, while PRE at 100 µg/mL showed cytotoxicity from 24 h, punicalagin 10 µg/mL a delayed cytotoxicity at 48 h (Figure 7.3A and 7.3H). All compound concentrations did not show a proliferative effect in any studied time-points. In addition, when PRE and punicalagin were combined with 0.1 mM Zn (II), they significantly decreased cell viability ($p<0.001$). However, treatment solely with Zn (II) 0.1 mM did not decrease HPGF viability at 24 h and 48 h ($p>0.05$; Figure 7.3D and 7.3E). Significantly decreased cell viability was observed at 72 h for Zn (II) 0.1 mM (Figure 7.3F).


24h
48h
72h
%cell viability
150
100
50
0
Control
100
10
1
0.1
[PRE] (μg/mL)
[PRE] (μg/mL) + Zn (II) 0.1 mM
Zn (II) 0.1 mM
(A)
(B)
(C)
24h
48h
72h
%cell viability
Concentration (mM)
Control
1
0.1
0.01
[Zn(II)]
(D)
(E)
(F)

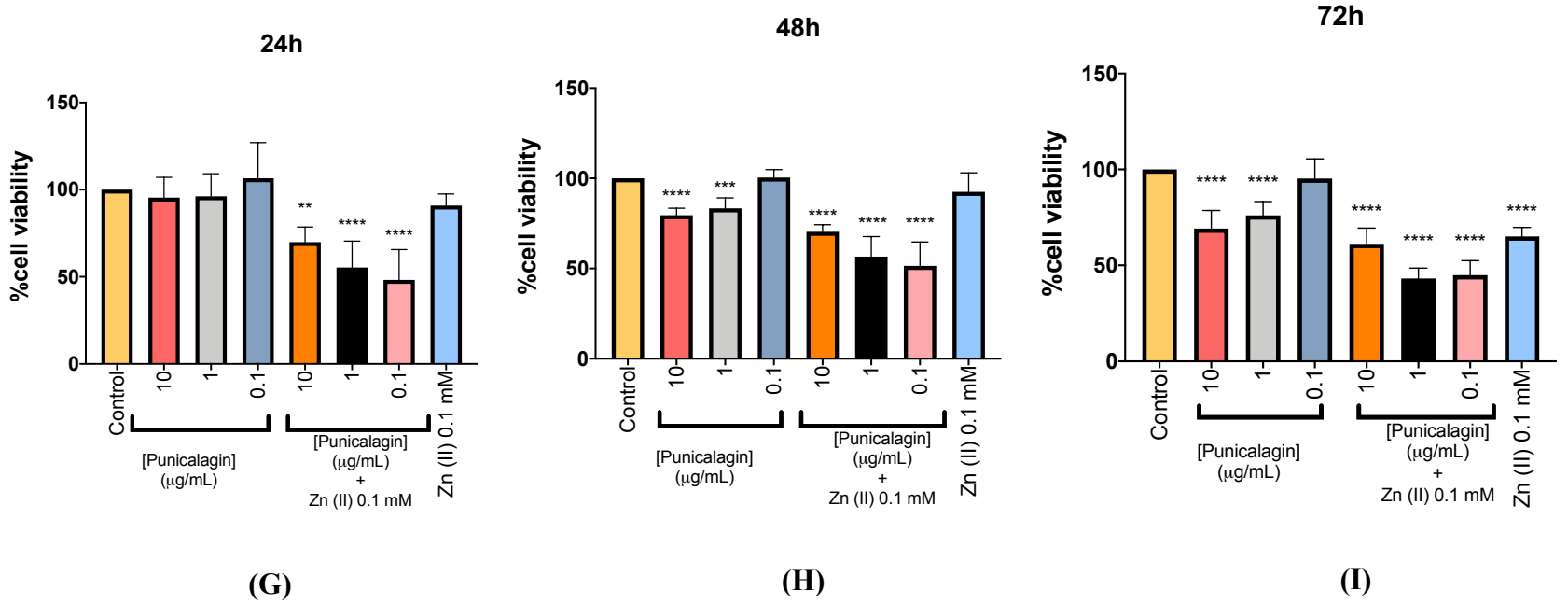


Figure 7.3: Effects of PRE (0.1-100 µg/mL), PRE in combination with Zn (II) 0.1 mM on HPGF viability at 24 h (A), 48 h (B) and 72 h (C). Effects of Zn (II) (0.01-1 mM) on HPGF viability at 24 h (D), 48 h (E) and 72 h (F). Effects of punicalagin (0.1-10 µg/mL) and punicalagin in combination with Zn (II) 0.1 mM on HPGF viability at 24 h (G), 48 h (H) and 72 h (I). Values were expressed as a mean percentage with standard error of the mean values. (N=3, average±SEM, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, p>0.05).

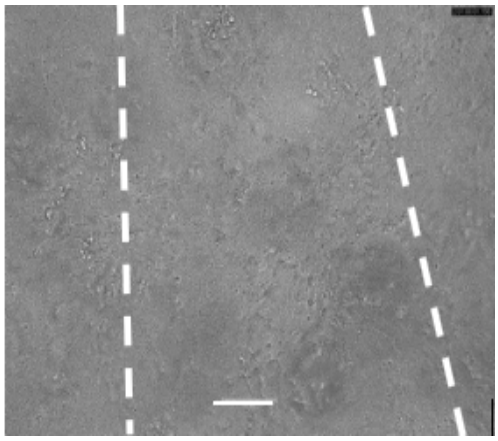
7.4.3 Effects of PRE, punicalagin, Zn (II) and PRE and punicalagin combination with Zn (II) on HaCaT and HPGF wound repopulation

7.4.3.1 HaCaTs

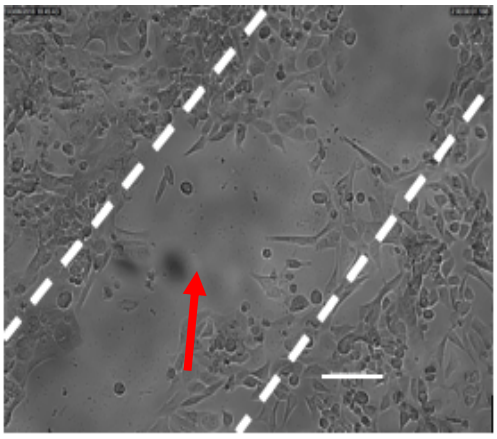
The scratch-wound assay was performed on HaCaTs cultured with different concentrations of PRE, punicalagin, Zn (II), PRE + Zn (II) and punicalagin+ Zn (II). A negative control group devoid of any of the compounds was included. The control group wound closed in less than 48 h (Figure 7.4A). Cells incubated with Zn (II) 0.1 mM did not close the scratch wound over 48 h (Figure 7.4B). However, combination of PRE and punicalagin with Zn (II) 0.1 mM led to a better closure in artificial wound than Zn (II) 0.1 mM alone. This is more apparent from the representative images of *in vitro* scratch assay of PRE (0.1 µg/mL), punicalagin (0.1 µg/mL and 1 µg/mL) in combination with Zn (II) 0.1 mM led to full closure of artificial wound (Figures 7.4H, 7.4l and 7.4N, respectively). However, when Zn (II) 0.1 mM was applied alone, the wound was not closed (Figure 7.4). combination of PRE and punicalagin with Zn (II) 0.1 mM led to a better closure in artificial wound than Zn (II) 0.1 mM.

The percentage of wound area at 24 h and 48 h showed that both PRE and punicalagin did not alter migration when applied alone. However, when applied with Zn (II) 0.1 mM, wound repopulation was significantly inhibited (Figure 7.5).

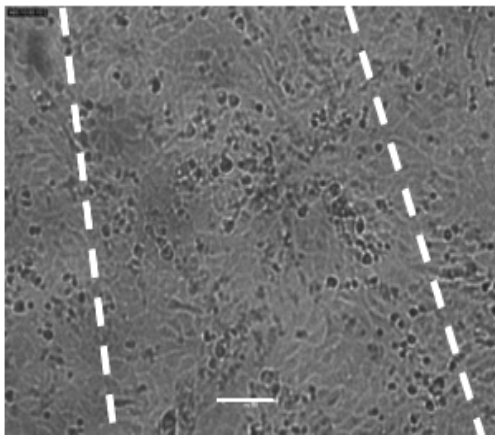
A Control



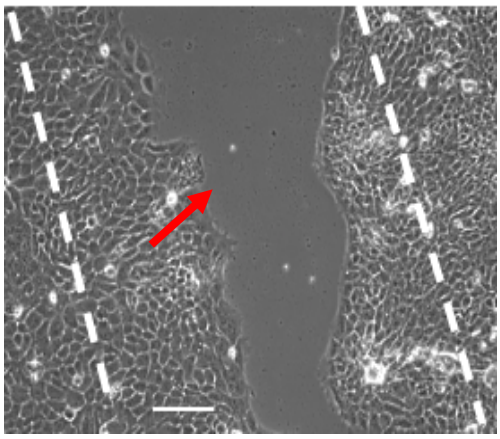
B Zn (II) 0.1 mM



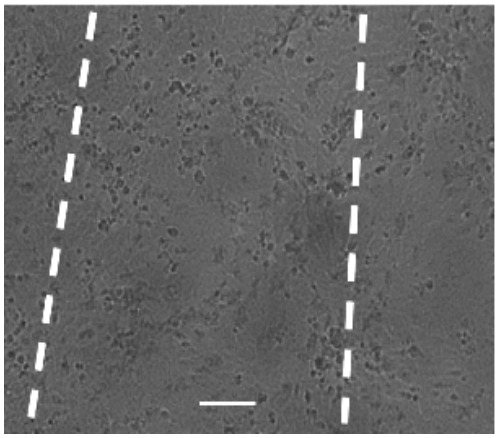
C PRE 10 µg/mL



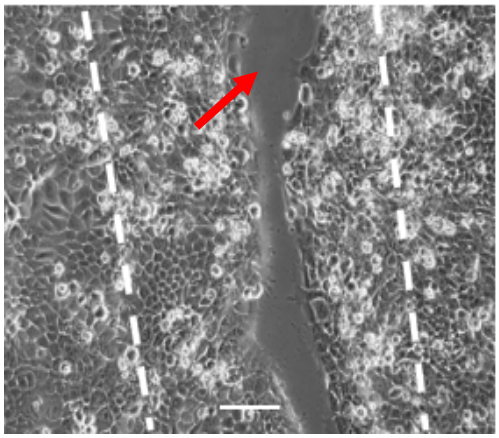
D PRE 10 µg/mL+ Zn (II) 0.1 mM



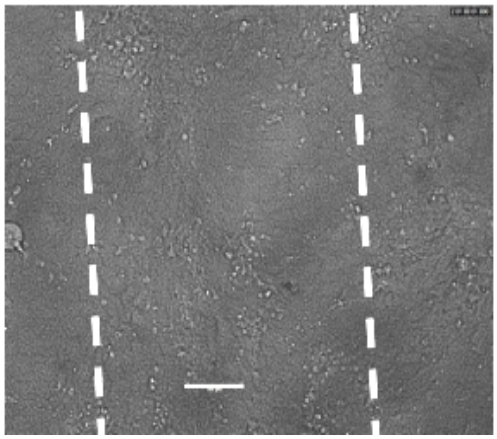
E PRE 1 µg/mL



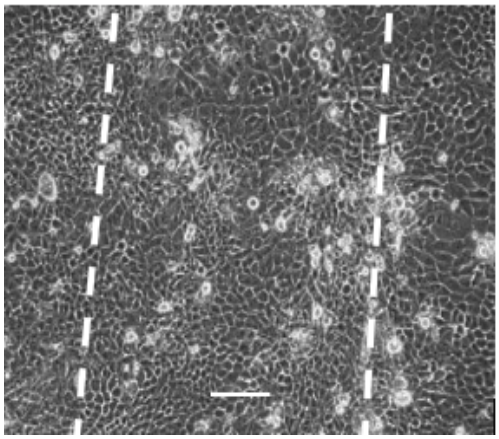
F PRE 1 µg/mL+ Zn (II) 0.1 mM



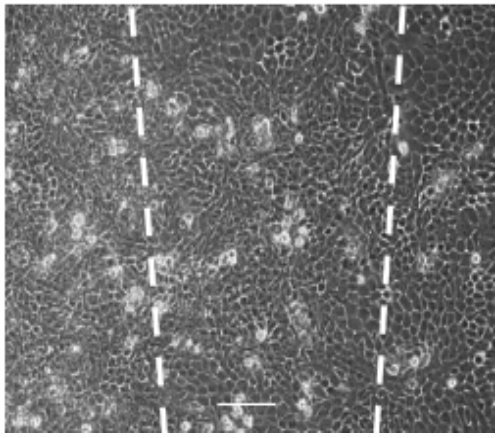
G PRE 0.1 µg/mL



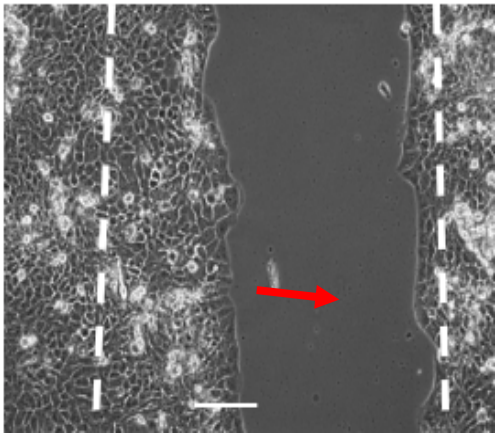
H PRE 0.1 µg/mL+ Zn (II) 0.1 mM



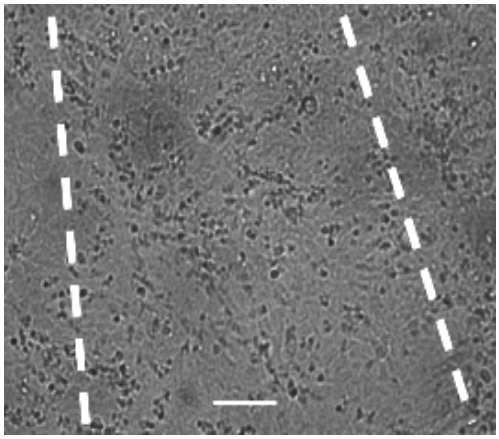
I Punicalagin 10 µg/mL



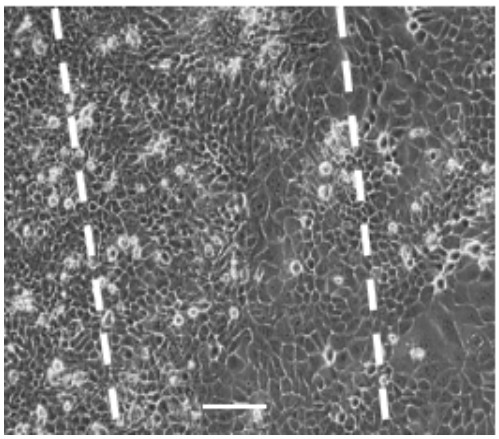
J Punicalagin 10 µg/mL+ Zn (II) 0.1 mM



K Punicalagin 1 µg/mL



L Punicalagin 1 µg/mL+ Zn (II) 0.1 mM



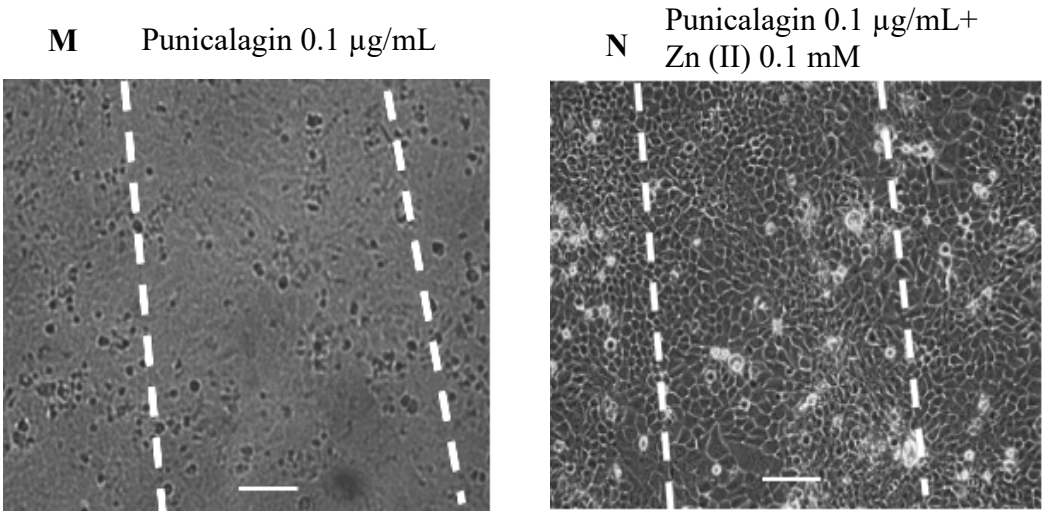


Figure 7.4: Representative images from *in vitro* scratch assay of HaCaTs over 48 h incubation with (A) Control (0 µg/mL), (B) Zn (II) 0.1 mM, (C) PRE 10 µg/mL, (D) PRE 10 µg/mL+ Zn (II) 0.1 mM, (E) PRE 1 µg/mL+ Zn (II) 0.1 mM, (F) PRE 1 µg/mL+ Zn (II) 0.1 mM, (G) PRE 0.1 µg/mL, (H) PRE 0.1 µg/mL+ Zn (II) 0.1 mM, (I) Punicalagin 10 µg/mL, (J) Punicalagin 10 µg/mL+ Zn (II) 0.1 mM, (K) Punicalagin 1 µg/mL, (L) Punicalagin 1 µg/mL+ Zn (II) 0.1 mM, (M) Punicalagin 0.1 µg/mL, (N) Punicalagin 0.1 µg/mL+ Zn (II) 0.1 mM . White dashed lines demark the scratch edges at 0 h. Red arrows show the unclosed wound areas and highlight effects of PRE and punicalagin with Zn (II) 0.1 mM on migration activity. Scale bar = 100 µm.

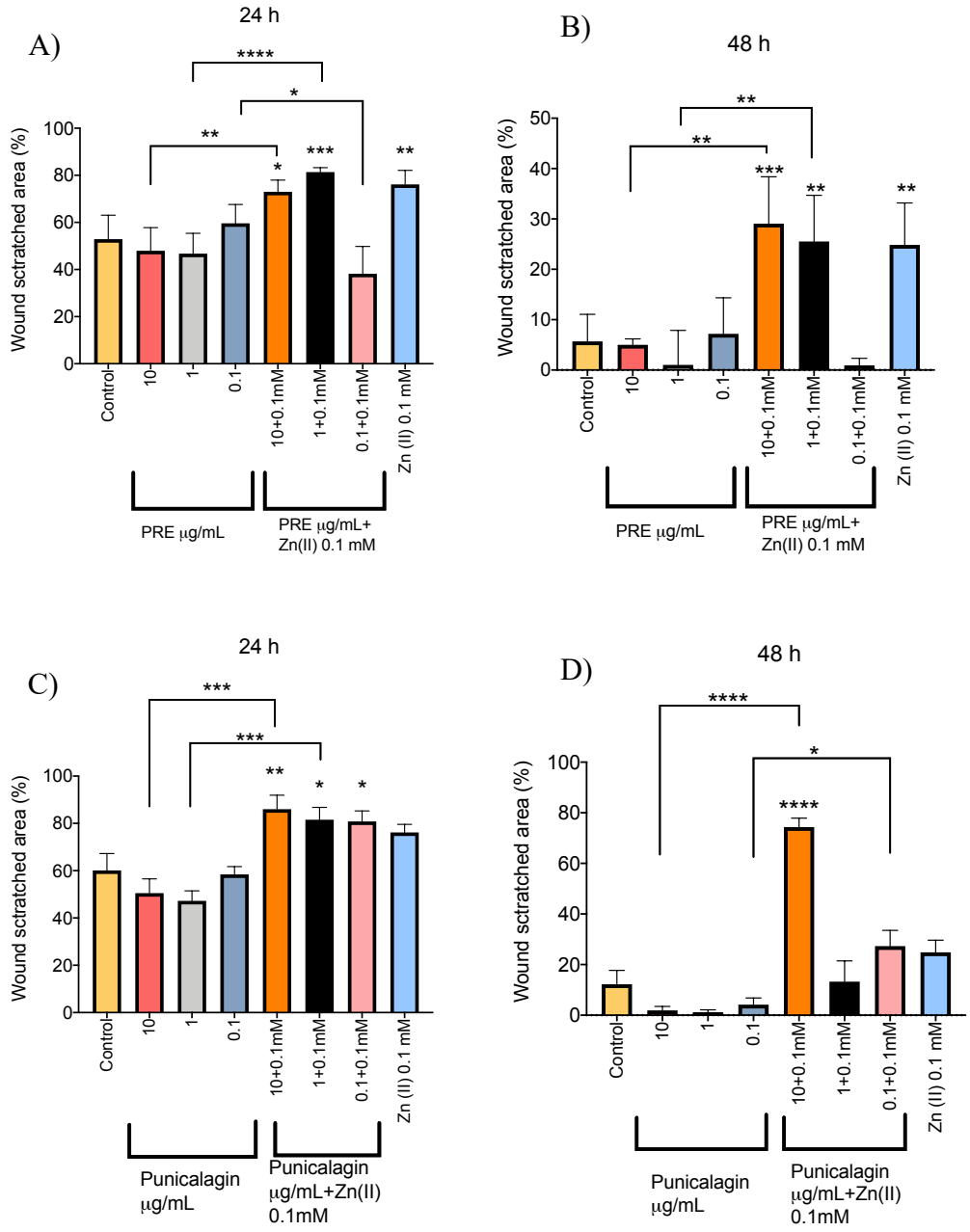


Figure 7.5: HaCaT scratch wound repopulation and closure analysis, in the presence of PRE (0µg/mL, 0.1µg/mL, 1µg/mL and 10µg/mL), and PRE (0µg/mL, 0.1µg/mL, 1µg/mL and 10µg/mL) combination with Zn (II) 0.1 mM at 24 h (A) and 48 h (B). Punicalagin (0µg/mL, 0.1µg/mL, 1µg/mL, and 10µg/mL), Zn (II) 0.1 mM and PRE (0µg/mL, 0.1µg/mL, 1µg/mL and 10µg/mL) combination with Zn (II) 0.1 mM and punicalagin (0µg/mL, 0.1µg/mL, 1µg/mL and 10µg/mL) combination with Zn (II) 0.1 mM at 24 h (C) and 48 h (D). (N=3, average±SEM, *p<0.05, **p<0.01, ***p<0.001,****p<0.0001).

7.4.3.2 HPGFs

Images obtained from time-lapse microscopy over 48 h are presented in Figure 7.6. These images show the repopulation of the denuded sites by HPGFs . The analysis of images is presented in Figure 7.7 and Figure 7.8.

The control group wound closed in less than 48 h (Figure 7.6A). HPGF cells incubated with PRE 10 µg/mL and punicalagin 10 µg/mL resulted with unclosed artificial wound with a few migrated cells (Figures 7.6D and 7.6J, respectively). However, PRE 10 µg/mL and punicalagin 10 µg/mL combinations with Zn (II) 0.1 mM led to more migrated HPGFs in the wounded area than their alone use (Figures 7.6E and Figure 7.6K). Similar closure behaviour was observed for punicalagin 1 µg/mL and punicalagin 1 µg/mL combination with Zn (II) 0.1 mM. Although punicalagin 1 µg/mL treated cells did not close the wounded area (Figure 7.6L), punicalagin 1 µg/mL combination with Zn (II) 0.1 mM led to a closure of artificial wound similar to control group (Figure 7.6M).

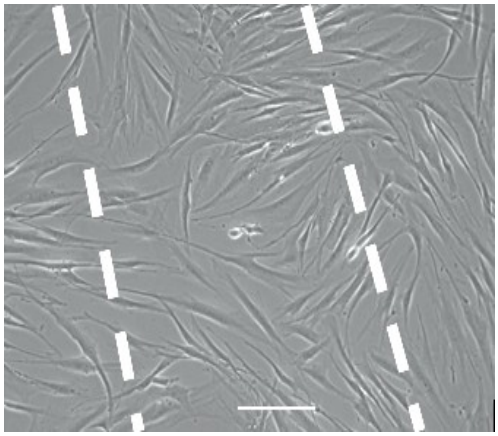
PRE at 100 µg/ml and punicalagin at 10 µg/ml significantly decreased cell speed (Tt/t; $p<0.05$; Figure 7.7B). However, this inhibition did not affect the distance travelled compared to the untreated control group. Other concentrations of PRE and punicalagin did not show any significant difference in the studied parameters ($p>0.05$; Figure 7.7B).

The combination of punicalagin at 0.1 µg/ml with Zn (II) 0.1 mM showed a significant enhancement in distance travelled (Tt) and speed (Tt/t) ($p<0.05$; Figure 7.8A and 7.8B), while there was no increase in the distance travelled (Tt) and speed (Tt/min) on HPGF when punicalagin 0.1 µg/mL or Zn (II) 0.1 mM were used alone ($p>0.05$) (Figure 7.7A and 7.7B, Figure 7.8A and 7.8B).

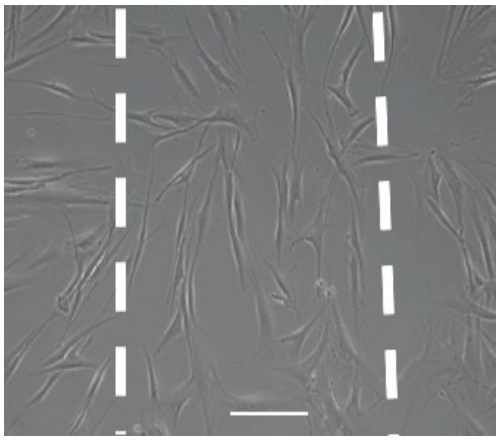
In addition, no inhibition was observed for punicalagin at 10 ug/mL in cell speed (Tt/t) when combined with Zn (II) 0.1 mM ($p>0.05$) (Figure 7.8B). However, when punicalagin was used alone, there was a significant inhibition in the cell speed (Tt/t) of HPGF cells (Figure 7.7B).

Probably, due to the high variability in the cell displacement (Td) and overall velocity (Td/t), HPGF cells under treatment did not exert any statistically significant differences compared to untreated control group ($p>0.05$; Figure 7.7C and 7.7D for PRE and punicalagin alone, and Figure 7.8 C and 7.8D for Zn (II) 0.1 mM, and PRE or punicalagin combinations with Zn (II) 0.1 mM treatments).

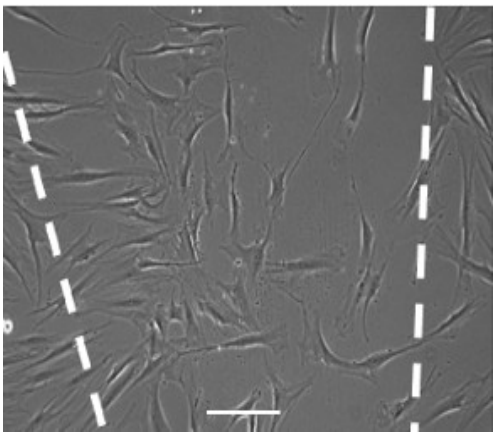
A Control

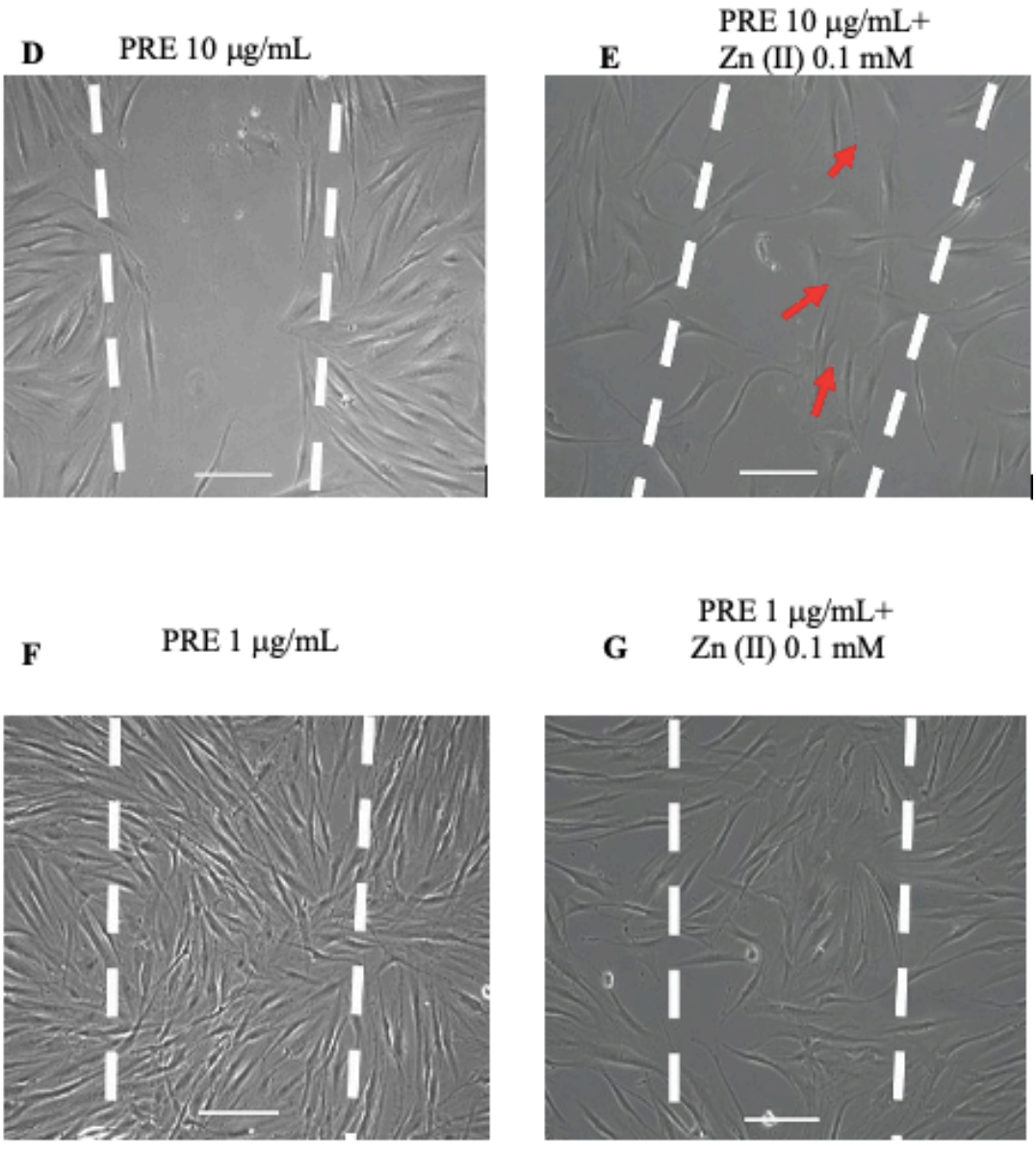


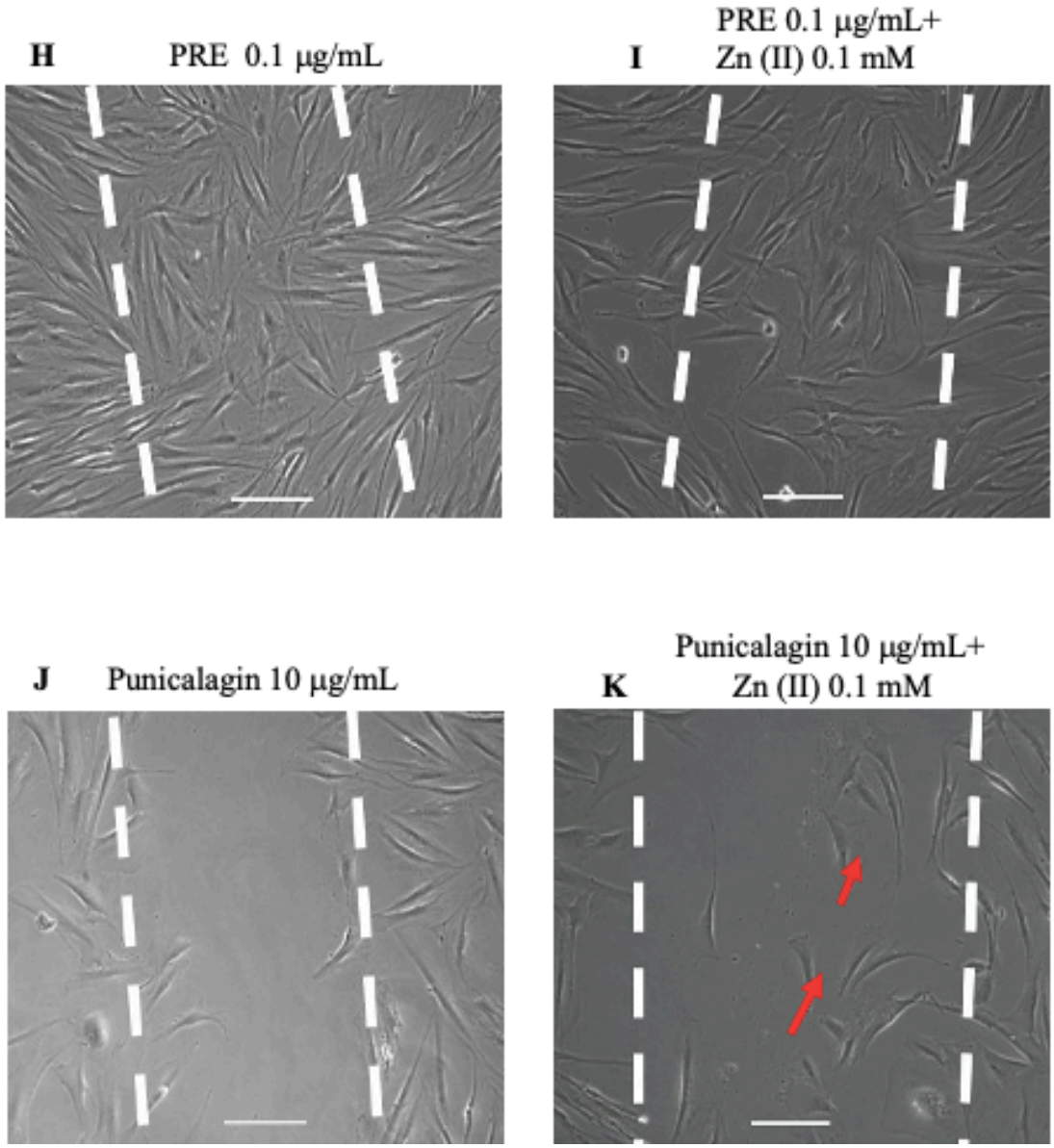
B Zn (II) 0.1 mM



C PRE 100 µg/mL




D
PRE 10 µg/mL
E
PRE 10 µg/mL+
Zn (II) 0.1 mM
F
PRE 1 µg/mL
G
PRE 1 µg/mL+
Zn (II) 0.1 mM


H
PRE 0.1 µg/mL
I
PRE 0.1 µg/mL+
Zn (II) 0.1 mM
J
Punicalagin 10 µg/mL
K
Punicalagin 10 µg/mL+
Zn (II) 0.1 mM

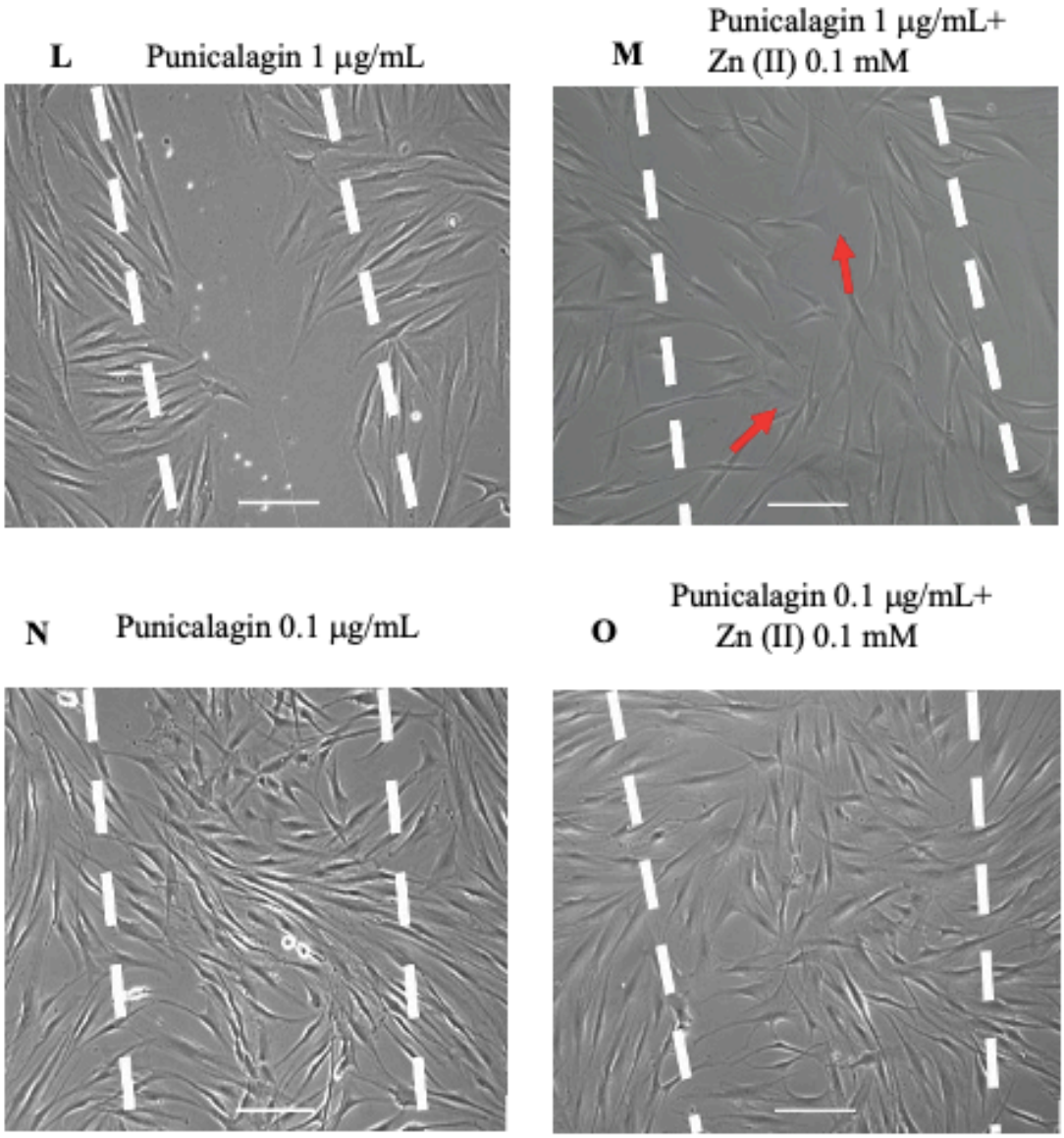


Figure 7.6: Representative images from *in vitro* scratch assay of HPGFs over 48 h incubation with (A) Control (0 µg/mL), (B) Zn (II) 0.1 mM, (C) PRE 100 µg/mL, (D) PRE 10 µg/mL, (E) PRE 10 µg/mL + Zn (II) 0.1 mM, (F) PRE 1 µg/mL, (G) PRE 1 µg/mL+ Zn (II) 0.1 mM, (H) PRE 0.1 µg/mL, (I) PRE 0.1 µg/mL+ Zn (II) 0.1 mM, (J) Punicalagin 10 µg/mL, (K) Punicalagin 10 µg/mL+ Zn (II) 0.1 mM, (L) Punicalagin 1 µg/mL, (M) Punicalagin 1 µg/mL+ Zn (II) 0.1 mM, (N) Punicalagin 0.1 µg/mL, (O) Punicalagin 0.1 µg/mL+ Zn (II) 0.1 mM . White dashed lines demark the scratch edges at 0 h. Red arrows show the unclosed wound areas and highlight effects of PRE and punicalagin with Zn (II) 0.1 mM on migration activity. Scale bar = 100 µm.

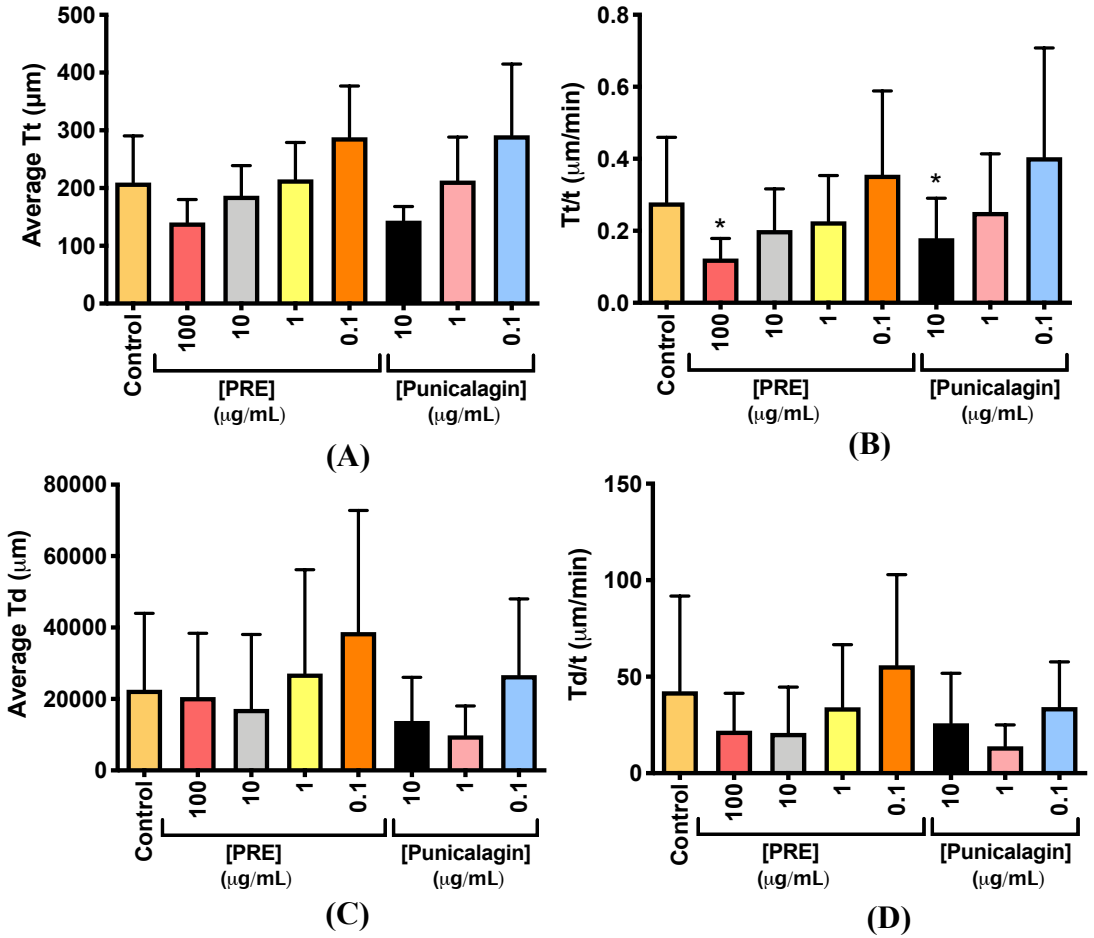


Figure 7.7: HPGF scratch wound migration analysis in the presence of PRE (0μg/mL, 0.1μg/mL, 1μg/mL and 10μg/mL, 100 μg/mL) and punicalagin (0μg/mL, 0.1μg/mL, 1μg/mL, and 10μg/mL). (A) distance travelled (Tt), (B) speed (Tt/min), (C) cell displacement (Td), and (D) overall velocity (Td/min) at 48 h (N=3, average±SEM, *p<0.05, **p<0.01, ***p<0.001).

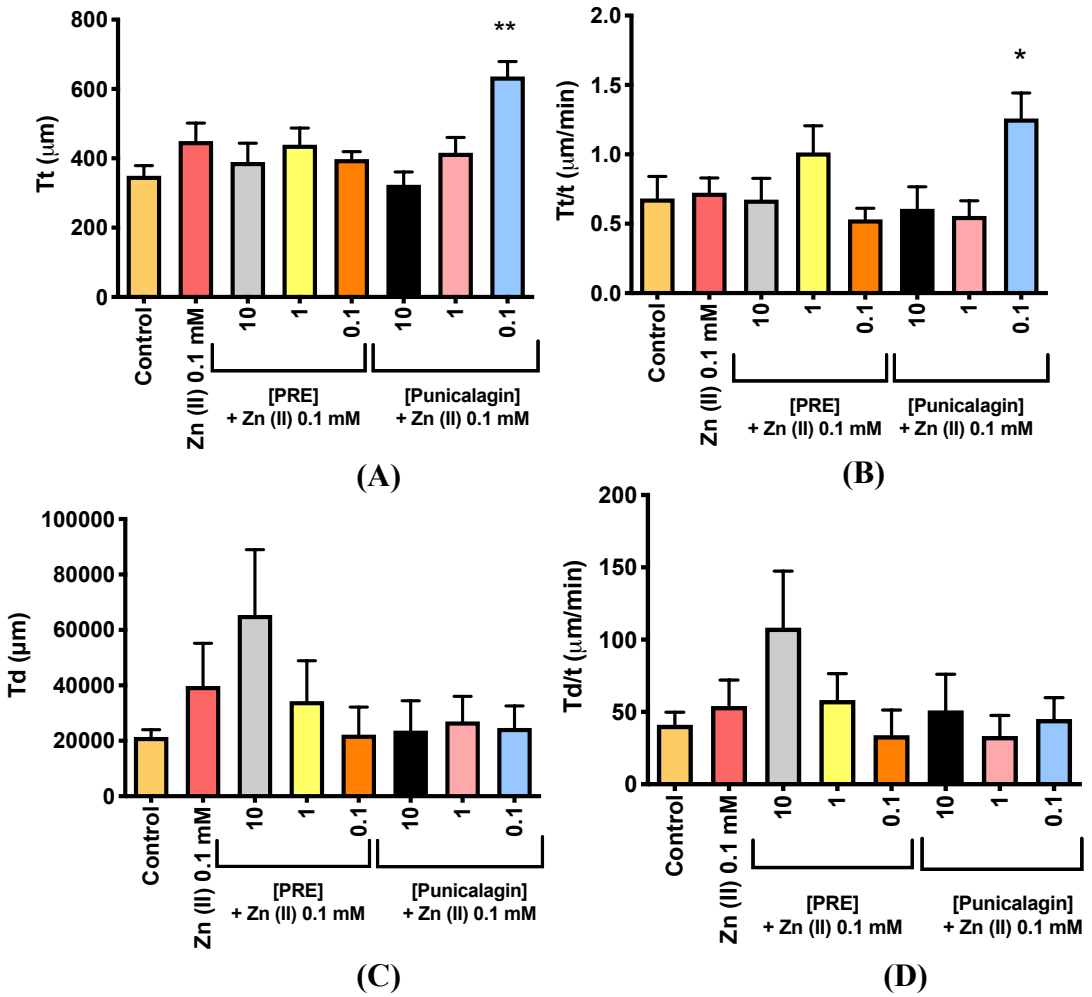


Figure 7.8: HPGF scratch wound migration analysis in the presence of PRE (0.1 - 100 µg/mL,) and punicalagin (0.1 - 10µg/mL) combination with Zn (II) 0.1 mM` compared to untreated control group (0 µg/mL). (A) distance travelled (Tt), (B) speed (Tt/min), (C) cell displacement (Td), and (D) overall velocity (Td/min) at 48 h (N=3, average±SEM, *p<0.05, **p<0.01, ***p<0.001).

7.5 Discussion

Pomegranate extract could be a promising alternative treatment for wound healing as it has been shown to possess many beneficial health activities such as antioxidant, anti-inflammatory, and antimicrobial capabilities (Kaneria et al. 2012; Celiksoy and Heard 2021). These biological activities of pomegranate are generally attributed to the phenolic contents in pomegranate extracts. Although the whole fruit comprises many phenolic compounds, including anthocyanins, gallotannins, hydroxycinnamic acids, hydroxybenzoic acids and hydrolysable tannins, pomegranate rind contains more than other parts of the fruit. The rind also contains the most promising phenolics, especially hydrolysable tannins i.e., punicalagin isomers. Punicalagin and other hydrolysable polyphenols are recognised as the most active antioxidants in pomegranate rind extract (Singh et al. 2002; Li et al. 2006, Orak et al. 2012; Akhtar et al. 2015; Derakhshan et al. 2018).

There are many reports showing the beneficial effect of PRE and punicalagin in the treatment of impaired wound healing in skin (Chidambara et al. 2004; Mo et al. 2014a; Mo et al. 2014b; Fleck et al. 2016; Tang et al. 2017; Nirwana et al. 2017; Lukiswanto et al. 2019). However, research into PRE and punicalagin in wound healing in oral cavity is limited. Periodontal diseases (periodontitis and gingivitis) are the most common oral diseases in humans and have become a major economic burden for healthcare providers (Tonetti et al. 2017). Periodontal disease prevalence has been also positively correlated with risk factors such as age and diabetes which are increasing in populations. There are many therapeutics available for periodontal disease. However, they generally have an antimicrobial or anti-inflammatory role and are not directly related to periodontal tissue healing, and only some have been used in routine clinical use (Rovai et al. 2016; Graziani et al. 2017). Hence, pomegranate could be an alternative prominent treatment with its antimicrobial, anti-inflammatory and pro-healing properties, as the reported side effects of commonly used therapeutics are a limitation for their routine use.

The periodontium is a specialised connective tissue that comprises the gum, periodontal ligament, cementum, and alveolar bone. The main role of this tissue is to

support the tooth in its alveolus (Guo and DiPietro 2010). A positive correlation between periodontitis and oxidative stress has been reported (Gjermo 1989). During inflammation of periodontium, PMNs release ROS as a response to dental plaque and is considered the main source of oxidative stress and tissue damage (Ghosh et al. 2013).

Some studies have shown the antioxidant activity of pomegranate rind extract (Negi et al. 2003; Ricci et al. 2006; Yasoubi et al. 2007). The potent antioxidant activity of pomegranate could play a role in periodontal wound healing, as excessive ROS production and oxidative stress induce host connective tissue damage (Waddington et al. 2000; Chapple et al. 2007). In this study, antioxidant activity was evaluated with *in vitro* DPPH and ABTS assays. In both assays, punicalagin showed significantly higher antioxidant activity than PRE, and Zn (II) and combating these agents did not affect the free radical scavenging capacity of punicalagin and PRE. Seeram et al. (2005) found that pomegranate juice had a higher antioxidant activity than punicalagin and suggested a synergistic/additive activity of polyphenols. However, it is difficult to assess antioxidant activity with a single method (Ciz et al. 2010). Discrepancy in results may arise because of extract and sample preparation, selection of endpoints and expression of results (Viuda-Martos et al. 2010). However, a correlation between phenolic content and antioxidant properties has been suggested (Gil et al. 2000; Seeram et al. 2005). In this study, the antioxidant assay results gave a dose- dependent response and could support the relationship between phenolic content and antioxidant activity for PRE and punicalagin. Punicalagin had a higher antioxidant activity than PRE in both DPPH and ABTS/TEAC assays. It could be suggested that the antioxidant activity of PRE is due to its punicalagin content, which is the main phenolic compound in PRE (Singh et al. 2009).

While the healing process of oral mucosal and dermal wounds are comprised of the same stages (homeostasis, inflammation, proliferation and remodelling of the collagen matrix) (Sciubba et al. 1978; Walsh et al. 1996), it should be noted that oral wound healing occurs faster with minimal scarring, compared to dermal wound healing (Szpaderska et al. 2003; Mak et al. 2009; Chen et al. 2010). Behind these differences, there are some intrinsic factors such as differences in growth factor production, stem

cell levels and cellular proliferation capacity (Angelov et al. 2004). In a study by Turabelidzade (2014), oral keratinocytes had an accelerated migratory and stimulated proliferative ability compared with epidermal keratinocytes. Similar behaviour was shown for fibroblast cells when oral fibroblasts and dermal fibroblasts were compared, and fibroblasts that have different anatomical origins exhibited intrinsic differences (Sandulache et al. 2005). For example , oral mucosal fibroblasts had an increased proliferative capacity and an altered contractibility profile (Lee et al. 1999). In the present study, the HaCaT was used for the assessment of compounds wound repopulation abilities. Oral primary keratinocytes, which might be considered more appropriate for this evaluation were initially used; however, these cells demonstrated low viability in tissue culture. HaCaTs are commonly used by many scientists to investigate drug candidate efficacy for treating periodontitis in *in vitro* studies (Becker et al. 2002; Beikler et al. 2008; Pereira et al. 2013; Koychev et al. 2017). HaCaT cells are a well characterised epithelial cell line and easily managed in *in vitro* conditions, making them suitable for this study (Boukamp et al. 1988).

To evaluate the effect of studied compounds on HPGF and HaCaT cells, an MTT assay was performed. For HPGF cells, no increase in cell proliferation was observed for any time point. In contrast, PRE and punicalagin significantly reduced cell viability when combined with Zn (II). A similar finding was shown in a study to evaluate the cytotoxicity of propolis, which has many beneficial bioactivities such as antimicrobial and antioxidant effects due to its polyphenols (Ghisalberti 1979; Tyzska- Czochara et al. 2014). In these studies, propolis, a polyphenol rich natural product, combined with zinc caused a significant decrease in cell viability. There are studies showing anti-proliferative or cytotoxic activity of Zn (II) application on keratinocyte proliferation (Lansdown 2007; Lin et al. 2018). In contrast to keratinocytes, negligible or inhibitory effects on fibroblast proliferative responses reported for Zn (II) (Aslam et al. 2006; Han et al. 2020). However, it should be noted that these activities are likely to be dose-dependent since there are reports of Zn (II) cytotoxicity at <500 mM (Gren and Mirastschicski 2004). There are many studies that show the anti-proliferative activity of polyphenols against cancer cells (Khan et al. 2009; Dai et al. 2010; Shirode et al. 2014; Shirode et al. 2015). It has been found that PRE has cytotoxic activity against oral cancer cells (Joel et al. 2019). For immortalised keratinocytes, a dose-dependent

response and PRE-treated cells showed a higher viability than punicalagin in this study. The interesting result from this present study is that pomegranate and punicalagin showed a greater decrease in proliferation for oral fibroblast cells than HaCaTs. Keratinocytes were less susceptible to studied compounds than fibroblasts in terms of cytotoxicity. Studied compounds showed a significant decrease in cell viability at higher concentrations of PRE, punicalagin and Zn (II) for oral fibroblasts (Figure 7.3). Moreover, it was found that there was a significant proliferative response with PRE and punicalagin for keratinocyte cells at lower concentrations. This response was similar to other studies that indicate natural products exert an effect on dermal cells (Moses et al. 2020). It was found that keratinocytes and fibroblasts had different cellular targets and acted differently with exogenous stimulus (Agyare et al. 2011; Deters et al. 2012). Previous research has shown that pomegranate juice extract from the whole fruit does not decrease in cell viability of HaCaT cells. In addition, pomegranate extract's protective activity has been studied on UV-irritated HaCaTs where is reduces MMP levels, induces tissue inhibitors of matrix metalloproteinase 1 (TIMP- 1) and inhibits the phosphorylation of MAPKs which have a large number of serine/threonine kinases. These enzymes are involved in the regulation of a wide array of cellular processes encompassing proliferation, differentiation, stress adaptation and apoptosis (Zaid et al. 2007).

The scratch-wound assay is a simple and inexpensive screening method for potential wound healing products. This assay assesses the proliferation and migration of fibroblasts and keratinocytes into a scratch wound area (Schafer and Warner 2007; Martinotti and Ranzato 2019). This assay consists of an *in vitro* scratched cell monolayer which is then incubated with studied compounds, providing the advantage being reliable and avoids of animal research and is a good alternative method to assess new wound healing treatments (Liang et al. 2007; Zhang et al. 2018). In this study, the effect of test compounds on HPGFs and HaCaTs cell migration speed (Tt/min), cell displacement (Td), overall velocity (Td/min) and distance travelled (Tt) and wound closure ratio was measured. Fibroblast cell migration and proliferation has a fundamental role in wound healing (Häkkinen et al. 2000; Posten et al. 2005). During the wound healing, fibroblasts essential activities comprise initial cellular migration, proliferation, cytokine/growth factor production, ECM synthesis and remodelling for

repairing damaged tissue (Smith et al. 1997; Liang et al. 2007). In the case of migration, diverse mechanisms involving expression and secretion of growth factors plays role (Häkkinen et al. 2000). It was reported that in an acute wound, fibroblast cells are activated and release cytokines including the interleukins IL-6 and IL-8 (Miller and Krangel 1992). Punicalagin at 0.1 µg/mL in combination with Zn (II) showed a significant increase in distance travelled (Tt) and cell speed (Tt/t). This significant enhancement could be due to potentiated activity of punicalagin with Zn (II) 0.1 mM. Punicalagin 10 µg/mL reduced cell speed, and the artificial scratch did not close as seen with the untreated control group. However, HPGF cells treated with the punicalagin 10 µg/mL and Zn (II) 0.1 mM did not show reduction in cell speed. It should be noted that in this wound scratch assay, the cell migration speed, cell displacement, overall velocity and distance travelled of HPGF cells under different treatments were evaluated after 48 h. From the cell proliferation assay, MTT, PRE, punicalagin, Zn (II), and both PRE/Zn (II) and punicalagin/Zn (II) did not show any significant cell proliferation compared to the untreated control group for any time interval, including 48 h. In contrast, PRE (100 µg/mL), punicalagin (10 µg/mL) and all concentrations of PRE (0.1-10 µg/mL) and punicalagin (0.1-10 µg/mL) with Zn (II) 0.1 mM significantly reduced cell proliferation at 24 h, 48 h and 72 h. Therefore, enhancement of distance travelled, and speed of cells treated with punicalagin 0.1 µg/mL in combination with Zn (II) 0.1 mM relates to HPGF cell migration, rather than proliferation. This study demonstrated that punicalagin and Zn (II) primarily induce oral fibroblast migration, rather than proliferation. While oral and dermal wound healing exhibit the same stages in wound healing, oral wounds display minimal inflammatory and angiogenic responses, rapid healing and minimal scar formation which is not seen dermal wounds (Szpaderska et al. 2003; Glim et al. 2013). These specific healing properties have been associated with specialised genotypic and phenotypic properties of oral fibroblasts. However, in chronic non-healing skin wounds such as pressure ulcers, cellular responses can be insufficient and could cause an unclosed wound (Diegelmann et al. 2004). Therefore, fibroblast properties including viability and migration have a critical role in the wound healing process. The differences between oral and dermal fibroblast responses to PRE and punicalagin concentrations could relate to well-defined in proliferation and migration (Glim et al. 2013). In the scratch wound assay, keratinocytes did not show any accelerated wound

healing. Furthermore, PRE (1 µg/mL and 10 µg/mL) with Zn (II) 0.1 mM and punicalagin (10 µg/mL)/Zn (II) (0.1 mM) did not lead to wound closure while in the absence of Zn (II) total closure was seen with these agents.

Generally, an effective wound-healing agent is linked with antimicrobial, anti-inflammatory activities in addition to enhanced proliferation, differentiation activities on fibroblasts or keratinocytes (Houghton et al. 2005). Therefore, herbal formulations have historically been considered in the treatment of a variety of skin diseases. Since bioactivities of such agents are well accepted by modern societies (Saraf et al. 2012; Ghosh and Gaba 2013). It has been suggested that medicinal plants have better healing effects and fewer adverse effects than other chemicals (Biswas and Mukherjee 2003; Davis and Perez 2009). Previous literature and research in this thesis supports both the antimicrobial and anti-inflammatory activity of PRE, punicalagin and PRE/Zn (II), along with the potentiated migration activity for punicalagin/Zn (II) (Menezes et al. 2006; Viuda-Martos et al. 2010; Houston et al. 2017a; Houston et al. 2017b; Houston et al. 2017c). Pomegranate is well placed to meet all these requirements and Zn (II) addition could potentiate these properties (McCarrell et al. 2008; Houston et al. 2017b; Houston et al. 2017c).

It is known that different parts of pomegranate have rich sources of secondary metabolites with potential biological activities (Bekir et al. 2013a). The rind is particularly rich in hydrolysable tannins (Singh et al. 2009). The wound healing activity of pomegranate, and in combination with Zn (II), has been shown in *in vivo* and *ex vivo* studies. A hydrogel formulation containing PRE and Zn (II) has a potentiated antiviral and anti-inflammatory activity as a topical agent (Houston et al. 2017a; Houston et al. 2017b; Houston et al. 2017c). In an *in vivo* study, *Punica granatum L.* peel extracts in a methanolic extract-based ointment, which substantially accelerated wound contraction, re-epithelisation, and biochemical and histopathological wound (Hayouni et al. 2011). Pomegranate peel extracts have an ability to expedite re-epithelialisation in rats (Nayak et al. 2013). Histologically of rat burns showed fewer inflammatory cells in tissue treated with pomegranate peel extract (Ma et al. 2015). In another study evaluating the cutaneous wound healing on diabetic rats, pomegranate peel polyphenols in a gel form increased fibroblast infiltration,

collagen regeneration and vascularisation in the wound area (Wei et al. 2013), and antioxidant activity of pomegranate rind provided a gastro-protective activity (Ajaikumar et al. 2005). Additionally, it has been shown that some phenolic compounds induce gene expression for connective tissue production and suppression of expression of genes which play a role in the regulation of collagen type I (Klass et al. 2010). Pomegranate peel extract with zinc oxide was also investigated in a clinical study of a 76-year-old woman with a one-year chronic leg ulcer, which could not be resolved with conventional drugs; treatment with 2% (w/w) pomegranate peel ethanolic extract hydrogel (PGMF) based on hydrophilic cream and zinc oxide achieved a good wound healing outcome (Fleck et al. 2016). However, a control group was not included and the PGMF in combination with zinc oxide treatment was applied in only on one patient. While positive dermal wound healing abilities of PRE and punicalagin have been demonstrated in some studies, in this present study the potential wound healing activities of punicalagin with Zn (II) was reported for the first time for alleviating ROS levels and oxidative stress and by stimulating migration of gingival fibroblasts.

Further *in vivo* and clinical studies are necessary to confirm the activity of PRE, punicalagin and heir combinations with Zn (II). *In vivo* studies, the microbial biofilm, ROS, and inflammatory response of the wound area will impact on wound healing process. However, *in vitro* studies could provide the first steps to search for the biological mechanisms of action of these natural compounds. Moreover, a deeper analysis is necessary to illuminate the mechanisms and pathways of wound healing activity by clarifying the molecular targets for punicalagin and punicalagin/Zn (II) combination at a cellular level.

7.5 Conclusion

In conclusion, enhanced migration activity was found with HPGF cells treated with punicalagin 0.1 µg/ml /Zn (II) 0.1 mM. Punicalagin in combination with zinc sulphate could accelerate the wound healing response by attenuating increased ROS activity in wounds and accelerating migration of gingival fibroblast to the wounded area. Higher concentrations of compounds inhibited proliferation and migration, with the lower

concentrations and combination of compounds causing toxicity on HPGF and HaCaTs. The antioxidant activity of both PRE and punicalagin was confirmed, and Zn (II) addition did not alter antioxidant activity. As a result, pomegranate rind extract and punicalagin and their Zn (II) combinations could be beneficial in wound healing due to their bioactivities in migration and antioxidant. The enhanced bioactivity of PRE and punicalagin with Zn (II) extract shows the potential of this natural treatment for oral cavity wounds.

Chapter 8: Effects of PRE, Zn (II) and PRE/Zn (II) on a 3-Dimensional *ex vivo* Rat Mandible Model

8.1 Introduction

In vitro systems aid our understanding of the bioactivities of candidate treatment and are simple, reproducible, and commonly used as a preliminary screening system. However, these systems often only use limited cell types. This simplicity means there is an inability to reproduce the *in vivo* spatial cell organisation and cellular interactions that might be evident in *in vivo* studies (Sloan et al. 2016). Also, important aspects such as drug diffusion, cannot be mimicked in the absence of a representative tissue structure. The gold standards are accepted to be *in vivo* model systems, which more realistically replicate the potential effects of treatment strategies. However, *in vivo* studies also have drawbacks. The first problem concerns ethical issues about *in vivo* studies, as large numbers of animals are required for these experiments and, secondly, there is a high cost associated with such studies. Finally, *in vivo* studies have lower throughput (Carbonell-Capella et al. 2014). Therefore, an *ex vivo* model system has been developed as an interim methodology. Physiological and pathological conditions can be studied using *ex vivo* models, since such systems retain relevance to *in vivo* scenarios. In such systems, cells and tissues can be cultured in the same spatial arrangement as found in *in vivo* situations, and the system may overcome some of these problems (Roberts et al. 2012).

An *ex vivo* culture model of the dentine-pulp complex was developed by Sloan et al. (1998), based upon 28-day-old-male Wistar rat incisor teeth. The model has extended viability and tissue organisation for up to 14 days in culture. This system has been extensively used to investigate dental pulp infections (Ayre et al. 2018), tissue repair processes (Sloan and Smith 1999; Sloan et al. 2000) and to evaluate the biocompatibility and cytotoxicity of dental treatments (Murray et al. 2000; Turner et al. 2002; Waddington et al. 2004). Extension of this tooth slice model has brought about the development of a mandible model, which has been used to examine the processes involved in bone repair (Smith et al. 2010). This system provides

information about the mechanisms underlying periodontal disease, bone tissue repair and the biocompatibility and cytotoxicity of treatment agents (Smith et al. 2010). Sloan et al. (2013) utilised the *ex vivo* mandible model for investigation of inflammatory bone destruction, which has been accepted as a feature of periodontitis, using lipopolysaccharide (LPS) and tumour necrosis factor-α (TNF-α), to induce infection and inflammatory responses, respectively. After administration of *P. gingivalis* LPS, an increase in osteoclasts, monocytes, neutrophils, and expression of pro-inflammatory cytokines by resident cells were observed in response to LPS stimulation. Additionally, a decrease in the viability of periodontal ligament fibroblasts and a loss in tissue structure were observed (Sloan et al. 2013).

In previous Chapters, the antimicrobial (in planktonic and biofilm forms) and anti-adhesive activities of PRE, Zn (II) and PRE/Zn (II) were investigated and found to be promising for use against microbes which play a role in oral infectious disease pathology. In this Chapter, the effects of PRE, Zn (II) and the PRE/Zn (II) combination on an infected *ex vivo* rat mandible model were investigated. This *ex vivo* model could provide additional validation of PRE, Zn (II) and PRE/Zn (II) efficacies and a basis for further translational potential before clinical use of these compounds.

8.2 Aims and objectives

In this Chapter, an infected *ex vivo* rat mandible tissue culture model was used to investigate the immune-inflammatory response following treatment with PRE, Zn and PRE/Zn (II). Specifically, this study:

- Examined the effects of different concentrations of PRE, Zn (II) and PRE/Zn (II) on the viability of rat mandible tissues, to determine the optimal concentration of compounds to apply to the *ex vivo* rat mandible model.
- Develop an infection of the rat mandible to stimulate periodontal diseases.
- Assess the effect of PRE, Zn (II) and PRE/Zn (II) on pro-inflammatory responses in the model.

8.3 Materials and methods

Equipment, glass and plastics, chemical consumables and assay kits used in this chapter were presented in Chapter 2, Section 2.1 in Table 2.1, Table 2.2, and Table 2.3, unless otherwise stated.

8.3.1 Cytotoxicity of PRE and Zn (II) on rat mandible tissue

8.3.1.1 Application of PRE, Zn (II) and PRE/Zn (II) to un-infected ex vivo rat mandible model

PRE, Zn (II) and PRE/Zn (II) were prepared in Green's medium as described in Chapter 2 (2.2.5.2). Then, different concentrations of PRE (5000 µg/mL, 1000 µg/mL, 100 µg/mL, 10 µg/mL, 1 µg/mL), Zn (II) (100 mM, 10 mM, 1 mM), and PRE/Zn (II) (1000 µg/mL+100 mM, and PRE 1000 µg/mL+10 mM) were applied to the rat mandible model for 30 min in a model described in Chapter 2 (Section 2.2.5.3, Figure 2.4A). The model with support ring and semi-solid agar model was not used because of the short time incubation at 37 ℃ in an aerobic environment. In addition, direct application was used to overcome any diffusion issues of PRE and Zn (II) through semi-solid agar and to simulate the clinical application. Negative (treated only with Green's medium) and a (treated with 12.5% H_2O_2) positive control groups were also used.

8.3.1.2 Quantitative lactate dehydrogenase (LDH) viability assay by evaluating LDH level in tissue culture supernatant

The Pierce Lactate Dehydrogenase (LDH) Cytotoxicity Assay Kit (ThermoFisher, Loughborough, UK), was used to assess the effects of PRE, Zn (II) and PRE/Zn (II) on the viability of rat mandible tissues.

After the incubation of tissue with different concentrations of PRE, Zn (II), PRE/ Zn (II) and untreated controls for 30 min, the supernatant of each tissue sample was placed into 1.5 mL centrifuge tubes and stored at -20 ℃. The protocol was followed according to the manufacturer's instructions (Figure 8.1), using the stored supernatant.

Briefly, 50 µL supernatant was added into wells in triplicate in a 96-well plate and 50 µL of reaction mixture added to each well and mixed gently with a pipette. The plates were incubated in the dark at room temperature for 30 min. Then, 50 µL of stop solution was added into the wells, and the colour change from clear to red was read using a plate reader with the 490 nm (background absorbance) and 680 nm (target absorbance). Supernatant from positive control (treated with H_2O_2 12.5%) was not included in this assay as H_2O_2 interfered with the assay progress and did not give a reliable result. The experiment was performed on three separate occasions, with three replicates for each sample and the result was graphically presented as direct absorbance readings for each sample and control, expressed as mean±SEM.

Results were analysed statistically using GraphPad Prism software (GraphPad Software, Version 8.2.1, San Diego, CA, USA), performing one-way ANOVA followed by post hoc Tukey test.

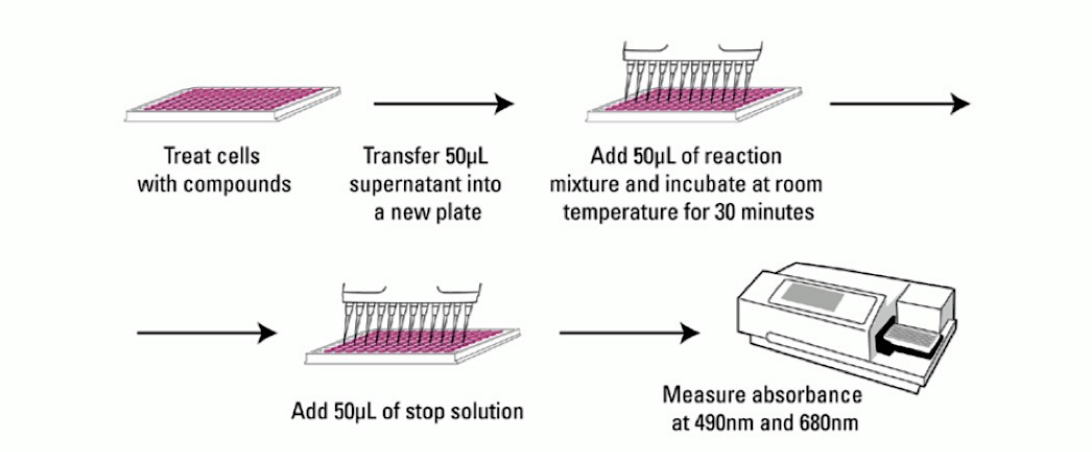


Figure 8.1: Schematic for the ThermoFisher Scientific Pierce LDH Cytotoxicity Assay (image was taken from the assay guideline of Pierce LDH Cytotoxicity Assay Kit, available at: https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0011851_Pierce_LDH_Cytotoxicity_Asy_UG.pdf).

8.3.1.3 Detection of DNA fragmentation by terminal deoxynucleotidyl transferase dUTP nick and labelling (TUNEL) method

Apoptosis detection was performed using a commercially available terminal deoxynucleotidyl transferase dUTP nick and labelling (TUNEL) Apoptosis Kit (Abcam, Cambridge, UK), according to the manufacturer's protocol. Terminal deoxynucleotidyl transferase (TdT) binds the exposed 3'-OH ends of DNA fragments in response to apoptosis signals and catalyses the addition of biotin- labelled deoxynucleotides. Biotinylated nucleotides were detected using a streptavidin-horseradish peroxidase (HRP) conjugate and DAB reacted with this HRP-labelled sample. On completion of this reaction, an insoluble brown coloured substrate was generated at the sites of DNA fragmentation. Samples were counterstained with Mayer's haematoxylin to give good contrast colour and for the morphological investigation of normal and apoptotic cells.

Results were presented as images (20x objective) and the number of apoptotic cells quantified. The number of nuclei on stained histological sections were counted using ImageJ analysis software. Images were captured at 20x objective, and five random areas (50 μm^2) were selected for each section and three sections were used for each treatment and control group. The mean number of apoptotic cell nuclei for each group was calculated and results were analysed statistically using GraphPad Prism software (GraphPad Software, Version 8.2.1, San Diego, CA, USA), performing one-way ANOVA followed by post hoc Tukey test, with results expressed as mean±SEM.

8.3.2 Infection of *ex vivo* rat mandible model

8.3.2.1 Determination of bacterial growth in an ex vivo model medium (Green's medium)

The growth of *S. gordonii* in Green's medium without antibiotics was investigated. The preparation of Green's medium for the *ex vivo* model was described in Chapter 2 (Section 2.2.5.2) but used without antibiotic. Bacterial suspensions of *S. gordonii* at 10^2 CFU/mL were prepared by the inoculation of Green's medium without antibiotics, with 5%, 10%, and 20% MH broth in Green's medium or MH broth only. Then, 200

µL of bacterial suspension from each condition was placed in the well of 96-well plates. MH broth only, without bacteria used as sterility controls. Plates were incubated in a plate reader in 5% CO_2, 95% air, at 37°C, and the absorbance values read over 20 h, at 600 nm (FLUOstar Omega, BMG Labtech, Ortenberg, Germany). After 20 h, the data was used to create the growth curve for *S. gordonii* in the modified culture medium. In this assay, six replicate wells were used for each condition, and the assay was repeated on three independent occasions.

8.3.2.2 Inoculation of bacteria to rat mandible tissue

The *ex vivo* rat mandible model was infected with *S. gordonii* and *P. gingivalis,* both separately and in combination, with the aim of simulating an oral infectious disease environment.

Rat mandible tissues were dissected and placed into semi-solid agar including Green's medium with antibiotics, as described in Chapter 2, Figure 2.4B. Firstly, tissues were pre-incubated for 4 h under these conditions and washed three times for 5 min with 5 mL PBS to eliminate trace antibiotics in the tissues. Then, tissues were used either for infection or as an uninfected control without antibiotics.

For infection under planktonic bacteria forms, cultures of *S. gordonii* and *P. gingivalis* were prepared as previously described in Chapter 2 (Section 2.2.3.6), and when used in dual species infection equal volumes (500 µL of each bacterium) of bacterial suspension were combined. The rat mandible tissues were infected directly with 20 µL of experimental culture of *S. gordonii, P. gingivalis*, and *S. gordonii* and *P. gingivalis* combined, by adding bacterial suspension(s) directly onto the tissues. Tissues were incubated at 37°C in a 5 % CO_2, 95% air for 20 min to allow bacterial attachment, before adding the tissues to the semi-solid agar medium. After placing tissues in 6-well plates, these were incubated for a further 20 h at 37 °C in 5% CO_2, 95% air.

S. gordonii biofilms were created by adding 0.5 mL of an overnight *S. gordonii* culture onto a sterile round filter paper, which was placed on FAA and incubated for 20 h at 37 °C in an aerobic cabinet. *P. gingivalis* biofilms were created by adding 0.5 mL of

P. gingivalis culture of onto a sterile filter paper placed again on FAA and incubated for 48 h in an anaerobic cabinet at 37 ℃. The dual species biofilm of *S. gordonii* and *P. gingivalis* was performed similarly by adding equal volumes (0.25 mL of each) of both bacterial cultures on sterile filter paper placed on FAA and incubated for 48 h in an anaerobic cabinet. After formation of mono-species and dual-species biofilms, sterilised rat mandible tissues were embedded with formed biofilms for 20 min for bacterial attachment. Uninfected controls without any bacterial inoculation were also included in all experiments. After infection, tissues were placed into semi-solid agar medium in the 3D *ex vivo* model for a further 20 h incubation at 37℃ and 5 % CO_2 in an incubator. Tissues were then collected from the *ex vivo* model and either stored in RNA Later Solution (Sigma-Aldrich Company Ltd., Poole, UK) at -20℃ for RNA extraction or fixed for immunohistochemistry (IHC) studies. The culture supernatants were collected and stored at -20 ℃ to use for cytokine quantification in culture media by ELISA.

8.3.2.3 Gram-staining of infected mandible to visualise attached bacteria

Gram-staining of histological rat mandible tissue sections was performed, using an optimised staining technique to detect *S. gordonii* and *P. gingivalis* within tissue from the infected *ex vivo* model. The assay was performed according to the method described by Sandra et al. (2016). Paraffin-embedded slides were prepared as described in Chapter 2 (Section 2.2.6). After deparaffinisation and rehydration through a series of xylene, 100%, 95% and 70% v/v ethanol for 5 min each then distilled water, tissue section slides were exposed to crystal violet for 5 min at room temperature and washed under running water to remove excess crystal violet. Gram's iodine was applied for 2 min and washed again with tap water. To remove any unspecific crystal violet staining, a Gram-decolouriser (acetone:ethanol, 1:1) was applied for 30 s and slides were washed immediately again with running tap water until the water ran clear. Then, tissue section slides were exposed to safranin for 2 min and dehydrated with 95 % and 100 % of ethanol for 5 min/each, respectively. Then slides were immersed in alcoholic saffron for 4 min, which served as a counterstain. The dehydration process continued with 100 % ethanol for 3 min, to remove any excess alcoholic saffron, and a xylene wash for 5 min. Finally, slides were mounted in DPX mounting solution and overlaid with coverslips for imaging by light microscopy.

8.3.3 Application of PRE, Zn (II) and PRE/Zn (II) treatments to *P. gingivalis* biofilm infected models

The *P. gingivalis* biofilm infected rat mandibles were prepared as described in Section 8.3.2.2.I Infected rat mandibles were incubated with PRE (1 mg/mL), Zn (II) (1.56 mM) and the PRE/Zn (II) (1 mg/mL and 1.56 mM) for 30 min, in a model without the support ring system illustrated in Chapter 2 (Figure 2.4A). PRE, Zn (II) and PRE/Zn (II) treatment solutions were prepared in Green's medium without antibiotics, using stock solutions of PRE and Zn (II) in phthalate buffer pH 4.5. For uninfected controls, 10% of phthalate buffer pH 4.5 was added in Green's medium to ascertain any effect from the phthalate buffer pH 4.5. After 30 min, tissues were collected and stored in RNA Later Solution at -20 ℃ for RNA extraction or fixed for IHC studies. The culture supernatants were collected and stored at -20℃ for cytokine quantification by ELISA.

8.3.4 RT-qPCR analysis for selected cytokines expression

Analysis of cytokine gene expression was performed in accordance with the Minimum Information for publication of Quantitative real-time polymerase chain reaction (PCR) experiments (MIQE) which is a collection of guidelines for carrying out and reporting quantitative real-time PCR studies and data (Bustin et al. 2009). The target genes are shown in Table 8.1. The primers were designed and validated by PrimerDesign (Southampton, UK).

Gene	TaqMan Probe (Assay ID)
IL-1β	Rn00580432_m1
IL-6	Rn99999011_m1
TNF-α	Rn01525859_g1
GAPDH (glyceraldehyde-3-phosphate dehydrogenase)	Rn99999916_s1

Table 8.1: Target genes used in qPCR assays.

8.3.4.1 Extraction of RNA from rat mandible tissue

RNA extraction from mandible tissues was performed using the PureLink RNA Mini Kit (ThermoFisher Scientific), according to the manufacturer's instructions using TRIzol lysing solution. Before RNA extraction, the workspace area was cleaned with IMS and RNaseZap, to ensure decontamination, including between handling different samples. Mandible tissue samples, stored in RNA Later Solution at -20°C, underwent tissue homogenisation using a mortar and pestle. The homogenised tissues were put in a 2 mL round bottom RNase-free tube with 1 mL of TRIzol reagent (ThermoFisher Scientific, Loughborough, UK) and the samples were left for 5 min at room temperature to allow complete dissociation of proteins and nucleic acids. Chloroform (200 µL) was added into each tube and shaken vigorously for 15 s, before being left for 2 min at room temperature. The samples were centrifuged at 12 000 x g for 15 min at 4°C to obtain three different layers. The colourless upper layer (400 µL) was carefully added to new RNase-free tubes, as this layer contained the RNA. The same volume of 70 % molecular grade ethanol was added to the RNA samples and was mixed by vortexing. Subsequently, 700 µL of the samples were transferred to spin cartridges inserted into collection tubes, centrifuged again at 12 000 x g for 15 s at room temperature and the flow-through was discarded and spin cartridges were re-inserted into the same collection tubes. This step was repeated until all samples had been processed. The bound RNA was washed by adding 700 µL of wash buffer I and centrifuged at 12 000 x g for 15 s at room temperature and the flow-through was discarded. The spin cartridges, which were inserted in same collection tubes, were washed with 500 µL of wash buffer II with 100% of ethanol (16 mL) and centrifuged

12 000 x g for 15 s at room temperature. After discarding the flow-through, this step was repeated. Then, the spin cartridges were inserted into recovery tubes to elute the RNA by adding 50 µL RNase-free water to the centre of the spin cartridges and incubated for 1 min at room temperature. At the end of the incubation period, these were centrifuged at 12 000 x g for 2 min at room temperature and the spin cartridges discarded. The recovery tubes contained the purified total RNA.

The quantity and quality of the purified total RNA from rat mandible tissues were determined using a Nanodrop D-800 Spectrometer (ThermoFisher Scientific, Loughborough, UK). RNA purity was evaluated using the ratio between absorbance values at 260 and 280 nm (260/280 ratio) and purities over 1.7 were accepted sufficient to use in qPCR assay. Purified RNA samples were stored at -80

8.1.1.1 ℃ until required . *Reverse transcription and synthesis of copy DNA (cDNA)*

The purified RNA was converted to complimentary DNA (cDNA), using High-Capacity cDNA Reverse Transcription Kits (ThermoFisher Scientific) according to the manufacturer's instructions. Briefly, 2x Reverse Transcription Master Mix was prepared (10X RT Buffer, 2 µL; 25X dNTP Mix (100mM), 0.8 µL; 10X RT Primers, 2 µL; Multiscribe Reverse Transcriptase, 1 µL; RNase Inhibitor, 0.5 µL; Nuclease-free H2O, 3.7 µL; in Kit) on ice and gently mixed. Then, 10 µL of 2x RT Master Mix was added into the tubes, with RNA samples and nuclease-free water added to a final volume of 10 µL. RNA sample volume was determined according to RNA concentration. Tubes were centrifuged briefly and placed into the Thermal Cycler (SimpliAmp Thermal Cycler, Applied Biosystems, ThermoFisher Scientific, Loughborough, UK), and it was programmed accordingly (37°C for 60 min, 95°C for 5 min and hold at 4 °C, until stored at -20 °C).

8.1.1.2 Quantitative polymerase chain reaction (qPCR) amplification and detection

The relative abundance of the transcripts of the candidate inflammatory genes were measured by quantitative real-time PCR (qPCR), using Taqman chemistry and

predesigned sets of primers and probes (Taqman Gene Expression Assays, Applied Biosystems, Warrington, UK) on a Quant Studio 6 qPCR. The reactions were performed in 96-well plates in a final reaction volume of 20 µL that included Taqman Fast Universal Master Mix (Applied Biosystems, Warrington, UK), cDNA (30 ng) of sample, Taqman gene expression master mix (Applied Biosystems, Warrington, UK) for each target gene and GADPH as a housekeeping gene. The plates were sealed and centrifuged at 10 000 x g for 2 min and transferred to the Quant Studio 6 qPCR system. Thermal cycling conditions were optimised as follows: 95°C for 20 s, followed by 40 cycles at 95 °C for 10 s and 60 °C for 20 s. The Comparative CT (ΔΔ CT) method was used to quantify levels of the genes of interest. For each sample, gene expression analysis was performed on three separate occasions. For each experimental condition three different rat mandible tissues were used for RNA extraction and then cDNA synthesis. Each qPCR assay was performed on three separate occasions with two internal replicates. GAPDH was used as a housekeeping gene to normalise the amount of messenger RNA (mRNA). Statistical analysis was performed using one-way ANOVA with Dunnett Multiple Comparisons Test analysis, using the untreated control as the control for comparison. Statistical significance considered at $p<0.05$, with results expressed as mean±SEM.

8.1.2 Assessment of infection on pro-inflammatory cytokine protein levels

The stored culture supernatant medium from the infected rat mandible cultures (in planktonic and biofilm forms of *S. gordonii, P. gingivalis,* and *S. gordonii* and *P. gingivalis*) and treated *P. gingivalis*-infected rat mandibles, were used to assess the protein levels of pro-inflammatory cytokines, interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumour necrosis factor α (TNF-α), pro-inflammatory mediator prostaglandin E2 (PGE-2), by ELISA. The cell culture supernatants were obtained as described in Sections 8.3.3 and Section 8.3.2.2. The frozen supernatants were thawed at room temperature and used according to the manufacturer's instructions for each ELISA Kit (IL-1β, IL-6 and TNF-α from PeproTech, London, UK; and PGE-2 from R&D Systems, Abingdon, UK) (see Appendix for detailed procedure of ELISA assays). The concentrations of IL-1β, IL-6, TNF-α and PGE-2 in each culture

supernatant were calculated according to calibration curves generated with standards provided for each Kit. Results were analysed statistically using GraphPad Prism software, performing one-way ANOVA followed by post hoc Tukey test, with results expressed as mean±SEM.

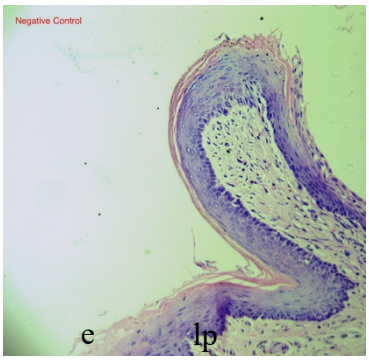
8.1.3 Imaging and statistical analysis

For imaging the tissue slides, an Olympus Ax70 Upright Fluorescent Microscope was used in the light mode. Images were taken with 20x, 40x, 60x and/or 100x objective. Statistical analysis of images was performed using one-way ANOVA with post-test Tukey analysis, and statistical significance considered at $p<0.05$.

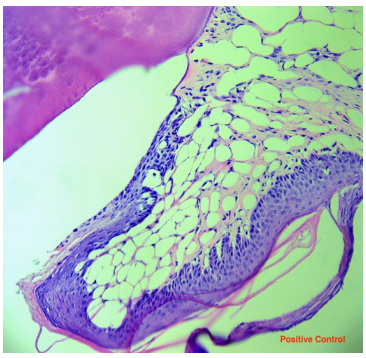
8.4 Results

8.4.1 Haematoxylin and eosin (H&E) staining of rat mandible sections

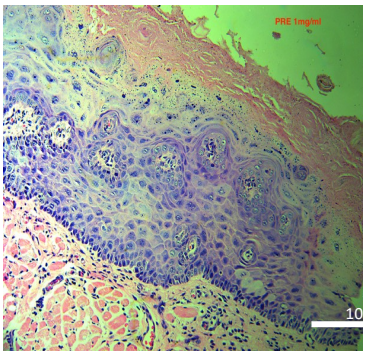
Healthy gingival tissue is characterised by three layers: keratinised layer, epithelium layer (e) and the basal layer attached to the underlying connective tissue of the lamina propria (lp) and are presented in Figure 8.2A for the negative control. With the exception of the positive control (Figure 8.2B (H_2O_2 12.5 %), the treated rat gingiva maintained structural integrity over 30 min with different concentrations of PRE, Zn (II) and the PRE/Zn (II). However, for the positive control (H_2O_2 12.5%), the connective tissue lost integrity, showing a highly damaged tissue compared to negative control, PRE, Zn (II) and PRE/Zn (II) treatment. However, for positive control, the epithelium was not shown to be affected as much as the connective tissue of the lamina propria. It showed similar epithelium in all cases including positive control (Figure 8.2A).


Negative Control
e
lp

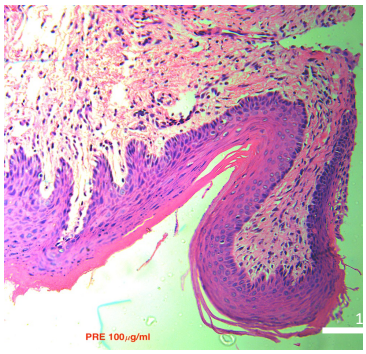
A


Positive Control

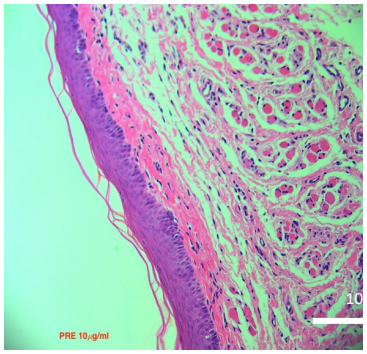
B


PRE 1mg/ml

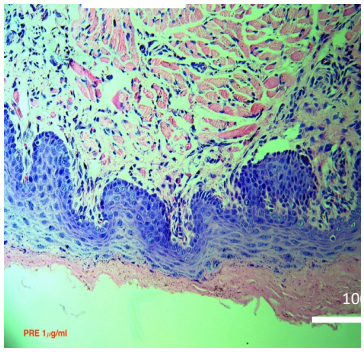
C


PRE 100μg/ml

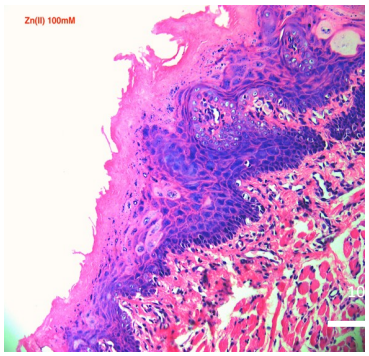
D


PRE 10μg/ml

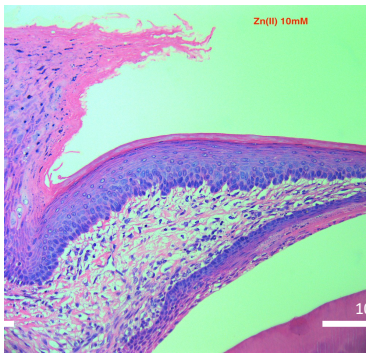
E


PRE 1μg/ml

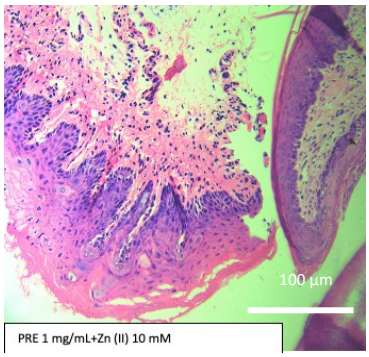
F


Zn(II) 100mM

G


Zn(II) 10mM

H



I

Figure 8.2: H&E staining of rat gingiva treated with different concentrations of PRE and Zn (II). A; untreated negative control, B; positive control (H_2O_2, 12.5 %), C; PRE 1000 µg/ml, D; PRE 100 µg/ml, E; PRE 10 µg/ml, F; PRE 1 µg/ml, G; Zn (II) 100 mM, H; Zn (II) 10 mM, I; PRE 1 mg/mL and Zn (II)10 mM. e: epithelium, lp: lamina propria. Red arrows indicate damage difference in connective tissue between positive and negative control. Scale bar = 100µm.

8.4.2 Cytotoxicity of PRE, Zn (II) and the PRE/Zn (II) in the *ex vivo* rat mandible

8.4.2.1 Quantitative LDH viability assay

Direct absorbance readings from the LDH assay are presented in Figure 8.3. A range of different concentrations of PRE and Zn (II) were, with mean LDH absorbance values for each treatment compound compared to untreated controls. PRE did not show any significant difference compared to the untreated control ($p>0.05$); only the highest concentration of Zn (II), 100 mM, showed a significant increase in LDH release compared to the untreated control ($p<0.01$).

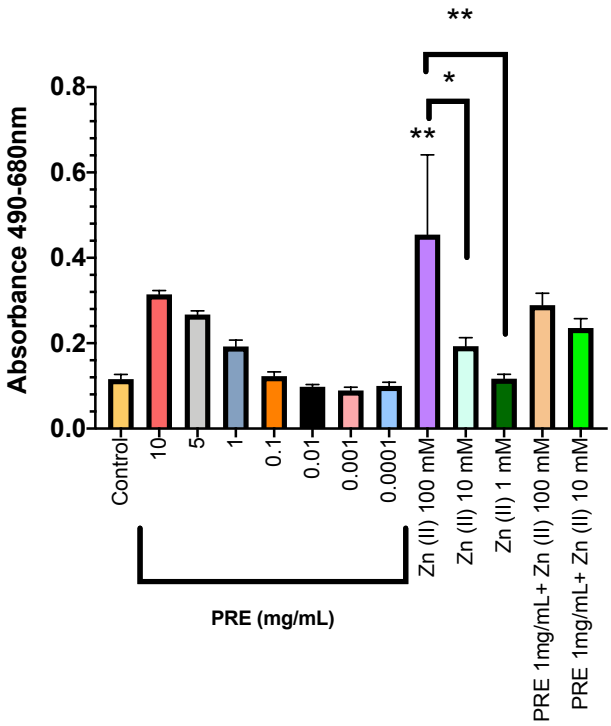
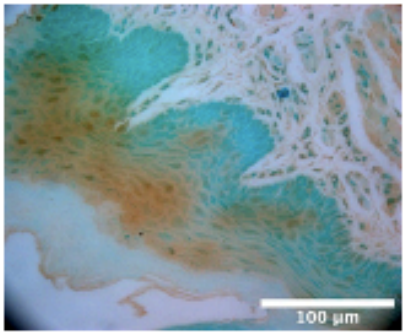


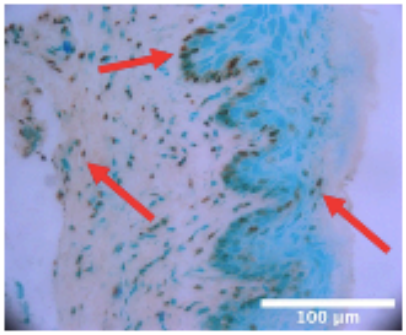
Figure 8.3: LDH assay results from supernatants of *ex vivo* rat mandible. A: absorbance reading of indicated concentrations of PRE, Zn (II) and PRE in combination with Zn (II). Data represented mean±SEM. N=3. *, $p < 0.05$; **, $p < 0.01$.

8.4.2.2 TUNEL assay analysis

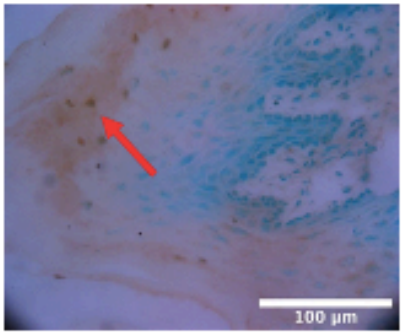
The TUNEL assay was considered useful for the localisation of apoptotic DNA fragmentation *in situ*. Thus, the TUNEL assay was used to investigate whether PRE, Zn (II) and PRE/Zn (II) combination were inducing apoptosis. In all groups, TUNEL-positive cells could be observed (Figure 8.4). Only 5 mg/mL PRE caused a statistically significant generation of TUNEL positive cells ($p<0.0001$) (Figure 8.5).



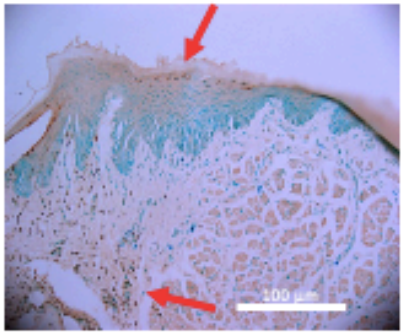
A



B



C



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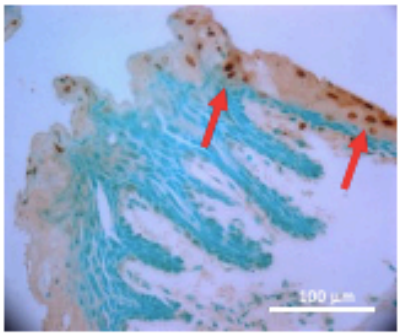

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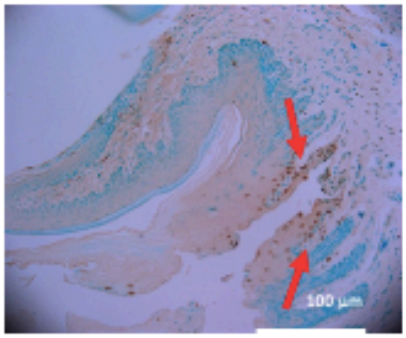
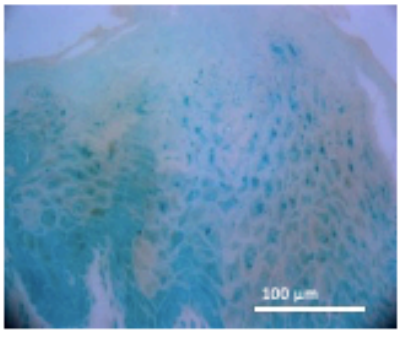
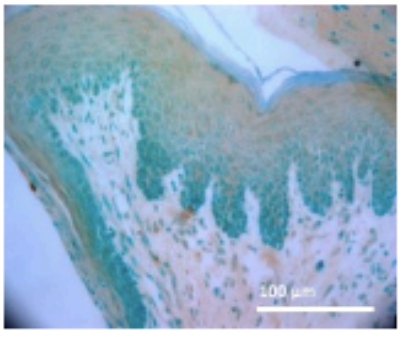


F



G



H

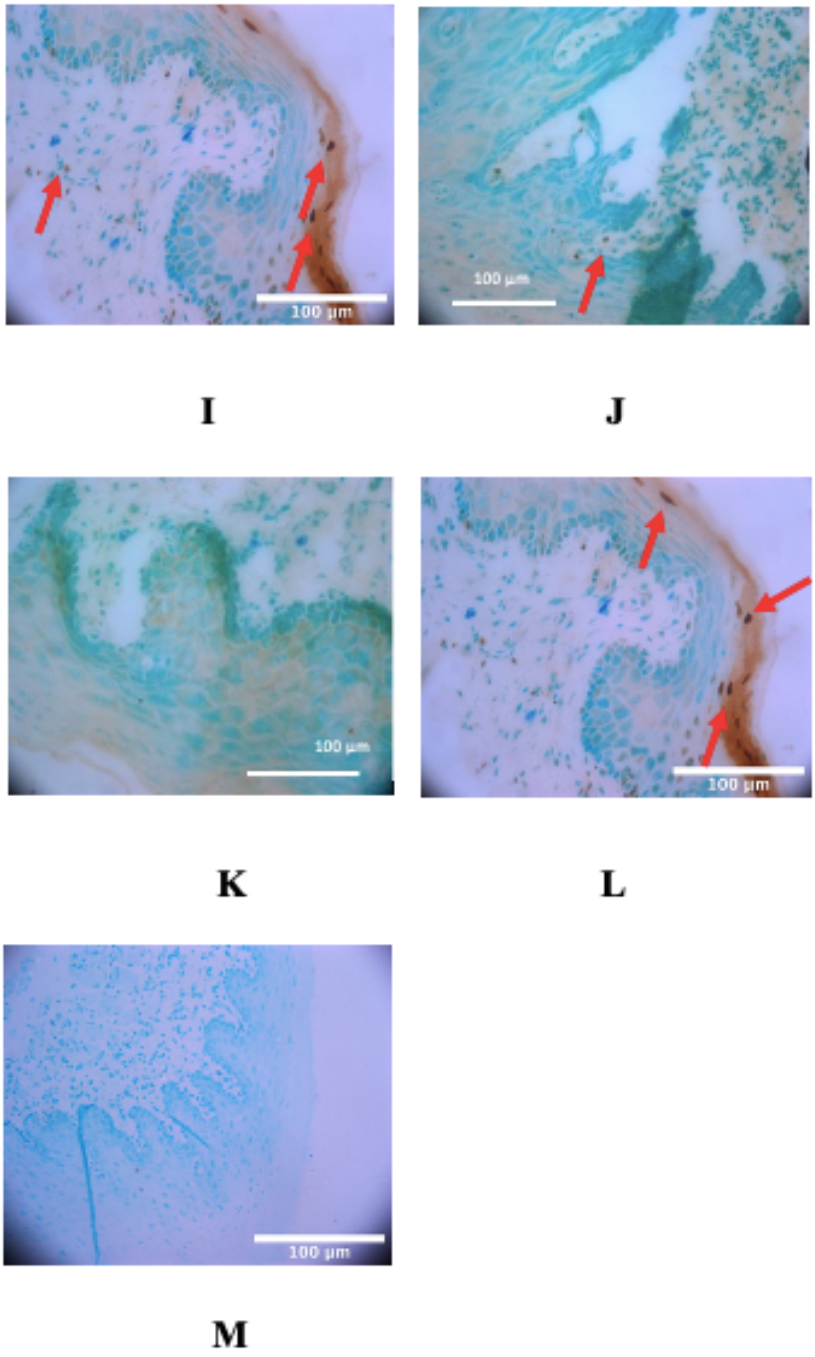


Figure 8.4: Images of TUNEL immunohistochemistry assay. Negative control was included in this IHC assay to demonstrate absence of non-specific binding between the tissue and antibodies. A; Untreated control, B; PRE 5 mg/mL, C; PRE 1 g/mL, D; PRE 0.1 mg/mL, E; PRE 0.01 mg/mL, F; PRE 0.001 mg/mL, G; PRE 0.0001 mg/mL, H; Zn (II) 100 mM, I; Zn (II) 10 mM, J; Zn (II) 1mM K; PRE 1mg/mL and Zn (II) 100 mM, L; PRE 1mg/mL and Zn (II) 10 mM, and M; no primary control. Red arrows on images indicates TUNEL positive stained cell nuclei.

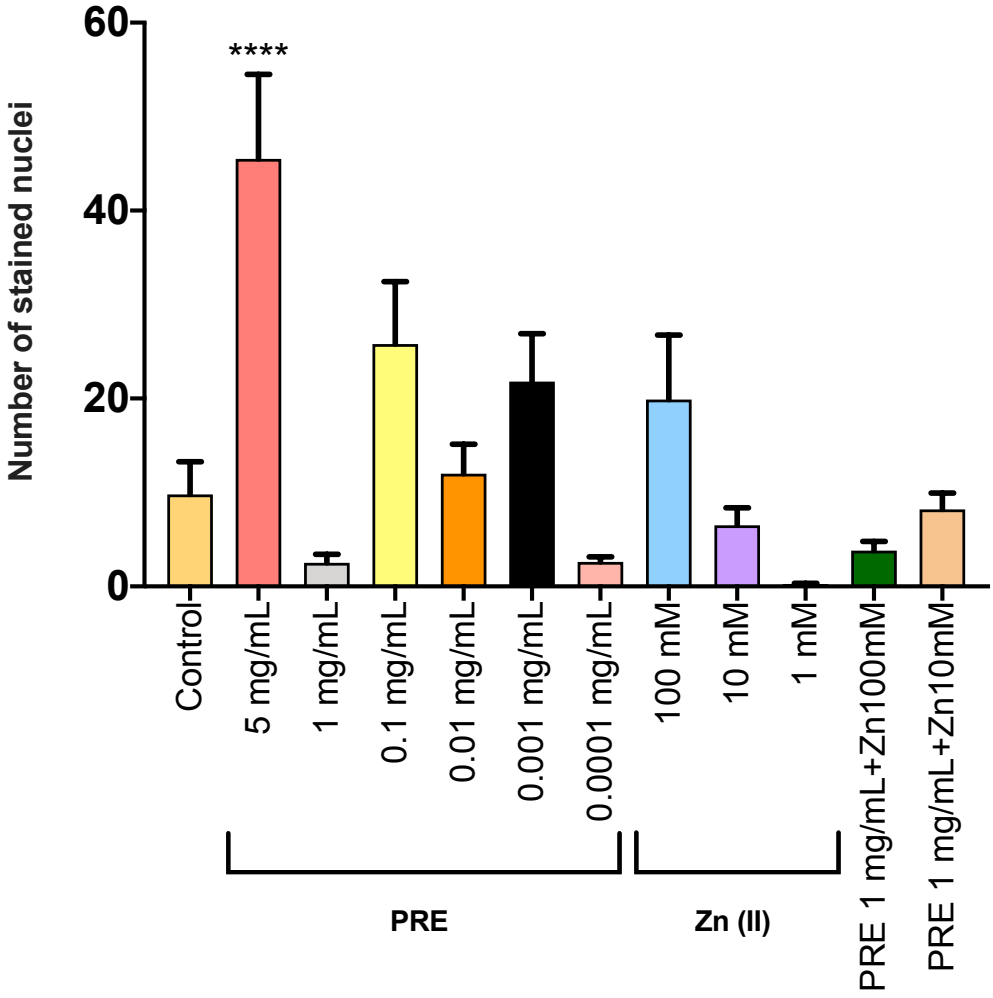


Figure 8.5: Semi-quantitative analysis of TUNEL assay images by counting stained nuclei with ImageJ. Data represents mean±SEM. N=3, ****, $p<0.0001$, compared to untreated controls.

8.4.3 Optimisation of infected *ex vivo* rat mandible model

8.4.3.1 The growth of S. gordonii in growth medium of ex vivo model

S. gordonii exerted a typical growth curve in Green's medium. Moreover, *S. gordonii* presented greater exponential phase in only Green's medium than Green's medium supplemented with MH broth and only MH broth (Figure 8.6). As *S. gordonii* grew better in Green's medium, this was used to infect the model. Therefore, Green's medium without antibiotic was used as a culture medium in the *ex vivo* rat mandible model. MH broth only, without *S. gordonii* inoculation, was used as a sterility control and no growth was observed.

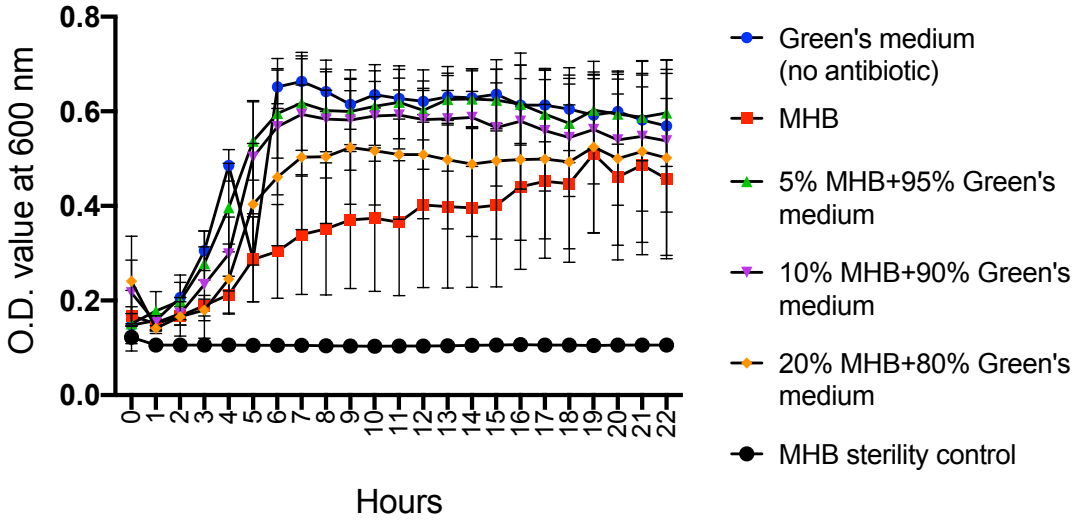


Figure 8.6: *S. gordonii* growth in *ex vivo* culture medium without antibiotic and modified with different ratios of MHB (MH broth) based on optical density (600 nm). Data presented as mean absorbance±*SEM* for three independent experiments.

8.4.3.2 Histology sections for infected ex vivo rat mandible model: Gram staining

S. gordonii exerted attached to rat mandible tissue in both planktonic and biofilm conditions (Figure 8.7 and Figure 8.8). In addition, when *S. gordonii* was combined with *P. gingivalis, S. gordonii* attachment was again observed in tissue sections (Figure 8.9 and Figure 8.10) and *S. gordonii* attachment was observed in both the epithelium and lamina propria of rat gingiva. However, *P. gingivalis* invasion in rat mandible tissue was not observed in this assay.

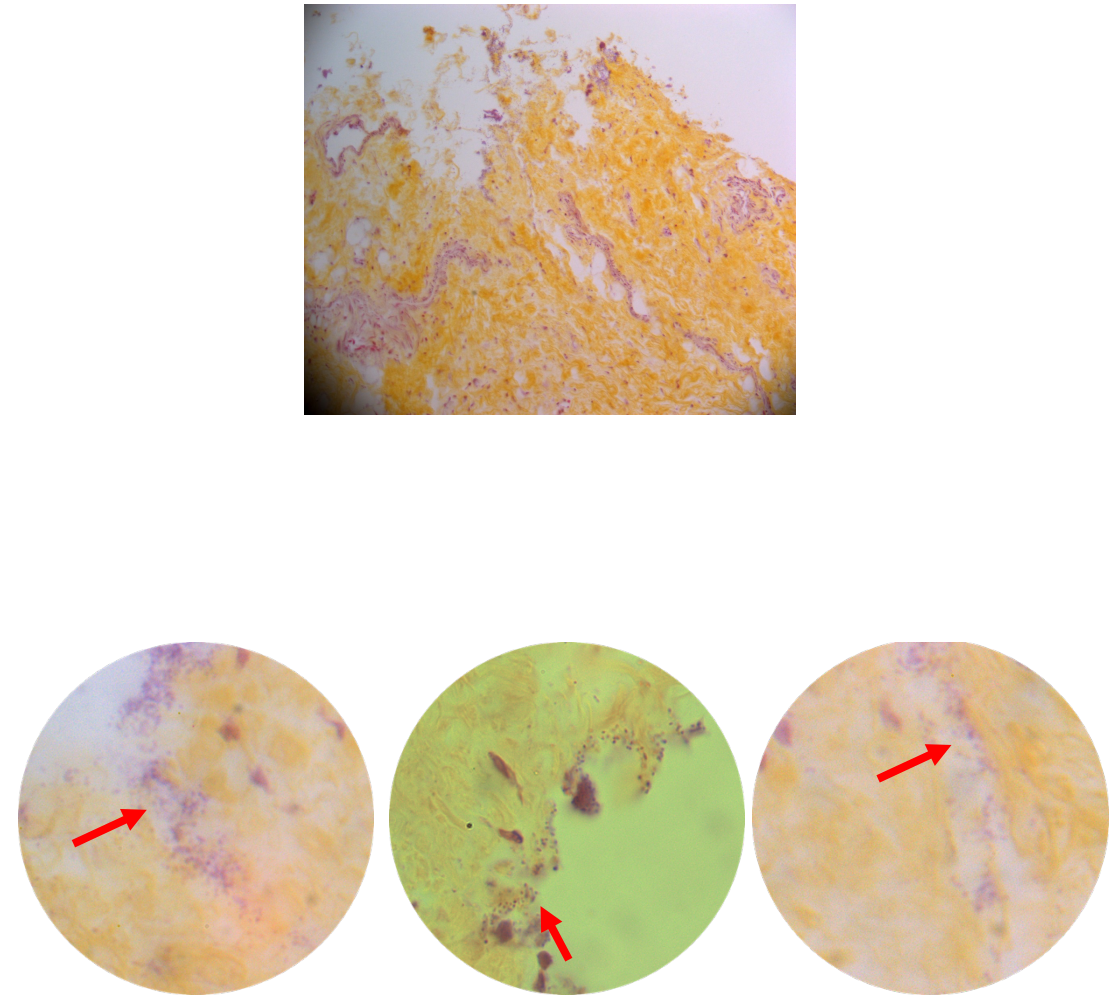


Figure 8.7: *S. gordonii* (planktonic) infected rat mandible sections after 20 h co-culturing. Top micrograph was taken at 60x objective. Three circle shaped micrographs were taken from different areas of 100x objective micrographs of the top micrograph. Red arrows show the Gram-stained *S. gordonii*. The micrographs are representative of three independent experiments.

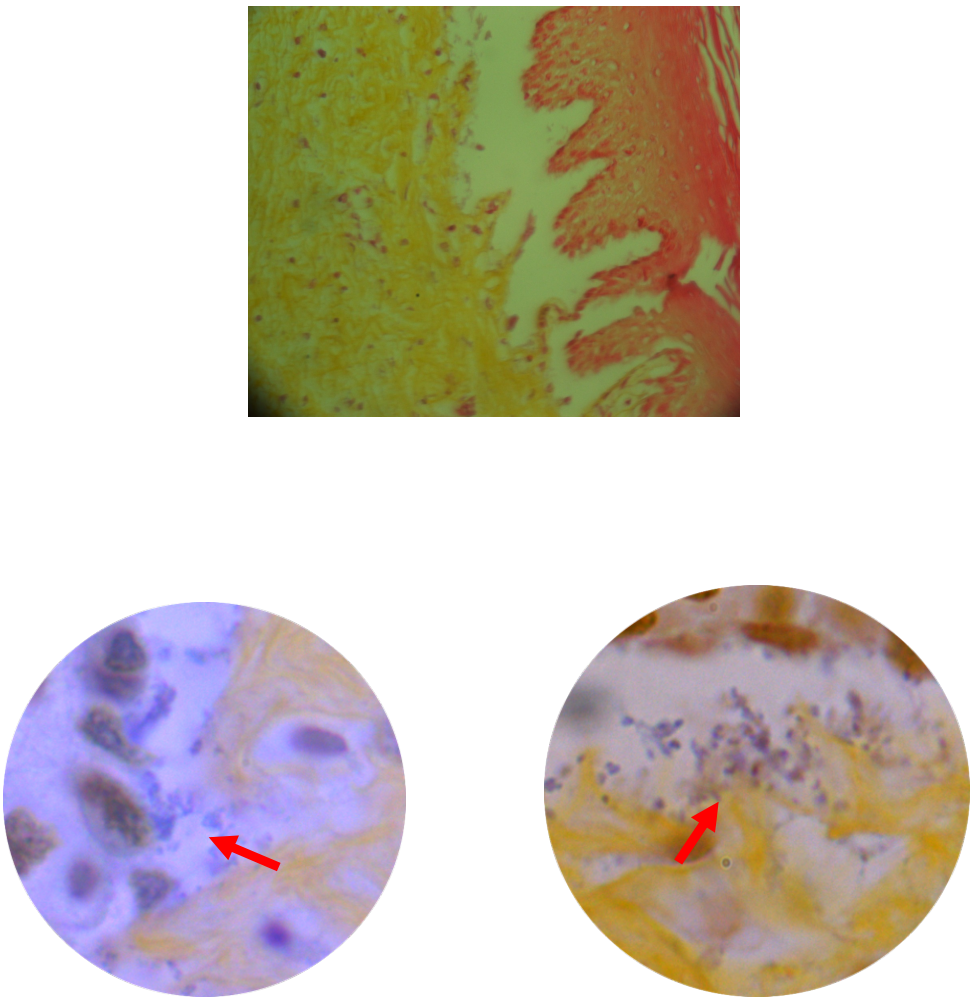


Figure 8.8: *S. gordonii* (biofilm) infected rat mandible sections after 20 h co- culturing. Top micrograph was taken at 60x objective. Three circle shaped micrographs were taken from different areas of 100x objective micrographs of the top micrograph. Red arrows show the Gram-stained *S. gordonii*. The micrographs are representative of three independent experiments.

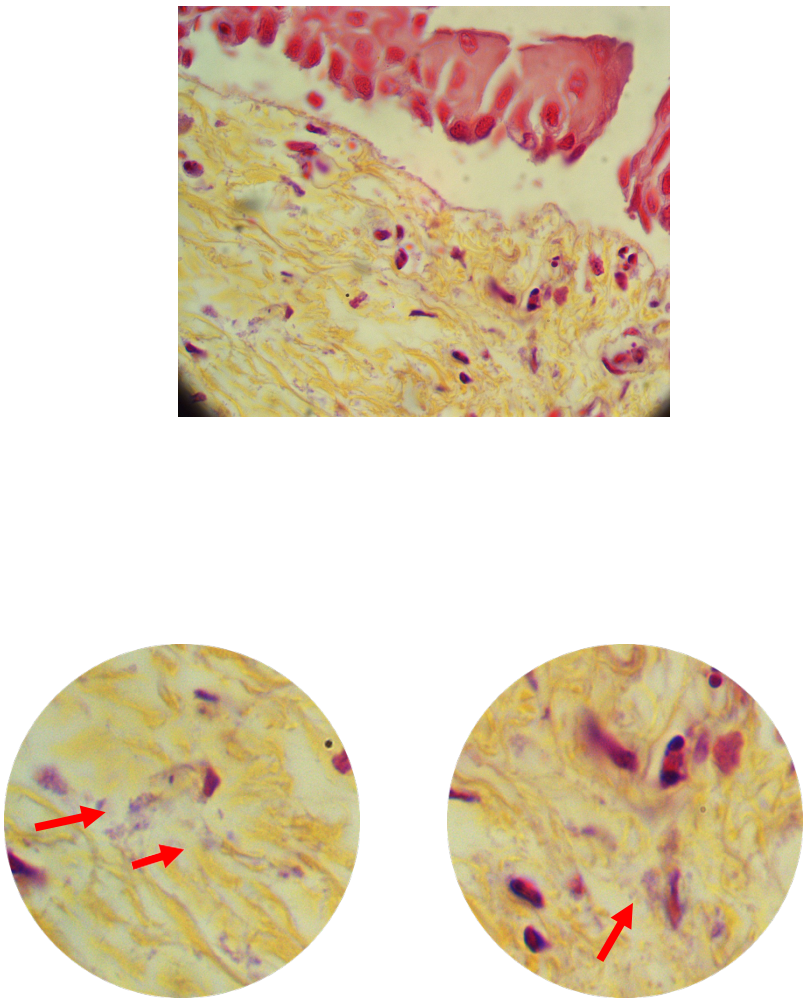


Figure 8.9: *S. gordonii* and *P. gingivalis* (planktonic) infected rat mandible sections after 20 h co-culturing. Top micrograph was taken at 60x objective. Three circle shaped micrographs were taken from different areas of 100x objective micrographs of the top micrograph. Red arrows show the Gram-stained *S. gordonii*. The micrographs are representative of three independent experiments.

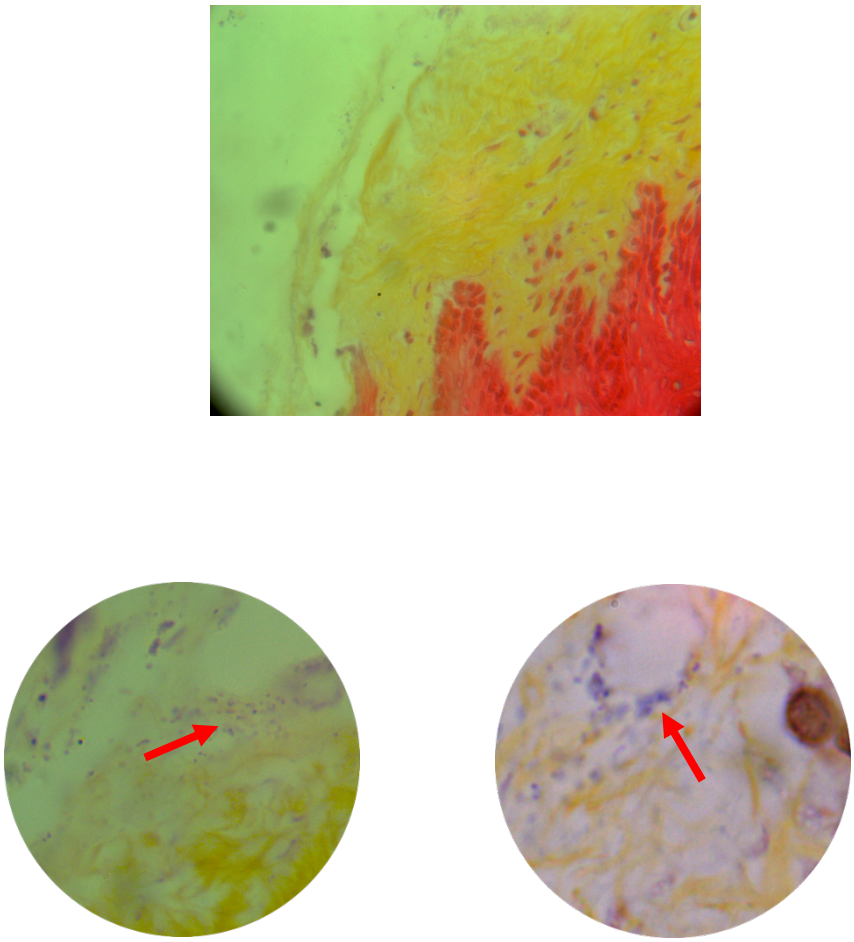
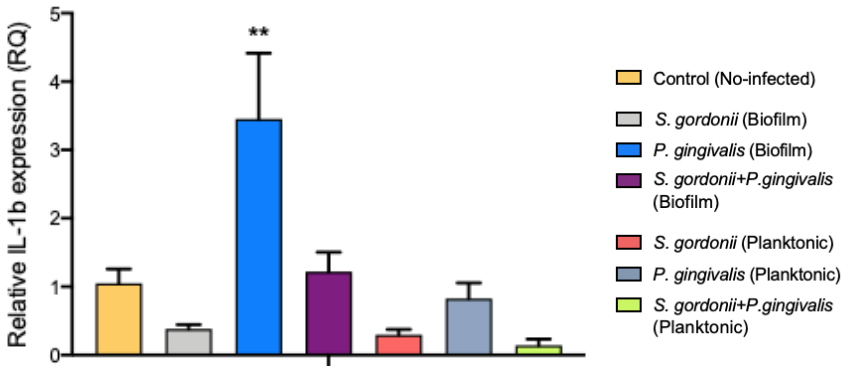


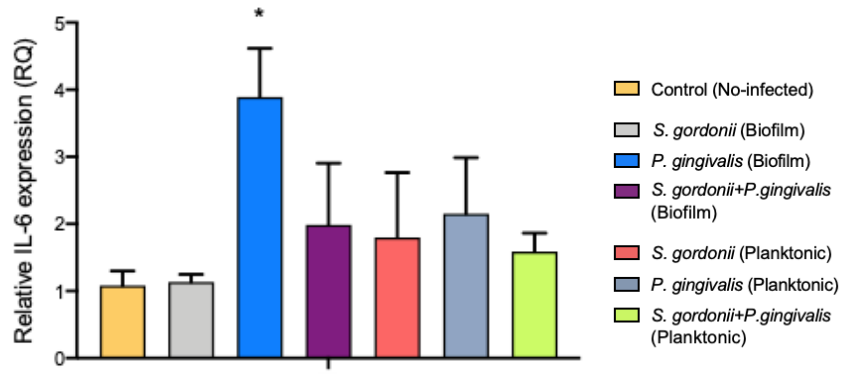
Figure 8.10: *S. gordonii* and *P. gingivalis* (biofilm) infected rat mandible sections after 20 h co-culturing. Top micrograph was taken at 60x objective. Three circle shaped micrographs were taken from different areas of 100x objective micrographs of the top micrograph. Red arrows show the Gram-stained *S. gordonii*. The micrographs are representative of three independent experiments.

8.4.4 RT-qPCR for pro-inflammatory cytokine gene expression level in co- cultured rat mandible tissue

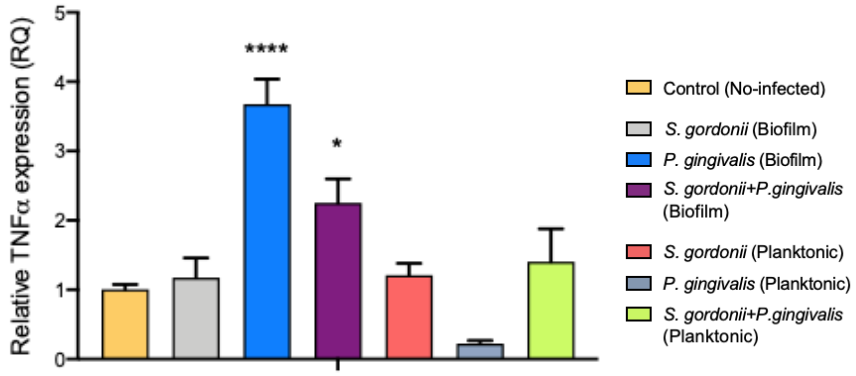
The pro-inflammatory markers were investigated in mandible tissues via mRNA gene expression for IL-1β, IL-6 and TNF-α , following 20 min direct application with *S. gordonii, P. gingivalis* and *S. gordonii* and *P. gingivalis*, in both planktonic and biofilm conditions and co-culturing with attached bacteria for 20 h (Figure 8.11A, B, C). Expression of all studied target genes, IL-1β, IL-6 and TNF-α, were significantly up-regulated by *P. gingivalis* biofilm infection ($p<0.01$ for IL-1β, $p<0.05$ for IL-6 and $p<0.0001$ for TNF-α). *P. gingivalis* infection under planktonic conditions did not up-regulate the gene expression of target genes, compared to uninfected controls ($p>0.05$). The dual species biofilm of *S. gordonii* and *P. gingivalis* significantly induced only TNF-α gene expression ($p<0.05$). However, it did not cause any statistically significant up-regulation in IL-1β or IL- 6 gene expression, compared to uninfected controls ($p>0.05$).



A



B



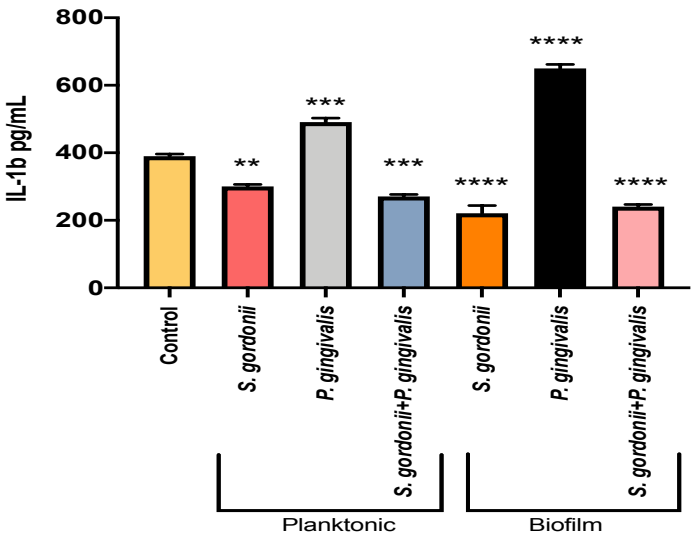
C

Figure 8.11: Pro-inflammatory gene expression in infected 3D *ex vivo* rat mandible model after 20 h infection of rat mandible tissue with *S. gordonii, P. gingivalis*, and *S. gordonii* and *P. gingivalis* in planktonic and biofilm conditions. A; IL-1β, B; IL-6, C; TNF-α. Statistical difference between *S. gordonii, P. gingivalis*, and *S. gordonii* and *P. gingivalis* in planktonic and biofilm conditions, compared to uninfected controls, are indicated on the bars. *; $p < 0.05$, **; $p < 0.01$ and ****; $p < 0.0001$, compared to uninfected controls.

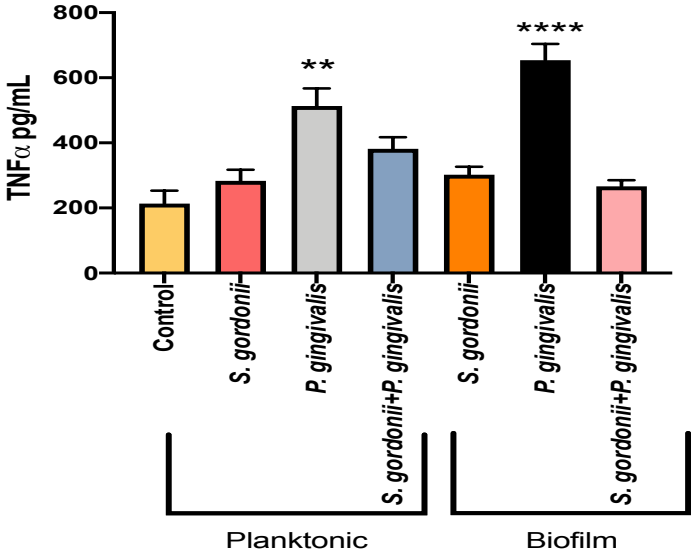
8.4.4.1 ELISA quantification of pro-inflammatory cytokines level in culture medium from co-cultured rat mandible tissues

The released pro-inflammatory cytokines from rat mandibles infected with *S. gordonii, P. gingivalis* and *S. gordonii* and *P. gingivalis* in both planktonic and biofilm conditions were evaluated via ELISA, using the collected supernatant (Figure 8.12 A,B,C, respectively). The levels of IL-1β, IL-6 and TNF-α were statistically higher than the uninfected controls ($p<0.0001$ for IL-1β and TNF-α, and $p<0.05$ for IL-6). Planktonic *P. gingivalis* also significantly increased IL-1β and TNF-α levels ($p<0.001$ and $p<0.01$, respectively). Although planktonic *P. gingivalis* increased IL-6 level, it was not statistically significant compared to uninfected controls ($p>0.05$). *S. gordonii* and *S. gordonii* and *P. gingivalis* infected rat mandibles did not elevate IL-1β, IL-6 or TNF-α levels ($p>0.05$). It was interesting to note that this bacterial combination significantly decreased IL-1β levels ($p<0.001$).

As seen with the mRNA gene expression data, ELISA quantification of selected pro-inflammatory markers, IL-1β, IL-6 and TNF-α, were the highest in *P. gingivalis* biofilm infected rat mandibles. Thus, the *P. gingivalis* biofilm infected rat mandible was used as an infected *ex vivo* model for the further assessment of PRE, Zn (II), and PRE/Zn (II) treatments.


IL-1b pg/mL
800
600
400
200
0
**
***
***
****
****
Control
S. gordonii
P. gingivalis
S. gordonii+P. gingivalis
S. gordonii
P. gingivalis
S. gordonii+P. gingivalis
Planktonic
Biofilm

A


TNFα pg/mL
800
600
400
200
0
**
****
Control
S. gordonii
P. gingivalis
S. gordonii+P. gingivalis
S. gordonii
P. gingivalis
S. gordonii+P. gingivalis
Planktonic
Biofilm

B

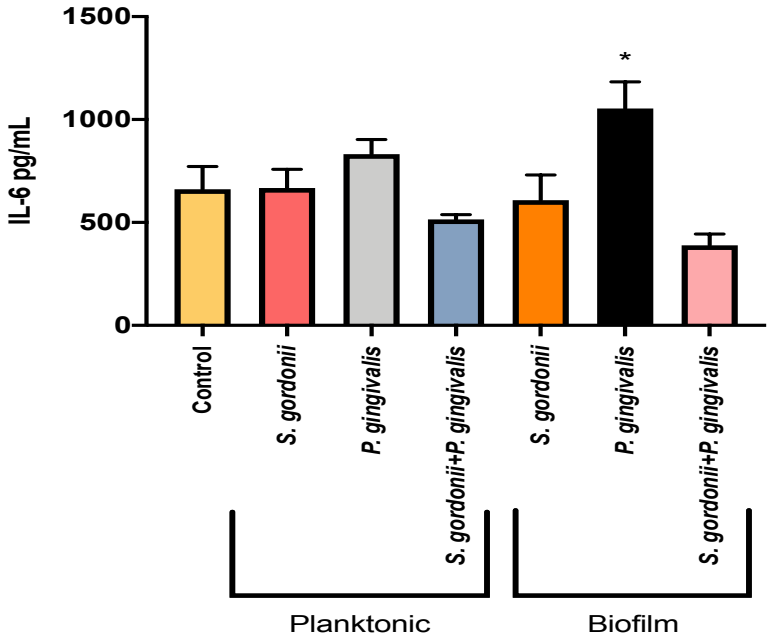
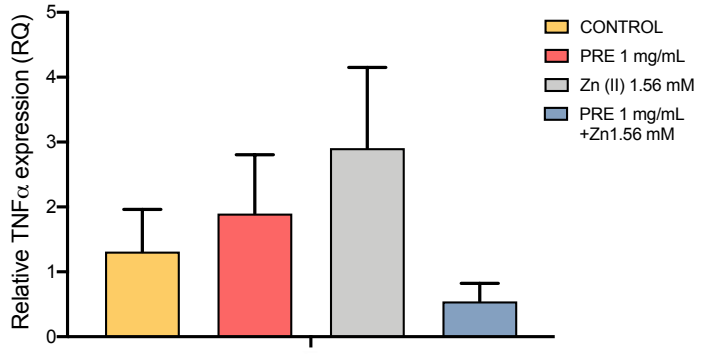


Figure 8.12: Pro-inflammatory cytokine release from infected 3D *ex vivo* rat mandibles after 20 h infection with *S. gordonii, P. gingivalis*, and *S. gordonii* and *P. gingivalis* under planktonic and biofilm conditions. A; IL-1β (pg/mL), B; TNF-α (pg/mL), C; IL-6 (pg/mL). Bar charts represent mean ± SEM, N=3. *; $p < 0.05$, **; $p < 0.01$ and ***; $p < 0.001$, ****; $p < 0.0001$, compared to uninfected controls.

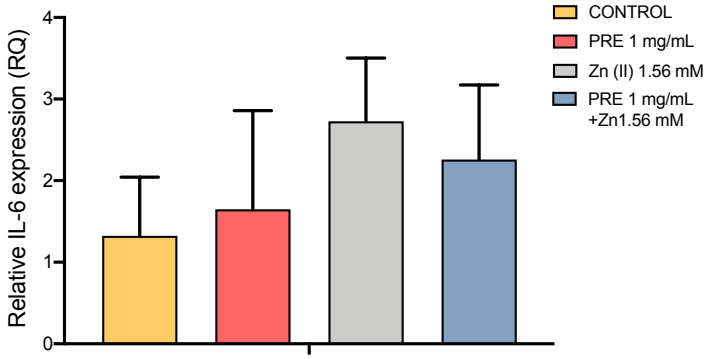
8.4.5 PRE, Zn (II) and PRE/Zn (II) treatment of *P. gingivalis* biofilm infected rat mandibles

8.4.5.1 Pro-inflammatory cytokine gene expression with PRE, Zn (II), PRE/Zn (II)

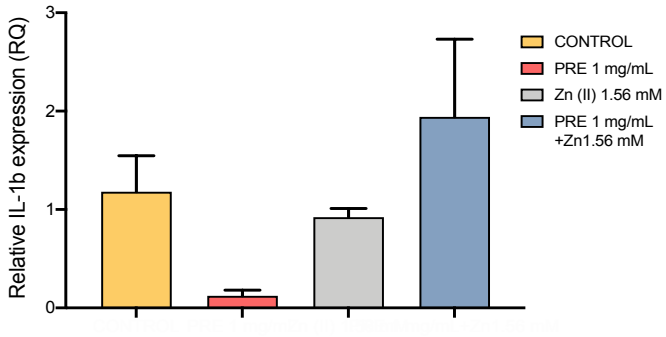
After treatment with PRE (1 mg/mL), Zn (II) (1.56 mM) and PRE/Zn (II) (1 mg/mL and 1.56 mM), pro-inflammatory gene expression of *P. gingivalis* biofilm infected rat mandibles were evaluated using RT-qPCR (Figure 8.13 A, B, C). After 30 min application of PRE (1 mg/mL), Zn (II) (1.56 mM) and PRE/Zn (II) (1 mg/mL and 1.56 mM), none of the treatment groups showed a statistically significant difference in gene expression (IL-1β, IL-6 and TNF-α), compared to untreated controls ($p>0.05$).



A



B

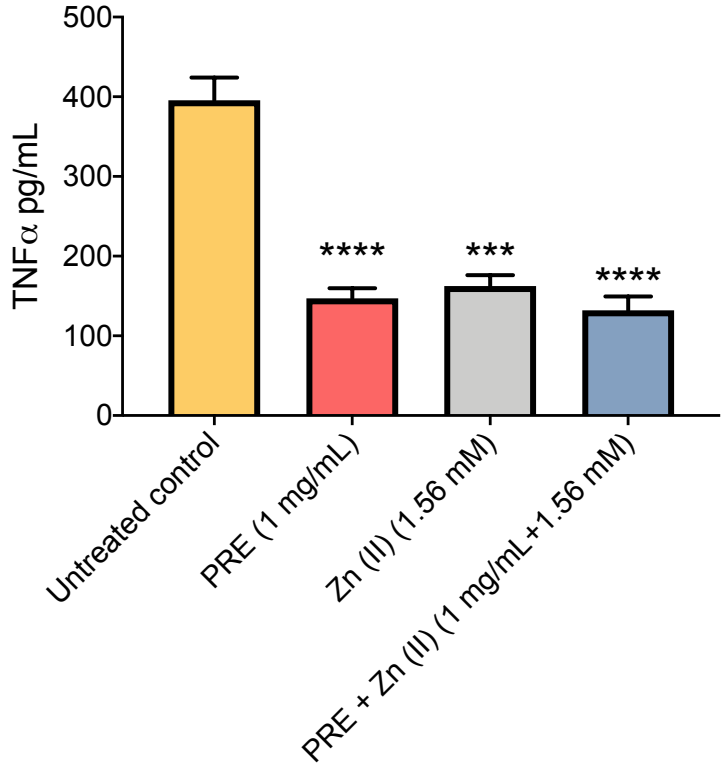


C

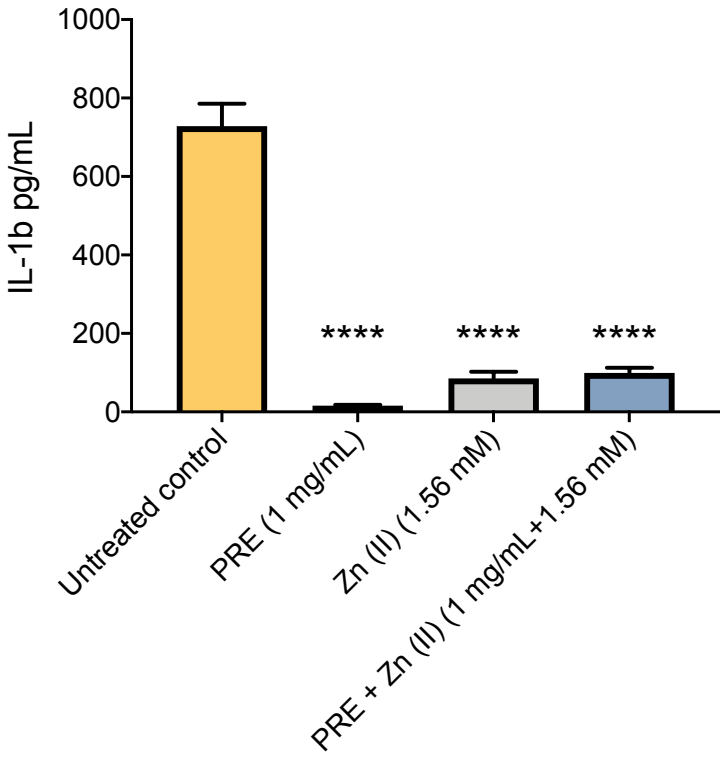
Figure 8.13: Pro-inflammatory gene expression in response to *P. gingivalis* biofilm infected 3D *ex vivo* rat mandible model treated with PRE (1 mg/mL), Zn (II) (1.56 mM) and PRE/Zn (II) (1 mg/mL and 1.56 mM). A; TNF-α, B; IL-6, and C; IL-1β. Bar charts represented as mean ± SEM, N=3.

8.4.5.1.1 ELISA quantification of pro-inflammatory cytokines and PGE2 after PRE, Zn (II), PRE/Zn (II) application

P. gingivalis biofilm infected rat mandibles were treated with PRE (1 mg/mL), Zn (II) (1.56 mM) and PRE/Zn (II) (1 mg/mL and 1.56 mM) for 30 min and their effect on pro-inflammatory cytokine release quantified by ELISA (Figure 8.14 A, B, C, D). PRE (1 mg/mL), Zn (II) (1.56 mM) and PRE/Zn (II) (1 mg/mL and 1.56 mM) significantly reduced IL-1β, IL-6 and TNF-α " cytokine levels, compared to untreated controls ($p<0.0001$). However, there were no significant differences between PRE (1 mg/mL), Zn (II) (1.56 mM) and PRE/Zn (II) (1 mg/mL and 1.56 mM) ($p>0.05$). Additionally, the PRE/Zn (II) did not show any IL-1β, IL-6 and TNF-α " cytokine levels, compared to treatment with PRE or Zn (II) alone ($p>0.05$). None of the treatment groups caused a significant alteration in released level of PGE2 ($p>0.05$).


TNFα pg/mL
500
400
300
200
100
0
****
***
****
Untreated control
PRE (1 mg/mL)
Zn (II) (1.56 mM)
PRE + Zn (II) (1 mg/mL+1.56 mM)

A


IL-1b pg/mL
1000
800
600
400
200
0
****
****
****
Untreated control
PRE (1 mg/mL)
Zn (II) (1.56 mM)
PRE + Zn (II) (1 mg/mL+1.56 mM)

B

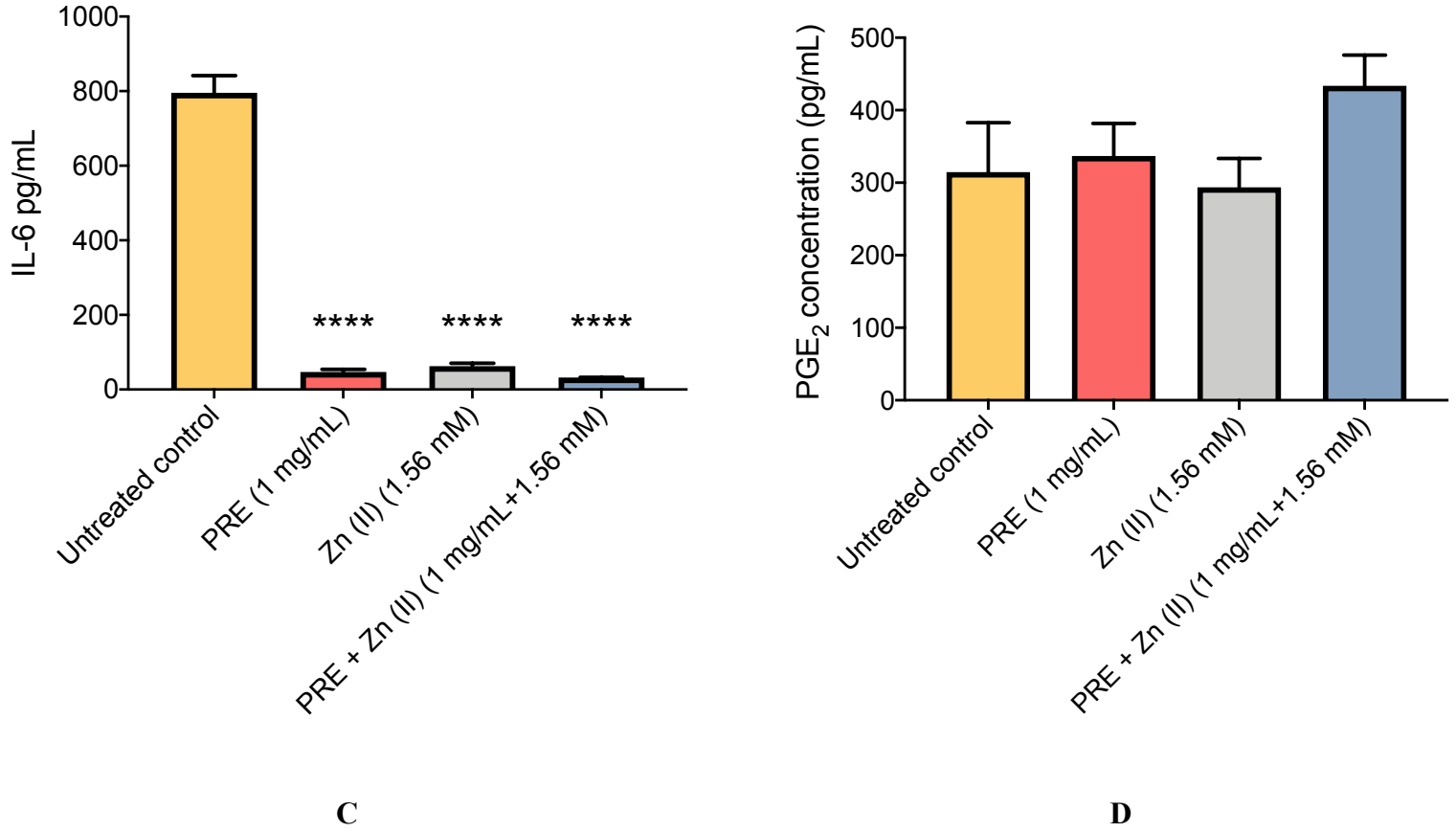


Figure 8.14: Pro-inflammatory cytokine release from *P. gingivalis* biofilm infected 3D *ex vivo* rat mandibles, treated with PRE (1 mg/mL), Zn (II) (1.56 mM) and PRE/Zn (II) (1 mg/mL and1.56 mM). A; IL-1β (pg/mL), B; TNF-α " (pg/mL), C; IL-6 (pg/mL), D; PGE 2 (pg/mL). Bar charts represented as mean ± SEM. *; $p < 0.05$, **; $p < 0.01$ and ***; $p < 0.001$, ****; $p < 0.0001$.

8.5 Discussion

Different research methods have been used to evaluate new therapies for infectious diseases. *Ex vivo* models have been used to investigate a wide variety of developmental, physiological, and pathological conditions. In an *ex vivo* model, cells can be cultured in a condition that protects cell spatial arrangement, and researchers face less systemic influences than *in vivo* research models. Furthermore, the number of required animals is considerably less for *ex vivo* models than for *in vivo* models.

Sloan et al. (1998) developed a rodent tooth slice culture model to study the process of dentinogenesis. It was shown that the dentine-pulp complex from a mature rodent tissue could be cultured for 14-21 days and was useful for investigation of not only dentinogenesis but also tissue repair processes (Sloan and Smith 1999). This model was used to investigate the effect of. different materials included in the formulation of dental products (Murray et al. 2000). Following this research, an *ex vivo* rodent mandible culture model was developed for bone repair investigation (Smith et al. 2000).

In this Chapter, the aim was to use a validated *ex vivo* co-culture model to study the effect of PRE, Zn (II) and PRE/Zn (II) combination for their bioactivity on pro- inflammatory cytokines. Moreover, the toxicity of PRE, Zn (II) and the PRE/Zn (II) were also investigated, before its co-culture with selected oral microbes under planktonic and biofilm conditions, to determine the optimal concentration of these compounds to apply to the infected *ex vivo* rat mandible model.

8.5.1 Evaluation of toxicity of PRE, Zn (II) and PRE/Zn (II) combination

Pharmacological and toxicological evaluations of medicinal plants are essential for drug development and cytotoxicity assessment of these compounds (Desta 1995). To use natural compounds as a topical agent, these topical agents should not induce cytotoxicity to the applied area (Kim et al. 2008). The optimal concentrations of PRE (1 mg/mL), Zn

(II) (1.56 mM) and PRE/Zn (II) (1mg/mL and 1.56 mM) were evaluated for cytotoxicity via LDH and TUNEL assays after 30 min direct application of treatment compounds. In addition, the morphology of rat gingival tissue was observed with H&E staining.

The major compounds in PRE are hydrolysable tannins, and these secondary metabolites can show some cytotoxic activity. Although many reports are available on the cytotoxicity of phenolic compounds, the mechanism of their cytotoxic activity is not fully known (Kulkarni et al. 2007). However, it has been suggested that both molecular weight and functional groups and their positions have an effect on cytotoxicity of hydrolysable tannins (Sakagami et al. 2000). It has been proposed that the protein binding capacity of phenolic compounds could be responsible for cytotoxic activity, as phenolic compounds can bind membrane proteins of cells that affects cell growth and viability (Damianaki et al. 2000). In addition, it should be noted that different tissue and cell types exhibit different cytotoxicity response against polyphenols (Kulkarni et al. 2007). For example, punicalagin showed cytotoxicity in different concentrations for different cell types; 450 μM for normal African green monkey kidney cell line, 740 μM for human larynx epithelial cancer cell line, and 830 μM for human small cell lung carcinoma cell line (Kulkarni et al. 2007). The LDH assay was used with supernatant of the tissue to determine the amount of LDH released from cells into the culture medium, whilst IHC was performed to evaluate localised LDH level in tissue sections. LDH is a soluble cytosolic enzyme that is released after disruption of cell membrane integrity. Therefore, LDH could be used as a marker of either apoptosis or necrosis and can be used to assess cytotoxicity (Leite et al. 2011). The results demonstrated that only the highest concentration of Zn (II) (100 mM) increased LDH levels in both spectrometric assays and IHC. PRE did not alter LDH levels in either assay. Similarly, lower concentrations of Zn (II) did not show a notable change in secreted LDH levels. Even though Zn (II) is an essential element for life and commonly used in clinical dentistry, it could show toxicity by inhibiting enzyme activation, disrupting the cell membrane, and competing with other essential cations such as Cu (II), Fe (II), Ca (II) (Donkin et al. 2000; Dietrich et al. 2016).

The apoptotic activity of PRE, Zn (II) and PRE/Zn (II) were determined using the TUNEL assay. The biochemical sign of apoptosis is degradation of DNA by endonucleases that cause double-stranded oligonucleosomal DNA fragments (Bortner et al. 1995; Saraste and Pulkki 2000). Zn (II) and PRE/Zn (II) did not increase the apoptotic cell nuclei number. Similarly, PRE did not increase apoptosis, except at the highest concentration of PRE (5 mg/mL. Although Zn (II) 100 mM significantly increased LDH levels, it did not cause a notable change in the number of TUNEL positive nuclei. As such, Zn (II) 100 mM may be considered as being non-apoptotic, but it caused more necrotic activity. Zinc can show both apoptotic and necrotic activity depending on the applied zinc concentration. In a study by Rudolf et al. (2003), zinc exhibited apoptotic activity at lower concentrations and necrotic activity at higher concentrations against Hep-2 cells. In contrast, the cytotoxicity result of this study should be taken carefully as application time and tissue type are important factors in the interpretation of results of this toxicity (Wolf and Eastman 1999).

PRE and Zn (II) showed antimicrobial activity at 4 mg/mL and 6.25 mM, respectively, against a range of oral microbes. PRE 1 mg/mL did not lead to an increased LDH level and at <5 mg/mL did not show apoptotic activity in TUNEL assay, compared to untreated controls. Therefore, the used concentration of PRE (1 mg/mL) applied to the infected *ex vivo* mandible model in this study was effective to cause growth inhibition of most bacteria, including *S. gordonii and P. gingivalis*. Similarly, Zn (II) 1.56 mM did not lead to an increased LDH level and apoptotic activity. As Zn (II) 1.56 mM was applied on *ex vivo* model, Zn (II) up to 1.56 mM could also exerted bacterial growth inhibition activities over the range of studied oral microbes. In addition to LDH and TUNEL assays, histopathological evaluation of rat mandible was also observed via H&E staining. The application of PRE, Zn (II) and the PRE/Zn (II) combination (30 min) did not cause any significant morphological changes.

Likewise, previously investigated, a pomegranate peel methanolic extract (224 mg/kg) administered by intradermal injection in mice, also caused no histopathological changes in sections of other soft tissues, such as tongue, trachea and larynx. In addition,

pomegranate peel was non-toxic in terms of glucose, cholesterol, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels as indicators of liver toxicity and did not cause local irritation of the oral mucosa (Jahromi et al. 2015). Furthermore, *in vivo,* and clinical studies showed that the oral ingestion of pomegranate ellagitannin-enriched polyphenol extract did not cause any adverse effects or alteration in haematology, serum chemistry (including ALT, AST levels),or urinalyses and in histopathological observations (Cerda et al. 2003; Heber et al. 2007). Acute and sub-chronic toxicity studies revealed that pomegranate extracts through oral administration were considered safe up to approximately 4,3 g/kg/day for rodents, and no observable adverse effects were reported (Cerda et al. 2003; Patel et al. 2008; Meerts et al. 2009).

8.5.2 Evaluation of infected *ex vivo* rat mandible models

In this Chapter, an *ex vivo* rat mandible model was infected with planktonic or biofilm single or dual species inocula of *S. gordonii* and *P. gingivalis* to simulate periodontal infectious disease. *P. gingivalis*, a Gram- negative obligate anaerobic bacterium, which has been extensively investigated due to its effect on inducing an immune response in the oral cavity (Slots et al. 1988, Holt and Bramanti 1991, Socransky et al. 1998, Byrne et al. 2009; Tribble et al. 2013). Although the exact role of *P. gingivalis* in initiation and progression of periodontal disease is still not clear, it has been widely accepted as a risk factor (Shi et al. 2007; Liu et al. 2013). It has been suggested that *P. gingivalis* virulence factors and extracellular proteases play a role in induction and progression of periodontitis. For example, LPS, fimbriae and gingipains released by *P. gingivalis* have all been associated with periodontal tissue destruction (Bostanci et al. 2012; Decaillet et al. 2012). *P. gingivalis* is a late or secondary coloniser in sub-gingival plaque development and requires a specific environmental and nutritional components for growth. Therefore, *P. gingivalis* exploits the colonisation and metabolic activity of other microorganisms to localise in biofilms (Hill and Marsh 1989). In the initial attachment of *P. gingivalis*, oral streptococci play an important role by providing a suitable attachment surface for *P. gingivalis*. Moreover, prevention of *P. gingivalis* interaction with some oral streptococci has been suggested as an ideal point for therapeutic intervention to control colonisation

or re-colonisation of oral tissues by *P. gingivalis* (Lamont et al. 2002; Park et al. 2005). Interaction between oral streptococci and *P. gingivalis* is a species-specific adherence and regulated by protein–protein interaction that occur between the minor fimbrial antigen (Mfa) of *P. gingivalis* and the antigen I/II (Ag I/II) polypeptide of streptococci (Daep et al. 2006, 2008, 2011). These interactions could be responsible for *P. gingivalis* adaptation into a biofilm (Maeda et al. 2008). Co-aggregation studies have demonstrated specific interaction between *P. gingivalis* and a variety of other oral microbes, such as *S. gordonii*. This co- aggregation study supports the idea that early colonisers provide an attachment site and possibly nutrition for secondary or late colonisers to become established within the biofilm (Hashimoto et al. 2003; Davey and Duncan 2020). The adhesion interaction between *P. gingivalis* and *S. gordonii* has been studied at a molecular level and multivalent interactions reported (Lamont et al. 1992; Amano et al. 1997; Brooks et al. 1997; Lamont et al. 2002). Hence, in addition to *P. gingivalis* infection under planktonic and biofilm conditions, the *ex vivo* rat mandible model was also infected dual species *S. gordonii* and *P. gingivalis*, to investigate whether potentiated inflammatory activity might occur due to addition of *S. gordonii,* increasing the biofilm forming ability of *P. gingivalis*.

P. gingivalis-only biofilm induced the largest increase in pro-inflammatory cytokine gene expression and released protein levels for IL- 1β, IL-6 and TNF-α. Dual species infection with *S. gordonii* and *P. gingivalis* did not stimulate infection and immune inflammatory responses as much as *P. gingivalis* biofilms in both planktonic and biofilm forms. Moreover, one unanticipated finding was that *P. gingivalis* attachment was not seen in modified Gram-stained tissue slides, when *P. gingivalis* was applied alone or in combination with *S. gordonii* under both planktonic and biofilm conditions. However, *S. gordonii* attachment to rat mandible tissue was observed when the model was infected either alone or in combination with *P. gingivalis,* again with both planktonic and biofilm forms. A possible explanation for the limited attachment could be that *P. gingivalis* is a late coloniser and needs a prepared environment for its attachment to host tissue, and in this model tissues interacted with *P. gingivalis* biofilm for only 20 min (Kolenblander et al. 2010). In addition, the *ex vivo* model used in this study was set up for a short time (20

h infection) and infected with a 48 h biofilm. It is possible that a more mature biofilm of *P. gingivalis* could be necessary to observe its attachment. As this is a 48 h biofilm, it could be possible that *S. gordonii* dominated this dual species biofilm. In addition, P. gingivalis is an obligate anaerobic bacterium and may lost its viability through incubation with rat mandible tissue in incubator with 5% CO_2, 95% air. Future studies are necessary with well-developed biofilm of *S. gordonii* and *P. gingivalis*. However, for a mature and a standardised dual species biofilm of *S. gordonii* and *P. gingivalis*, a considerable amount of additional time would be necessary, and this study could be considered as a pilot for a dual species infected *ex vivo* rat mandible model.

The highest levels of pro-inflammatory cytokines observed with *P. gingivalis* biofilms, could be due to a higher level of virulence factors produced by *P. gingivalis*. *P. gingivalis* could be considered the main pathogenic microbe, and when combined with *S. gordonii* it could show less virulence in this *ex vivo* model. Moreover, *P. gingivalis* biofilms caused a greater increase in the level of pro- inflammatory cytokines than in planktonic form, and biofilm infections have been considered more relatable to clinical oral infectious diseases with a greater LPS production (Marsh et al. 2009). Hence, *P. gingivalis* biofilm infected *ex vivo* rat mandible tissues were used to evaluate the effect of PRE, Zn (II) and the PRE/Zn (II) combination on pro-inflammatory cytokine levels.

P. gingivalis LPS has often been used to simulate and initiate oral tissue infections in various research models (Moue et al. 2008; Jiang et al. 2013; Xiao et al. 2018). Previously, LPS has been used in this *ex vivo* rat mandible model and found to initiate the host response and tissue destruction (Sloan et al. 2013). Similarly, in the current study, *P. gingivalis* biofilm infected rat mandible tissues induced the highest levels of pro-inflammatory cytokine expression and protein levels, compared to uninfected controls. It has been shown that after the colonisation of a 'keystone' pathogen, such as *P. gingivalis*, such colonisation could induce pathogenicity within oral biofilms and disrupt periodontal tissue homeostasis (Hajishengallis et al. 2012). In addition to *P. gingivalis* virulence factors, the increased ratio of *P. gingivalis* can cause an alteration in oral cavity microbiota, and this imbalance could initiate and induce inflammation in the periodontium

(Olsen et al. 2017). The initiation and progression of periodontal diseases are associated with different factors, the most important of which are the oral microbiota and the host immune response (Hajishengallis 2014). In the host immune response, cytokines play a critical role during periodontitis progression, as they act as first responders against pathogens (Boukortt et al. 2015). Cytokine secretion is activated through interaction between microbiomes and host cells. Representatives of these cytokines are the IL-1 family, the IL-6 family and TNF family. It has been reported that IL-1β is involved in the pathogenesis of periodontitis, as increased levels of IL-1β have been detected following host-microbiota interaction (Ben-Sasson et al. 2009). Moreover, the total amount of IL-1β in gingival crevicular fluid (GCF) has been associated with the severity of periodontitis (Reis et al. 2014). IL-6, secreted by many types of activated immune cells, has been well established in chronic inflammation and an inflammatory booster and a meta-analysis study showed an elevated IL-6 in the GCF of chronic periodontitis patients (Stadler et al. 2016). TNF-α is one of the main pro-inflammatory cytokines in the progression of periodontitis. In addition to its role in cell death induction, TNF-α plays a role in pro-inflammatory responses and cellular communication (Brenner et al. 2015). Increased levels of TNF-α were reported in GCF and serum of chronic periodontitis patients (Gorska et al. 2003; Madureira et al. 2018). Therefore, the protein and gene expression level of IL-1β, IL-6 and TNF-α were investigated to choose the most prominent infection method, using the *ex vivo* rat mandible model.

8.5.3 Evaluation of PRE, Zn (II) and PRE/Zn (II) in *P. gingivalis* biofilm infected *ex vivo* mandible model

P. gingivalis biofilm infected rat mandible tissues resulted in the expression of a panel of pro-inflammatory cytokines, indicative of a pro- inflammatory response. The initiation and progression of inflammation are mainly related to the local microbiota and host immune responses (Hajishengallis 2014). Over-activation of the host response takes place after progression of infection with immune cell infiltration and osteoclastic activity activation. The primary host response occurs as a result of interaction between the oral microbiome and host cells which includes periodontal tissue cells such as mucosal

epithelial cells and gingival fibroblasts, and other immunocytes (Pan et al. 2019). Then, elevated inflammation finally concludes with both soft and hard tissues damage in periodontium (Moutsopoulos and Konkel 2018). Cytokines play a critical role in the progression of periodontitis and has been evidenced in human and animal studies (Alayan et al. 2007; Eskan et al. 2012). In the current study, mandible tissues infected with *P. gingivalis* biofilm was treated with PRE (1mg/mL), Zn (II) (1.56 mM), and PRE/Zn (II) (1 mg/mL and 1.56 mM) for 30 min. The anti-inflammatory properties of PRE, Zn (II) and PRE/Zn (II) were validated against the *P. gingivalis* biofilm infected mandible tissues. After 30 min treatment, all groups caused a significant reduction in pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α). However, these treatment regimens did not influence the levels of released PGE2 levels, compared to untreated controls. Furthermore, no potentiated anti-inflammatory activity was seen with the PRE/Zn (II) within the infected *ex vivo* model. In contrast to the ELISA findings, the qPCR data did not show any significant alteration in gene expression of IL-1β, IL-6 and TNF-α after 30 min treatment with PRE (1mg/mL), Zn (II) (1.56 mM), and PRE/Zn (II) (1 mg/mL and 1.56 mM. The difference between the ELISA and qPCR results from PRE, Zn (II) and PRE/Zn (II) evaluation on pro-inflammatory cytokine levels could be related to the composition of PRE. As it is well known that tannins in PRE can interact with proteins (Papadopoulou and Frazier 2004), possible interactions between secreted cytokines (IL-6 and IL-8) and a chemokine (MCP-1) and punicalagin were investigated by ELISA without cell culture. The findings showed that studied pro-inflammatory cytokines and a chemokine concentration decreased in a dose-dependent manner with applied punicalagin (Hollebeeck et al. 2012). This suggested an interaction between tannins in PRE and proteins could be the reason for the discrepancy between mRNA gene expression (qPCR) and protein level (ELISA) in this study. While there have been no reported studies for Zn (II) and its possible interactions with pro-inflammatory cytokines, it is well known that Zn (II). plays both structural and catalytic cofactor roles for many proteins (Haase and Rink 2009). Although molecular sensors for Zn (II) are not known, approximately 2800 proteins can bind Zn (II) and this could result in either a direct or an indirect effect of the function, or loss of function, of one or many of these proteins (Andreini et al. 2006). Hence, there could be a similar possible interaction between Zn (II) and these cytokines.

Another explanation for the discrepancy between protein level and gene expression results could be the observed high deviation in the gene expression assay. While all gene expression assays are validated in terms of cDNA concentration and quality of extracted RNA, different animals were used for the different groups through experimental repeats. In addition, it is possible that nutrition and oxygen supplementation could show differences depending on the size and geometry of tissue, and this could affect the immune response of tissue (Shi et al. 2019). However, while PRE and PRE/Zn (II) application were not effective in suppressing gene expression of pro-inflammatory cytokines, they did lower in protein levels of pro-inflammatory cytokines. This could be the result of PRE and PRE/Zn (II) interference effect on downstream signalling pathways and/or pro-inflammatory cytokines related receptor complexes. Simply, there is a possibility that PRE and PRE/Zn (II) may disrupt cytokine activity and inflammatory cell signalling, but more studies are necessary to investigate this. This interaction between cytokines and PRE, and possibly Zn (II), could have an important role in periodontal infectious diseases. An implication of this is the possibility that PRE and Zn (II) can decrease the secreted level of cytokines in the oral cavity and in GCF (Reis et al. 2014; Brenner et al. 2015; Stadler et al. 2016).

The anti-inflammatory properties of pomegranate polyphenols have been reported and associated with a high reduction in pro-inflammatory cytokines (mRNA and protein levels). This has supported the potential of PRE as an anti-inflammatory agent in the Caco- 2 cell line and in an *ex vivo* stimulated pig colonic tissue explant model (Hollebeeck et al. 2012; Mastrogiovanni et al. 2019). It was also reported that pomegranate peel polyphenols down-regulated expression of IL-1β, IL-6 and TNF-α and reduced secreted levels of these pro-inflammatory cytokines in a murine macrophage cell line (Du et al. 2019). The anti-inflammatory properties of PRE are most likely driven via suppression of the nuclear factor kappa B (NF-κβ) and mitogen-activated protein kinase (MAPK) pathways. The NF-κβ pathway has a crucial role in the inflammatory response, especially in modifying cytokine expression (Libermann et al. 2006; Lawrence 2009; Zhang et al. 2016). While NF- κβ and MAPK are suggested mechanisms for polyphenols' anti-inflammatory properties, the exact underlying mechanisms of action are still not

clear. This anti- inflammatory activity of PRE has been highly associated with hydrolysable tannins in its content. Punicalagin is the most abundant tannin in PRE, and the punicalagin's effect on the NF-κβ pathway was observed through suppression of the phosphorylation of IκBα and p65, thus inhibiting the activation of LPS-induced NF-κβ (Xu et al. 2014). Moreover, it was reported that UVB-induced inflammation was inhibited through NF-κB and MAPK signalling in oral dosing of pomegranate to mice (Khan et al. 2012). In addition to PRE, punicalagin pre-treatment (5–40 μM) had inhibitory activity on TNF-α, IL-6 and PGE2 production, and also reduced COX-2 expression in protein and mRNA in LPS- stimulated rat primary microglia (Olajide et al. 2014).

The absence of down-regulated gene expression in this study could be due to the exposure time of treatment groups or extraction method differences in pomegranate peel extract preparation and longer exposure time can be adopted for future studies. In this study, rat mandible tissues were exposed to treatment groups for only 30 min as a post-treatment after infection, and this time may not be sufficient for PRE, Zn (II) and PRE/Zn (II) to inhibit gene expression of studied pro-inflammatory cytokines. In a study by Hollebeeck et al. (2012), pomegranate polyphenolic extract was applied for 1 h as a pre-treatment before LPS-infection of the Caco-2 cell line, in which pomegranate extract and compounds down-regulated pro-inflammatory cytokine and chemokine gene expression levels (IL-6, MCP-1). This pre-exposure of cell and/or tissue to treatment compounds could provide an enhanced activity of treatment groups, as more diffusion of the agent into cells/tissue could occur. However, pre-exposure of PRE and PRE/Zn (II) is not applicable in terms of therapeutic viewpoint for gingivitis, and periodontitis, where patients apply treatment after infection. In addition to above mentioned studies, there is a high possibility PRE and PRE/Zn (II) may disrupt cytokine activity and inflammatory cell signalling that make PRE and PRE/Zn (II) treatment a promising anti-inflammatory agent. In this Chapter, the PRE/Zn (II) anti-inflammatory activity was also investigated to determine whether there were any potentiated anti-inflammatory activities. However, at both gene expression and protein levels, this combined application did not show any potentiated activity, compared to their respective applications alone. A similar result was shown in a study where PRE, Zn (II) and PRE/Zn (II) had anti-inflammatory activities in

an *ex vivo* porcine skin model. PRE and the PRE/Zn (II) were shown to exert an anti-inflammatory activity after 6 h, which lasted up to 24 h on COX-2 level (Houston et al. 2017c). Houston et al. (2017a) reported that PRE caused a reduction in expression of COX-2, but Zn (II) did not alter its expression. Furthermore, when Zn (II) was applied in combination with PRE, this combination showed similar reduction in COX-2 level, indicating no potentiated activity with Zn (II) addition (Houston et al. 2017a). Here, PGE2, a product of COX-2 was studied, and absence of change in levels was unexpected, but could be to due single and short time exposure of PRE, or an anti-inflammatory response to PRE and PRE/Zn (II) combination could show differences in different tissue types (Kulkarni et al. 2017).

8.6 Conclusion

An existing *ex vivo* rat mandible model was adopted and infected with planktonic and biofilm forms of *S. gordonii, P. gingivalis*, *and S. gordonii* and *P. gingivalis* bacteria. For these different infections, gene expression and protein levels were compared for pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α). *P. gingivalis* biofilm infection significantly increased IL-1β, IL-6 and TNF-α expression and protein levels, compared to the uninfected control or the other infection groups. This model was employed to assess PRE (1 mg/mL, Zn (II) (1.56 mM) and PRE/Zn (II) (1 mg/mL and 1.56 mM) effect on induction of pro-inflammatory cytokines. Concentrations of PRE, Zn (II) and PRE/Zn (II) were chosen according to LDH and TUNEL assay data. All treatment groups significantly reduced secreted protein levels of IL-1β, IL-6 and TNF-α. However, no alteration was observed in the gene expression of IL-1β, IL-6 and TNF-α. At both protein level and gene expression, no potentiated or antagonistic anti-inflammation activity was observed for PRE/Zn (II) compared to their single applications.

General Discussion

9.1 Overview

The background for this research was the clinical need for oral care products with improved activity against planktonic and biofilm mediated periodontal. Periodontal diseases are multifactorial polymicrobial infections and have been characterised as destructive inflammatory processes affecting the periodontium, which comprises the teeth-supporting structures: gingiva, cementum, periodontal ligament, and alveolar bone (Marsh et al. 2009). It is estimated that there are 3.5 billion people suffering from periodontal diseases across the world (Niemetz and Gross 2005). In many countries, periodontal infectious diseases, which affect people from their childhood to death, are an economic burden for healthcare systems (Flemming et al. 2021). Despite a number of preventive measures for periodontal disease, the priority of prevention has been neglected or under-emphasised. Hence, gingivitis, defined as inflammation of gingivae, is considered one of the most common diseases, affecting 20-50% of the dentate population globally (Mariotti 1999; Nazir 2017). Gingivitis is a reversible condition and to prevent its progression to periodontitis and eventually tooth loss, a comprehensive home-based regular plaque control and non- surgical periodontal therapy is necessary (Axelsson and Lindhe 1982; Lang et al. 2009). Antiseptics (e.g., chlorohexidine, benzydamine, and doxycycline), anesthetics (e.g., benzocaine, lidocaine), corticosteroids (e.g., dexamethasone, triamcinolone) and immunomodulatory agents (e.g., amlexanox, colchicine, cyclosporine, cyclophosphamide, and methotrexate) are the most widely used topical agents in periodontal infectious diseases (Belenguer-Guallar et al. 2014). Despite their common use in clinical treatment, their side effects and risks should not be over-looked. One of the main disadvantages of broad spectrum antimicrobials is the increase of antimicrobial resistant microbes and hypersensitivity. In addition, the systemic adsorption of corticosteroids highlights another risk for prolonged use of topical corticosteroids (Rogers III 1997). These problems demonstrate the need for an alternative treatment for periodontal diseases.

Previous work suggested that a natural product, PRE, had potential as the basis of an alternative therapeutic, with its multi-therapeutic benefits such as antimicrobial, anti-inflammatory and wound-healing bioactivities. Moreover, based on previous reports, PRE in combination with Zn (II), could provide a synergistic and/or potentiated bioactivity in the treatment of periodontal diseases (Houston et al. 2017a; Houston et al. 2017b; Alrashidi 2020; Celiksoy et al. 2021).

Punica granatum L. (*Puniceae*), widely known as pomegranate, has a long history in ethnopharmacy. It has been used as a folkloric medicine in different cultures, including traditional Chinese medicine, Ayurveda in the treatment of traumatic haemorrhage, ulcers and infections, and disorders of the digestive tract, such as diarrhoea and dysentery (National Pharmacopoeia Committee, 2010). Pomegranate rind is a by-product of the juice processing industry and comprises approximately 30-40% of the whole fruit (Cam et al. 2014; Gullon et al. 2016). While pomegranate rind is a waste product of industrial manufacturing and processing, it is a rich source of bioactive compounds, such as flavonoids, phenolic acids, and tannins (ellagitannins, such as punicalin and punicalagin) (Türkyılmaz et al. 2013). In the last thirty years, research on pomegranate extracts has attracted increasing attention, and pomegranate rind is considered a promising natural therapeutic with its antimicrobial, antioxidant, anti-inflammatory, and anticancer properties (Viuda and Martos et al. 2010; Pirzadeh et al. 2021). PRE/Zn (II) was investigated in this thesis, due to previously reported synergistic antiviral and antibacterial activities by Houston et al. (2017b) and Alrashidi (2020), respectively. Therefore, PRE, Zn (II), and PRE/Zn (II) were assessed for their antimicrobial, anti-inflammatory and wound-healing activities, to investigate their potential use in the treatment of periodontal diseases. Thus, considering the bioactivities of PRE and the synergistic antimicrobial activity of PRE/Zn (II), the investigation of PRE and PRE/Zn (II) for potential use in periodontal infectious diseases is a necessity in terms of fighting antimicrobial resistance, providing efficient, economical, and safe treatment to patients, along with reducing the burden on the environment utilising a waste product.

9.2 PRE composition

Aqueous pomegranate rind extract was prepared as described by Houston (2011) and Al Alrashidi (2020). The extraction method could be described as a simple, rapid, and low-cost strategy, due to using water as a solvent and decoction as a conventional extraction technique. Water is a more environmentally friendly and a safer extraction solvent compared to methanol and other organic solvents (Panda et al. 2019; Rajha et al. 2019). In this study, the total polyphenol content of PRE was found to be 496 mg tannic acid equivalent/g freeze-dried PRE. Moreover, PRE composition was investigated for secondary compounds using mass spectra and compared with the previous literature using fragmentation patterns, both in positive and negative ionisation modes. Results corroborated previous studies indicating that ellagitannins were the major phenolic compounds in pomegranate rind, comprising over 99 % of the total content of pomegranate phenolics (Fischer et al. 2011; Qu et al. 2012; Romeo et al. 2015). A list of tentatively characterised compounds was presented in Chapter 3, Table 3.4 and Table 3.5. Punicalagin, which has been commonly considered as the most abundant compound in PRE, was found to comprise almost 20 % of the total extract. Thus, according to analytical results of PRE, this conventional short time (10 min boiling) extraction method with water solvent could be considered a good method to reveal secondary compounds, especially hydrolysable tannins, in pomegranate rind (Turrini et al. 2020).

9.3 Antimicrobial activity

Antimicrobial agents are important in the treatment of periodontal diseases, because of the high association between dental plaque accumulation and periodontal infection induction. The antimicrobial activity of pomegranate has been extensively studied and found antimicrobial against bacteria, virus, yeasts and parasites (Fahmy et al. 2009; Haidari et al. 2009; Sundararajan et al. 2010; Endo et al. 2012, Foss et al. 2014; Celiksoy and Charles 2021). PRE and metal salts, including Zn (II), have also been studied for their potentiated/synergistic antimicrobial activity against a range of microbes and viruses (Stewart et al. 1998; McCarrell et al. 2008; Gould et al. 2009; Houston et al. 2017b;

Celiksoy et al. 2021). Although there were previous studies on pomegranate extracts antimicrobial activity, there were no studies for PRE/Zn (II) activity against oral microbes. In this study, PRE was antimicrobial against planktonic streptococcus species and *Candida albicans*. Synergistic antimicrobial activity of PRE/Zn (II) was detected against *S. gordonii* and *C. albicans,* whereas additive activity was found against *S. mutans*, which is an important aetiologic factor in dental caries (Koo and Bowen 2014).

In addition to planktonic cultures, PRE, Zn (II) and PRE/Zn (II) were investigated for their antibiofilm activities. Microorganisms are often found in biofilms in clinical cases, and this provides advantages to a microbe, such as increased resistance against antimicrobial agents and hostile environmental conditions (Sreenivasan and Gaffar 2002). PRE was effective in eradicating biofilms, including 24 h mono-species biofilms of *S. sanguinis, S. gordonii, S. mutans, C. albicans* and *P. gingivalis*, but addition of Zn (II) to PRE did not show any synergistic or antagonistic activity in biofilm eradication. *P. gingivalis* biofilm eradication by PRE is important because of its virulence in periodontitis. Therefore, such findings on biofilm eradication by PRE and PRE/Zn (II) shows the possible value of using these agents in clinical periodontal infectious diseases. While there is a lack of *in vitro* biofilm studies to investigate pomegranate extract activity, findings from clinical studies have been published supporting the use of pomegranate extract in the treatment and management of periodontal diseases (Prasad and Kunnaiah 2014; Eltay et al. 2021; Tyagi et al. 2021). It has been reported that pomegranate extract mouthwash led to a reduction of gingival bleeding, pain and healing time, and controlled the adhesion of *S. mutans, S. sanguis* and *S. mitis* to the surface of the oral cavity in clinical studies (Vasconcelos et al. 2003; Ghalayani et al. 2013; Batista et al. 2014). Structure-bioactivity studies were not assessed in this study, but different mechanisms have been suggested for the antimicrobial activity of PRE. The polyphenol constituents of PRE can damage the cytoplasmic membrane, inhibit nucleic acids, disrupt energy metabolism and interfere with the synthesis of the cell membrane and cell wall (Cowan 1999; Cushnie and Lamb 2011). In terms of biofilm eradication, it is possible that there is more than one factor and mechanism involved. Polyphenols have been reported for their activities, such as changing bacterial surface inhibition of quorum sensing and inhibition of the synthesis

of constituents for biofilm formation, such as adhesins (O'May et al. 2011; Sarabhai et al. 2013).

9.4 Anti-adhesive activity

In the oral cavity, microbes colonise quickly after cleaning, on biotic and abiotic surfaces. Biofilms in the oral cavity are considered the main aetiological factors of periodontal diseases, such as caries, periodontitis and denture stomatitis (Yang et al. 2011). Microbial biofilms maintain structure with the help of extracellular polymeric substances (EPS). Thus, considering the importance of microbial attachment to surfaces and EPS for the development of biofilms, prevention of microbial adhesion could be considered a favourable strategy to prevent biofilm formation and thus dental plaque-associated oral diseases (Stewart 2001; Davies 2003; Cegelski et al. 2008). Moreover, restricting initial biofilm formation can impede the advantageous biofilm environment for microbes, such as increased resistance to antimicrobial agents and hostile environmental conditions (Pontefract 1991; Dunne 2002).

Therefore, the anti-adhesive activity of PRE, Zn (II) and PRE/Zn (II) was assessed on glass surfaces coated with PRE, Zn (II) and PRE/Zn (II). Glass surfaces have been considered as a standard model for the hard surface of teeth in previous studies (Hamada and Torii 1978; Hamada and Slade 1980; Mattos-Graner et al. 2000; Carter et al. 2001; Tao and Tanzer 2002; Sasaki et al. 2004; Furiga et al. 2008). PRE- and PRE/Zn (II)-coated surfaces caused a significant reduction in biomass of attached microbes for *S. mutans* and *C. albicans.* Both species have a critical role in dental caries aetiology and prevention of these microbial attachment to enamel could limit microbial composition changes from health to disease (Metwalii et al. 2013; Kooand Bowen 2014). This inhibition of biomass in the initial attachment of microbes on the surfaces coated with PRE and PRE/Zn (II) is likely to be due to the presence of PRE, due to no difference observed between PRE and PRE/Zn (II), and inactivity of Zn (II) alone. The anti-adhesive activity of PRE has been associated with its high polyphenolic composition, such as tannins, anthocyanins, and flavonoids, since these polyphenols are well known for their potential interaction with

macromolecules, including carbohydrates and proteins (Janecki and Kolodziej 2010). In addition, these interactions could occur between PRE and enzymes/proteins from both host and microorganisms, such as saliva and adhesins, respectively, which might be involved in attachment. Similar results were observed in this study for PRE at inhibiting attachment of *S. gordonii, S. mutans* and *C. albicans* to glass surfaces. Pomegranate tannins were also effective at inhibiting glucosyltransferase and glucan synthesis of *S. mutans* (Menezes et al. 2006; Smullen et al. 2007). Moreover, alteration in bacterial cell surface hydrophobicity and surface chemistry, due to pomegranate polyphenols, has been considered as reasons for anti-adhesive activity (Lei et al. 2011). It has been reported that the hydrophobic surface properties of the bacterial cell wall have an indirect role in the adhesion of bacteria to the acquired pellicle on the tooth surface (Nesbitt et al. 1982). These hydrophobic properties of bacteria have been associated with different factors, such as appendages like fimbriae, structural components like amphipathic lipoglycan lipoteichoic acid, and structurally related polypeptides. Thus, interaction of pomegranate polyphenols with these hydrophobic properties could prevent the adhesion between surface and microbes and eventually biofilm formation (Janecki and Kolodziej 2010).

9.5 Antimicrobial activity relationship between PRE and Zn (II)

In previous studies, synergistic antimicrobial activity has been shown against viruses and bacteria (Houston et al. 2017b; Alrashidi 2020; Celiksoy et al. 2021). In this thesis, the PRE/Zn (II) demonstrated synergistic antimicrobial activity in *in vitro* checkerboard and time-kill assays against *C. albicans*. Moreover, a potentiated/synergistic anti-adhesive activity of PRE/Zn (II) was observed against *C. albicans*, through a significant reduction in collected viable CFU from PRE/Zn (II)-coated surfaces.

While there are a few suggested mechanisms behind PRE/Zn (II) synergistic antimicrobial activity, no studies have investigated their mechanisms from a ROS perspective. Therefore, addition of Zn (II) to PRE was studied in relation to anti-/pro-oxidant activity, and whether this contributed to synergistic antifungal activity against *C. albicans*. Excessive ROS can cause a deleterious effect on the viability of bacteria through enzyme

inactivation, membrane disruption and cell death (Kobayashi et al. 2002). This study showed that PRE/Zn (II) induced higher intracellular ROS levels in both planktonic and biofilms after 24 h treatment, compared to PRE or Zn (II) only treatments. This increased ROS level could be the reason for the synergistic antifungal activity with PRE/Zn (II). In addition, perturbation of the redox potential at sites in the oral cavity could impact on the composition and metabolism of the microbial community. Therefore, increasing redox reagents has been considered a novel approach in the treatment of periodontal infections, since it was reported that the increased redox level in sub-gingival plaque could become less suitable for obligate anaerobes that dominate in periodontal pockets (Marsh et al. 2009). However, future studies are necessary to ascertain the exact mechanism between PRE and Zn (II) in an environment with microbes and host cells.

9.6 Anti-inflammatory activity

In addition to anti-microbial effects, a highly desirable attribute in a new oral care product would be the reduction or amelioration of gum inflammation. Periodontal diseases are a set of inflammatory conditions that affect tissues surrounding the teeth. The immuno-inflammatory response within the periodontium starts with the accumulation of dental plaque, with a prolonged or dysfunctional immuno-inflammatory response potentially leading to alterations in the structures and functions of tissues which protect and support the teeth (Loos 2005). As mentioned before, the first step of this inflammation is gingivitis, accompanied by sensitive, red and bleeding gums. If gingivitis is untreated, it proceeds to periodontitis. During periodontitis progression, inflammation and destruction of tissues goes deeper from the gingival tissue to alveolar bone and migration of the junctional epithelium towards to root. Finally, periodontal pocket formation results with associated bone loss and tooth exfoliation (Lang et al. 2009; Kurgan and Kantarci 2018). The modulation and resolution of the immuno- inflammatory response could ultimately contribute to the prevention of periodontitis and eventual tooth loss. Previously, a defined *ex vivo* rat mandible model developed by Sloan et al (2013) was infected with a *P. gingivalis* biofilm, leading to inflammatory response initiation. Such a model was found appropriate to use in this study, for an initial assessment of the anti-inflammatory

activities of PRE, Zn(II) and PRE/Zn (II) against periodontal disease. Application of PRE (1 mg/mL), Zn (II) (1.56 mM) and PRE/Zn (II) (1 mg/mL+1.56 mM) (all 30 min), significantly reduced detectable levels of released pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α), compared to untreated controls. However, the gene expression of these pro-inflammatory cytokines did not show any significant alterations. The discrepancy between gene expression and protein levels could be associated with several factors. One of the possible reasons could be the reaction between released cytokines and polyphenols (Hollebeeck et al. 2012). In addition, differences in size and geometry of dissected rat mandible tissues could be the reason for high deviation observed in the gene expression results. The diffusion pattern of PRE, Zn (II) and PRE/Zn (II) through to tissue could affect the gene expression levels in these treatment groups. However, the inhibitory activity of compounds for pro- inflammatory cytokines released (IL-1β, IL-6 and TNF-α) in culture medium have been reported in the literature and this property could be of critical importance and a promising approach to prevent inflammation of the periodontium. The inhibition in released pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) in culture medium could be the result of disruption of cytokines and inflammatory cell signalling by PRE and PRE/Zn (II). Inhibition of pro-inflammatory cytokines in GCF could reduce excessive inflammatory response of the host during periodontal disease (Guentsch et al. 2011; Barros et al. 2016, Pan et al. 2019).

9.7 Wound-healing activity

Impaired wound healing is a clinical problem that effects many people worldwide. In the oral cavity, wounds can occur as a result of periodontal diseases, traumas, aphthae and traumatic ulcers. These oral wounds cause pain to the patient and affect quality of life, in terms of food intake and speaking (Mortavazi et al. 2016; Minhas et al. 2019). Anti-inflammatory therapies, such as corticosteroids, are the most common agents used to treat oral wounds. However, there is no standard treatment plan and corticosteroids could cause a delay in the re-epithelisation of open wounds. Additionally, systemic influences, such as renal and liver damage, could occur as a result of high-dose and/or long-term therapy (Levin and Maibach 2002; Gutkowski et al. 2011). In addition to antimicrobial and anti-

inflammatory activities, PRE and punicalagin have been reported as promising agents for treatment of dermal wounds (Chidambara et al. 2004; Mo et al. 2014a; Fleck et al. 2016; Nirwana et al. 2017; Tang et al. 2017; Lukiswanto et al. 2019). Although the oral mucosa demonstrates a faster healing response than other dermal sites, they possess similar wound healing stages (Serra et al. 2017; Iglesias-Bartolome et al. 2018). In this thesis, PRE, punicalagin and their combination with Zn (II) did not exert any significant increase in the proliferative ability of keratinocyte cell line (HaCaTs) and human primary gingival fibroblasts (HPGFs). In contrast, higher concentrations of these agents caused a reduction in HaCaT and HPGF proliferation. However, it was found that 0.1 μg/mL punicalagin in combination with Zn (II), significantly increased the distance travelled (Tt) and cell speed (Tt/t) of HPGFs. Fibroblast migration and proliferation have a fundamental role in successful wound healing (Häkkinen et al. 2000; Posten et al. 2005). Initial fibroblast migration, proliferation, cytokine/growth factor production can all aid extracellular matrix (ECM) synthesis and remodelling for repairing damaged tissue (Smith et al. 1997; Liang et al. 2007). Furthermore, the antioxidant activity of PRE, punicalagin and their combination with Zn (II) was assessed and punicalagin exhibited higher antioxidant ability than PRE, as shown by *in vitro* radical scavenging assays, ABTS and DPPH. The addition of Zn (II) to PRE and/or punicalagin did not alter the antioxidant capacity of these compounds. From these results, it could be concluded that punicalagin in combination with Zn (II) could accelerate the wound healing response by accelerating migration of gingival fibroblasts to the wounded area.

9.8 Future perspectives

The health benefits of pomegranate extracts obtained using different extraction methods and solvents have been shown in different areas, such as antimicrobial, anti-inflammatory, anticancer, wound healing and antioxidant activities. The water extract of pomegranate rind prepared with the decoction method in these studies showed antimicrobial, anti-inflammatory and antioxidant properties. However, there are a lack of studies comparing bioactivities of pomegranate rind extract with different solvents and extraction methods. For this aim, a precisely standardised extraction method from the most suitable cultivar

of pomegranate fruit with same harvesting time could be developed. In this way, variability between different batches of extracts could be prevented as this is one of the main limitations for research with plant extracts. Moreover, PRE comprises many secondary compounds, as with other raw plant extracts. Thus, studies for characterisation and quantification of these secondary compounds is important for further structure-activity studies. After developing a standardised extraction method, secondary compounds could be extracted with a preparative HPLC method and characterisation could be determined via nuclear magnetic resonance (NMR). The quantification of these secondary compounds could be achieved using standard compounds via analytical methods, such as high-performance liquid chromatography (HPLC). However, it should be recognised that bioactivities of plant extracts could be the result of a single compound or a synergistic effect from many secondary compounds in the extract. Analytical studies are important in both scenarios, whether a single or multiple compounds are responsible for bioactivities. These analytical studies can provide the different secondary compounds' ratio in the extract and could enhance understanding of PRE composition for any further studies from structure-activity to formulation. For example, punicalagin, the most abundant compound in pomegranate rind, has been strongly associated with bioactivities of pomegranate rind extract. Thus, a standardised preparative method could be developed to purify punicalagin from PRE. Then bioactivities of PRE and PRE/Zn (II) could be investigated with punicalagin instead of the whole extract PRE. This could provide more information for structure-activity relationships. In addition, it would help to establish the necessity of using the whole extract or a single compound with or without Zn (II). In addition to punicalagin, other individual compounds could be screened for their antimicrobial, anti-biofilm, antiadhesive, anti-inflammatory and pro-wound healing responses.

In this thesis, PRE and PRE/Zn (II) were evaluated for potential use in the treatment of periodontal disease. Both PRE and PRE/Zn (II) demonstrated antimicrobial activity against oral microbes in planktonic and biofilm cultures. However, biofilm studies were conducted with single species and 24 h biofilms. While this biofilm eradication activity of PRE and PRE/Zn (II) is promising, using a multi-species oral biofilm would be a more

representative method for periodontal infectious diseases. For this aim, a multi- species biofilm could be generated using a range of commensal and pathologic oral microbes and cultured in a biofilm device, such as a Calgary device for a longer time period, to obtain a thick and well-formed biofilm for use in the investigation of antibiofilm activity of PRE and PRE/Zn (II).

Biofilm formation is a multifactorial process involving different steps which are regulated by distinct physical and chemical processes; and commencing with initial attachment. Thus, investigation of anti-adhesive properties of an agent could be assessed via different methods for the proper assessment of anti-adhesive activity of PRE and PRE/Zn (II). In this study, initial attachment of *C. albicans, S. gordonii* and *S. mutans* was inhibited by coating a glass surface with PRE and PRE/Zn (II). Further studies could be done to investigate the mechanism of anti-adhesive activity, such as effects on microbial cells, including cell surface hydrophobicity and charge, cell morphology, membranes, membrane proteins. In the evaluation of microbial cells, techniques such as gene expression and protein level assays could be used to identify the effect of compounds on the adhesive properties (Kelly and Younson 2000). Anti-adhesive activity of PRE and PRE/Zn (II) could be studied using periodontal surfaces and characterisation of surfaces with these agents could be optimised. For this aim, human teeth and/or hydroxyapatite discs could be coated with PRE and PRE/Zn (II) would be more suitable method with *in vivo* conditions (Furiga et al. 2009; Song et al. 2012). Research with different and relative surfaces would confirm the efficiency of this anti-adhesive activity as surface properties, such as hydrophobicity, electric charge and surface roughness.

Impact on the initial attachment (Sterzenbach et al. 2020). After an optimised surface coating process, physiochemical properties could be included in the research to stimulate the *in vivo* environment. Fluid properties and environmental conditions, including polarity, flow velocity, pH, ionic strength, temperature, atmosphere, presence of salts, antimicrobials, nutrient availability and different media are considered the main physiochemical properties (Klancnik et al. 2013; Klancnik et al. 2021). Therefore, the distribution and penetration of PRE and PRE/Zn (II) could be investigated in biofilm EPS.

EPS is an important factor in the adhesion and maintenance microbes and may play a role in biofilm structure. Moreover, it has been suggested that EPS could act as a barrier against diffusion of antimicrobial agents in the biofilm (Corbin et al. 2011).

It has been hypothesised that addition of Zn (II) to PRE would lead to a synergistic antimicrobial activity against oral microbes. This hypothesis was based on previously shown synergistic activity of PRE/Zn (II) combination against viruses and bacteria, such as methicillin resistance *Staphylococcus aureus* and *S. epidermidis* (Houston et al. 2017b; Alrashidi 2020). In this study, this synergistic activity was observed only for *S. gordonii* and *C. albicans.* The effects of this combination on ROS level were investigated against *C. albicans* and it was found that PRE/Zn (II) could increase ROS level and cause a deleterious effect on *C. albicans* cells. It is an important finding in the explanation of synergistic antimicrobial activity for PRE/Zn (II), but further studies are necessary to investigate this combination effect on *C. albicans* oxidative defence system. In addition, more studies are needed against other microbes. PRE/Zn (II) could also be assessed to determine if a new combination of substances or a simple complexation develops, and such the binding interactions can be studied using isothermal titration calorimetry (ITC). In addition, binding interactions between PRE and Zn (II) could be investigated in culture media and bodily fluids, such as saliva and GCF, to find any possible interactions in these environments.

The wound-healing studies were performed for PRE, punicalagin and their Zn (II) combinations using HaCaTs and HPGFs. The oral and mucosal wound healing processes exhibit similar healing stages (Walsh et al. 1996). However, oral wound healing is clinically different from skin healing as oral wounds heal faster and with minimal scar formation compared to skin wounds (Whitby and Ferguson 1991). It has been reported that while gene expression patterns are similar in oral and dermal wound healings, they are not identical (Chen et al. 2010). These differences between dermal and oral healing have been linked to both environmental and anatomical differences. For instance, the skin but not the mucosa, has hair follicles and sweat glands, whereas the taste buds are present in the mucosa, but not in the skin (Turabelidze et al. 2014). Additionally, differences

between keratinocytes are important as oral keratinocytes have a greater proliferative capacity than that of skin keratinocytes. The disparities in the reaction of the tissue to injuries in the mucous membrane and skin suggests that keratinocytes residing in different tissue sites could differ inherently both at baseline and in response to trauma (Turabelidze et al. 2014). Therefore, it would be necessary to use human primary gingival keratinocytes in future studies. Punicalagin with Zn (II) combination causes an increase in the cell speed and distance travelled. of HPGFs. It is a promising activity in terms of wound healing activity for this combination. Further studies could be conducted to identify other properties and mechanisms behind this increased migration activity. It would be beneficial to investigate the effect of this combination on extracellular matrix production and remodelling by proteinases, which are important during the wound healing processes (Hochstein and Bhatia 2014).

As previously discussed, an increase in pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) occurs in periodontal disease. *P. gingivalis* biofilm infection significantly elevated pro-inflammatory cytokines in the *ex vivo* rat mandible model. It was hypothesised that *S. gordonii* and *P. gingivalis* biofilms could cause higher pro-inflammatory cytokine levels (Cook et al. 1998; Lamont et al. 2002; Park et al. 2005). However, this dual species biofilm did not demonstrate increases in pro- inflammatory cytokines (IL-1b, IL-6 and TNF-α) compared with *P. gingivalis* biofilms. In future studies, the culture and infection time with *S. gordonii* and *P. gingivalis* biofilms could be increased and more defined and identified dual species biofilms could be tested.

The *ex vivo* rat mandible model infected with *P. gingivalis* biofilm was treated with PRE and PRE/Zn (II) and this lowered pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α). Further studies are necessary to investigate the anti-inflammatory activity of PRE and PRE/ Zn (II) and their effects on COX-2, NF-kB and MAPK signalling pathways, as it has been stated that PRE has an inhibitory effect on these (Afaq et al. 2005; Khan et al. 2012; Houston et al. 2017a; Houston et al. 2017c). There are numerous studies indicating the anti-inflammatory activity of PRE. Thus, further studies on anti-inflammatory cytokines, such as IL-2, IL-4, IL-5, IL-6, IL-10 and IL-13, could also be assessed with

molecular biology and immunohistochemistry methods (Bozkurt et al. 2006). Lack of anti-inflammatory cytokines could facilitate the inflammation process and a destructive effect on bone and collagen in periodontal diseases (Katsikis et al. 1994; Van Roon et al. 1996; Walmsley et al. 1996; Gemmel and Seymour 2004).

PRE and PRE/Zn (II) induced anti-inflammatory activities that could also be conducted in an *in vivo* periodontitis model, to obtain more relative results for clinical conditions. In that vein, an experimentally induced periodontitis or acute periodontal defect model could be used, as pre-clinical *in vivo* models are necessary, along with proof-of-concept and/or feasibility studies to enhance our understanding of substances/biomaterials for periodontal regeneration (Stavrapoulos et al. 2000).

The ultimate aim of this research with PRE and PRE/Zn (II) would be the development of a pharmaceutical formulation, such as a mouthwash, toothpaste or hydrogel formulation, to provide an inexpensive, safer and effective treatment for patients. However, there are some steps in the formulation that should be carefully considered. For instance, components to be included in the formulation should be evaluated in terms of their effect on PRE and PRE/Zn (II) combination bioactivities. Addition of other components could cause reduced or increased bioactivity and toxicity of the main agents, PRE and PRE/Zn (II) combination, in the formulation. In addition, PRE and PRE/Zn (II) combination drug release from the formulation and penetration ability at the applied body site should be carefully considered and investigated.

Overall, this research indicated that PRE and PRE/Zn (II) are promising agents in the development of oral care products with demonstrable properties, such as antioxidant, antimicrobial, anti-inflammatory and wound healing, to prevent and treat periodontal diseases.

Bibliography

Abdallah, F.B., Chaieb, K., Zmantar, T., Kallel, H. and Bakhrouf, A., 2009. Adherence assays and slime production of Vibrio alginolyticus and Vibrio parahaemolyticus. *Brazilian Journal of Microbiology*, *40*, pp.394-398.

Abdollahzadeh, S.H., Mashouf, R.Y., Mortazavi, H., Moghaddam, M.H., Roozbahani, N. and Vahedi, M., 2011. Antibacterial and antifungal activities of Punica granatum peel extracts against oral pathogens. *Journal of Dentistry (Tehran, Iran)*, *8*(1), p.1.

Abid, M., Yaich, H., Cheikhrouhou, S., Khemakhem, I., Bouaziz, M., Attia, H. and Ayadi, M.A., 2017. Antioxidant properties and phenolic profile characterization by LC–MS/MS of selected Tunisian pomegranate peels. *Journal of food science and technology*, *54*(9), pp.2890-2901.

Abusleme, L., Dupuy, A.K., Dutzan, N., Silva, N., Burleson, J.A., Strausbaugh, L.D., Gamonal, J. and Diaz, P.I., 2013. The subgingival microbiome in health and periodontitis and its relationship with community biomass and inflammation. *The ISME journal*, *7*(5), pp.1016-1025.

Abusleme, L., Hoare, A., Hong, B.Y. and Diaz, P.I., 2021. Microbial signatures of health, gingivitis, and periodontitis. *Periodontology 2000*, *86*(1), pp.57-78.

Acevedo, A.C., 2010. Saliva and oral health. *Revista da Associação Médica Brasileira*, *56*(1), pp.2-2.

Ackermans, F., Klein, J.P. and Frank, R.M., 1981. Ultrastructural location of Streptococcus mutans and Streptococcus sanguis antigens in carious human dentine. *Journal de biologie buccale*, *9*(3), pp.203-217.

Adams, L.S., Seeram, N.P., Aggarwal, B.B., Takada, Y., Sand, D. and Heber, D., 2006. Pomegranate juice, total pomegranate ellagitannins, and punicalagin suppress inflammatory cell signaling in colon cancer cells. *Journal of agricultural and food chemistry*, *54*(3), pp.980-985.

Afaq, F., Saleem, M., Krueger, C.G., Reed, J.D. and Mukhtar, H., 2005. Anthocyanin-and hydrolyzable tannin-rich pomegranate fruit extract modulates MAPK and NF-κB pathways and inhibits skin tumorigenesis in CD-1 mice. *International journal of cancer*, *113*(3), pp.423-433.

Agarwal, S., Reynolds, M.A., Pou, S., Peterson, D.E., Charon, J.A. and Suzuki, J.B., 1991. The effect of sanguinarine on human peripheral blood neutrophil viability and functions. *Oral microbiology and immunology*, *6*(1), pp.51-61.

Aguilar-Zárate, P., Wong-Paz, J.E., Michel, M., Buenrostro-Figueroa, J., Díaz, H.R., Ascacio, J.A., Contreras-Esquivel, J.C., Gutiérrez-Sánchez, G. and Aguilar, C.N., 2017. Characterisation of pomegranate-husk polyphenols and semi-preparative fractionation of punicalagin. *Phytochemical Analysis*, *28*(5), pp.433-438.

Agyare, C., Lechtenberg, M., Deters, A., Petereit, F. and Hensel, A., 2011. Ellagitannins from Phyllanthus muellerianus (Kuntze) Exell.: Geraniin and furosin stimulate cellular activity, differentiation and collagen synthesis of human skin keratinocytes and dermal fibroblasts. *Phytomedicine*, *18*(7), pp.617-624.

Ahmad, M.S., Fazal, F., Rahman, A., Hadi, S.M. and Parish, J.H., 1992. Activities of flavonoids for the cleavage of DNA in the presence of Cu (II): correlation with generation of active oxygen species. *Carcinogenesis*, *13*(4), pp.605-608.

Ahmad, N. and Sharma, S., 2012. Biosynthesis of silver nanoparticles from biowaste pomegranate peels. *International Journal of Nanoparticles*, *5*(3), pp.185-195.

Ahmad, N., Sharma, S. and Rai, R., 2012. Rapid green synthesis of silver and gold nanoparticles using peels of Punica granatum. *Adv. Mater. Lett*, *3*(5), pp.376-380.

Ahn, S.J., Ahn, S.J., Wen, Z.T., Brady, L.J. and Burne, R.A., 2008. Characteristics of biofilm formation by Streptococcus mutans in the presence of saliva. *Infection and immunity*, *76*(9), pp.4259-4268.

Ahuja, S., 2006. High-pressure liquid chromatography. *Comprehensive Analytical Chemistry*, *47*, pp.485-559.

Ainsworth, E.A. and Gillespie, K.M., 2007. Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin–Ciocalteu reagent. *Nature protocols*, *2*(4), pp.875-877.

Ajaikumar, K.B., Asheef, M., Babu, B.H. and Padikkala, J., 2005. The inhibition of gastric mucosal injury by Punica granatum L.(pomegranate) methanolic extract. *Journal of Ethnopharmacology*, *96*(1-2), pp.171-176.

Akbarpour, V., Hemmati, K. and Sharifani, M., 2009. Physical and chemical properties of pomegranate (Punica granatum L.) fruit in maturation stage. *American-Eurasian Journal of Agricultural & Environmental Sciences*, *6*(4), pp.411-416.

Akhtar, S., Ismail, T., Fraternale, D. and Sestili, P., 2015. Pomegranate peel and peel extracts: Chemistry and food features. *Food chemistry*, *174*, pp.417-425.

Al-Rawahi, A.S., Edwards, G., Al-Sibani, M., Al-Thani, G., Al-Harrasi, A.S. and Rahman, M.S., 2014. Phenolic constituents of pomegranate peels (Punica granatum L.) cultivated in Oman. *European Journal of Medicinal Plants*, pp.315-331.

Al-Wazni, W.S. and Hadi, B.S., 2016. Antivirulence effects of pomegranate peel extracts on most common urinary tract infection pathogens in pregnant women. *Journal of Contemporary Medical Sciences*, *1*(4), pp.7-12.

Al-Zoreky, N.S., 2009. Antimicrobial activity of pomegranate (Punica granatum L.) fruit peels. *International journal of food microbiology*, *134*(3), pp.244-248.

Alayan, J., Gemmell, E., Ford, P., Hamlet, S., Bird, P.S., Ivanovski, S. and Farah, C.S., 2007. The role of cytokines in a Porphyromonas gingivalis-induced murine abscess model. *Oral microbiology and immunology*, *22*(5), pp.304-312.

Aldhaher, Z.A., Mahmood, M.A., Taha, G.I. and Shaker, R.M., 2015. The effect of pomegranate peels aqueous extract against Streptococcus mutans and the adherence to tooth surface in comparison to chlorhexidine gluconate (in Vitro study). *Adv Life Sci Technol*, *35*, pp.28-32.

Alexander, M.B. and Damoulis, P.D., 1994. The role of cytokines in the pathogenesis of periodontal disease. *Current opinion in periodontology*, pp.39-53.

Ali, A., Chen, Y., Liu, H., Yu, L., Baloch, Z., Khalid, S., Zhu, J. and Chen, L., 2019. Starch-based antimicrobial films functionalized by pomegranate peel. *International journal of biological macromolecules*, *129*, pp.1120-1126.

Alighourchi, H., Barzegar, M. and Abbasi, S., 2008. Anthocyanins characterization of 15 Iranian pomegranate (Punica granatum L.) varieties and their variation after cold storage and pasteurization. *European Food Research and Technology*, *227*(3), pp.881-887.

Allam, J.P., Niederhagen, B., Bücheler, M., Appel, T., Betten, H., Bieber, T., Berge, S. and Novak, N., 2006. Comparative analysis of nasal and oral mucosa dendritic cells. *Allergy*, *61*(2), pp.166-172.

Allam, J.P., Novak, N., Fuchs, C., Asen, S., Bergé, S., Appel, T., Geiger, E., Kochan, J.P. and Bieber, T., 2003. Characterization of dendritic cells from human oral mucosa: A new Langerhans' cell type with high constitutive FcεRI expression. *Journal of Allergy and Clinical Immunology*, *112*(1), pp.141-148.

AlMatar, M., Var, I., Kayar, B., Eker, E., Kafkas, E., Zarifikhosroshahi, M. and Köksal, F., 2019. Evaluation of polyphenolic profile and antibacterial activity of pomegranate juice in combination with rifampin (R) against MDR-TB clinical isolates. *Current pharmaceutical biotechnology*, *20*(4), pp.317-326.

Almatroudi, A., Tahir, S., Hu, H., Chowdhury, D., Gosbell, I.B., Jensen, S.O., Whiteley, G.S., Deva, A.K., Glasbey, T. and Vickery, K., 2018. Staphylococcus aureus dry-surface biofilms are more resistant to heat treatment than traditional hydrated biofilms. *Journal of Hospital Infection*, *98*(2), pp.161-167.

Almzaiel, A.J., Billington, R., Smerdon, G. and Moody, A.J., 2015. Hyperbaric oxygen enhances neutrophil apoptosis and their clearance by monocyte-derived macrophages. *Biochemistry and Cell Biology*, *93*(4), pp.405-416.

Alpaslan, G., Nakajima, T. and Takano, Y., 1997. Extracellular alkaline phosphatase activity as a possible marker for wound healing: a preliminary report. *Journal of oral and maxillofacial surgery*, *55*(1), pp.56-62.

Alrashidi, A.A.R., 2020. *Potentiation of pomegranate rind extract (PRE) bactericidal activity by ZnSO4 combination* (Doctoral dissertation, Cardiff University).

Amano, A., Fujiwara, T., Nagata, H., Kuboniwa, M., Sharma, A., Sojar, H.T., Genco, R.J., Hamada, S. and Shizukuishi, S., 1997. Porphyromonas gingivalis fimbriae mediate coaggregation with Streptococcus oralis through specific domains. *Journal of dental research*, *76*(4), pp.852-857.

Ambigaipalan, P., de Camargo, A.C. and Shahidi, F., 2017. Identification of phenolic antioxidants and bioactives of pomegranate seeds following juice extraction using HPLC-DAD-ESI-MSn. *Food chemistry*, *221*, pp.1883-1894.

Amri, Z., Zaouay, F., Lazreg-Aref, H., Soltana, H., Mneri, A., Mars, M. and Hammami, M., 2017. Phytochemical content, fatty acids composition and antioxidant potential of different pomegranate parts: comparison between edible and non edible varieties grown in Tunisia. *International journal of biological macromolecules*, *104*, pp.274-280.

Anderl, J.N., Franklin, M.J. and Stewart, P.S., 2000. Role of antibiotic penetration limitation in Klebsiella pneumoniae biofilm resistance to ampicillin and ciprofloxacin. *Antimicrobial agents and chemotherapy*, *44*(7), pp.1818-1824.

Andersen, R.N., Ganeshkumar, N. and Kolenbrander, P.E., 1993. Cloning of the Streptococcus gordonii PK488 gene, encoding an adhesin which mediates coaggregation with Actinomyces naeslundii PK606. *Infection and immunity*, *61*(3), pp.981-987.

Andrade, M.A., Lima, V., Silva, A.S., Vilarinho, F., Castilho, M.C., Khwaldia, K. and Ramos, F., 2019. Pomegranate and grape by-products and their active compounds: Are they a valuable source for food applications?. *Trends in Food Science & Technology*, *86*, pp.68-84.

Andreini, C., Banci, L., Bertini, I. and Rosato, A., 2006. Counting the zinc-proteins encoded in the human genome. *Journal of proteome research*, *5*(1), pp.196-201.

Angelov, N., Moutsopoulos, N., Jeong, M.J., Nares, S., Ashcroft, G. and Wahl, S.M., 2004. Aberrant mucosal wound repair in the absence of secretory leukocyte protease inhibitor. *Thrombosis and haemostasis*, *92*(08), pp.288-297.

Angelova Volponi, A., Kawasaki, M. and Sharpe, P.T., 2013. Adult human gingival epithelial cells as a source for whole-tooth bioengineering. *Journal of dental research*, *92*(4), pp.329-334.

Anibal, P.C., Peixoto, I.T.A., Foglio, M.A. and Höfling, J.F., 2013. Antifungal activity of the ethanolic extracts of Punica granatum L. and evaluation of the morphological and structural modifications of its compounds upon the cells of Candida spp. *Brazilian Journal of Microbiology*, *44*(3), pp.839-848.

Annachhatre, A.P., 1996. Anaerobic treatment of industrial wastewaters. *Resources, conservation and recycling*, *16*(1-4), pp.161-166.

Antoci Jr, V., Adams, C.S., Parvizi, J., Davidson, H.M., Composto, R.J., Freeman, T.A., Wickstrom, E., Ducheyne, P., Jungkind, D., Shapiro, I.M. and Hickok, N.J., 2008. The inhibition of Staphylococcus epidermidis biofilm formation by vancomycin-modified titanium alloy and implications for the treatment of periprosthetic infection. *Biomaterials*, *29*(35), pp.4684-4690.

Aparecida Procópio Gomes, L., Alves Figueiredo, L.M., Luiza do Rosário Palma, A., Corrêa Geraldo, B.M., Isler Castro, K.C., Ruano de Oliveira Fugisaki, L., Jorge, A.O.C., de Oliveira, L.D. and Junqueira, J.C., 2016. Punica granatum L.(Pomegranate) extract: in vivo study of antimicrobial activity against Porphyromonas gingivalis in Galleria mellonella model. *The Scientific World Journal*, *2016*.

Ara, T., Kurata, K., Hirai, K., Uchihashi, T., Uematsu, T., Imamura, Y., Furusawa, K., Kurihara, S. and Wang, P.L., 2009. Human gingival fibroblasts are critical in sustaining inflammation in periodontal disease. *Journal of periodontal research*, *44*(1), pp.21-27.

Arancibia, R., Oyarzún, A., Silva, D., Tobar, N., Martínez, J. and Smith, P.C., 2013. Tumor necrosis factor-α inhibits transforming growth factor-β–stimulated myofibroblastic differentiation and extracellular matrix production in human gingival fibroblasts. *Journal of periodontology*, *84*(5), pp.683-693.

Archambaud, C., Sismeiro, O., Toedling, J., Soubigou, G., Bécavin, C., Lechat, P., Lebreton, A., Ciaudo, C. and Cossart, P., 2013. The intestinal microbiota interferes with the microRNA response upon oral.

Archibald, L.K. and Gaynes, R.P., 1997. Hospital-acquired infections in the United States: the importance of interhospital comparisons. *Infectious disease clinics of North America*, *11*(2), pp.245-255.

Arciola, C.R., Campoccia, D. and Montanaro, L., 2018. Implant infections: adhesion, biofilm formation and immune evasion. *Nature Reviews Microbiology*, *16*(7), pp.397-409.

Ardila, C.M. and Bedoya-García, J.A., 2020. Antimicrobial resistance of Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis and Tannerella forsythia in periodontitis patients. *Journal of global antimicrobial resistance*, *22*, pp.215-218.

Arif, H., Sohail, A., Farhan, M., Rehman, A.A., Ahmad, A. and Hadi, S.M., 2018. Flavonoids-induced redox cycling of copper ions leads to generation of reactive oxygen species: A potential role in cancer chemoprevention. *International journal of biological macromolecules*, *106*, pp.569-578.

Arnao, I., Seminario, J., Cisneros, R. and Trabucco, J., 2011, December. Potencial antioxidante de 10 accesiones de yacón, Smallanthus sonchifolius (Poepp. & Endl.) H. Robinson, procedentes de Cajamarca-Perú. In *Anales de la Facultad de Medicina* (Vol. 72, No. 4, pp. 239-243). UNMSM. Facultad de Medicina.

Ascón-Cabrera, M.A., Ascón-Reyes, D.B. and Lebeault, J.M., 1995. Degradation activity of adhered and suspended Pseudomonas cells cultured on 2, 4, 6-trichlorophenol, measured by indirect conductimetry. *Journal of applied bacteriology*, *79*(6), pp.617-624.

Asen, S., Stewart, R.N. and Norris, K.H., 1972. Co-pigmentation of anthocyanins in plant tissues and its effect on color. *Phytochemistry*, *11*(3), pp.1139-1144.

Aslam, M.N., Lansky, E.P. and Varani, J., 2006. Pomegranate as a cosmeceutical source: pomegranate fractions promote proliferation and procollagen synthesis and inhibit matrix metalloproteinase-1 production in human skin cells. *Journal of ethnopharmacology*, *103*(3), pp.311-318.

Åsman, B., Wijkander, P. and Hjerpe, A., 1994. Reduction of collagen degradation in experimental granulation tissue by vitamin E and selenium. *Journal of clinical periodontology*, *21*(1), pp.45-47.

Asres, K., Bucar, F., Knauder, E., Yardley, V., Kendrick, H. and Croft, S.L., 2001. In vitro antiprotozoal activity of extract and compounds from the stem bark of Combretum molle. *Phytotherapy Research*, *15*(7), pp.613-617.

Atabek, A., 2006. *Investigating bacterial outer membrane polymers and bacterial interactions with organic molecules using atomic force microscopy* (Doctoral dissertation, Worcester Polytechnic Institute).

Atamas, S.P., 2002. Complex cytokine regulation of tissue fibrosis. *Life sciences*, *72*(6), pp.631-643.

Athanasiadou, S., Kyriazakis, I., Jackson, F. and Coop, R.L., 2001. Direct anthelmintic effects of condensed tannins towards different gastrointestinal nematodes of sheep: in vitro and in vivo studies. *Veterinary parasitology*, *99*(3), pp.205-219.

Avilés-Reyes, A., Miller, J.H., Lemos, J.A. and Abranches, J., 2017. Collagen-binding proteins of Streptococcus mutans and related streptococci. *Molecular oral microbiology*, *32*(2), pp.89-106.

Axelsson, P. and Lindhe, J., 1981. The significance of maintenance care in the treatment of periodontal disease. *Journal of clinical periodontology*, *8*(4), pp.281-294.

Axelsson, P., Albandar, J.M. and Rams, T.E., 2002. Prevention and control of periodontal diseases in developing and industrialized nations. *Periodontology 2000*, *29*(1), pp.235-246.

Ayad, S., Boot-Handford, R., Humphries, M., Kadler, K. and Shuttleworth, A., 1998. *The extracellular matrix factsbook*. Elsevier.

Ayukekbong, J.A., Ntemgwa, M. and Atabe, A.N., 2017. The threat of antimicrobial resistance in developing countries: causes and control strategies. *Antimicrobial Resistance & Infection Control*, *6*(1), pp.1-8.

Azmi, A.S., Bhat, S.H. and Hadi, S.M., 2005. Resveratrol–Cu (II) induced DNA breakage in human peripheral lymphocytes: implications for anticancer properties. *FEBS letters*, *579*(14), pp.3131-3135.

Badria, F.A. and Zidan, O.A., 2004. Natural products for dental caries prevention. *Journal of Medicinal Food*, *7*(3), pp.381-384.

Baehni, P.C. and Takeuchi, Y., 2003. Anti-plaque agents in the prevention of biofilm-associated oral diseases. *Oral diseases*, *9*, pp.23-29.

Baker, J.L. and Edlund, A., 2019. Exploiting the oral microbiome to prevent tooth decay: has evolution already provided the best tools?. *Frontiers in microbiology*, *9*, p.3323.

Balasundram, N., Sundram, K. and Samman, S., 2006. Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence, and potential uses. *Food chemistry*, *99*(1), pp.191-203.

Baldwin Jr, A.S., 1996. The NF-κB and IκB proteins: new discoveries and insights. *Annual review of immunology*, *14*(1), pp.649-681.

Baltimore, D., 2009. Discovering NF-κB. *Cold Spring Harbor perspectives in biology*, *1*(1), p.a000026.

Bao, B., Prasad, A.S., Beck, F.W. and Godmere, M., 2003. Zinc modulates mRNA levels of cytokines. *American Journal of Physiology-Endocrinology and Metabolism*, *285*(5), pp.E1095-E1102.

Bar-Ya'akov, I., Tian, L., Amir, R. and Holland, D., 2019. Primary metabolites, anthocyanins, and hydrolyzable tannins in the pomegranate fruit. *Frontiers in plant science*, *10*, p.620.

Barnett, M.L., 2006. The rationale for the daily use of an antimicrobial mouthrinse. *The Journal of the American Dental Association*, *137*, pp.S16-S21.

Baronetti, J.L., Villegas, N.A., Paraje, M.G. and Albesa, I., 2011. Nitric oxide-mediated apoptosis in rat macrophages subjected to Shiga toxin 2 from Escherichia coli. *Microbiology and immunology*, *55*(4), pp.231-238.

Barritt, B.H. and Einset, J., 1969. Inheritance of 3 major fruit colors in grapes. *Journal of the American Society for Horticultural Science*, *94*(2), p.87.

Barros, S.P., Williams, R., Offenbacher, S. and Morelli, T., 2016. Gingival crevicular fluid as a source of biomarkers for periodontitis. *Periodontology 2000*, *70*(1), pp.53-64.

Barry, K.M., Davies, N.W. and Mohammed, C.L., 2001. Identification of hydrolysable tannins in the reaction zone of Eucalyptus nitens wood by high performance liquid chromatography–electrospray ionisation mass spectrometry. *Phytochemical Analysis: An International Journal of Plant Chemical and Biochemical Techniques*, *12*(2), pp.120-127.

Bartold, P.M., Marshall, R.I. and Haynes, D.R., 2005. Periodontitis and rheumatoid arthritis: a review. *Journal of periodontology*, *76*, pp.2066-2074.

Bassiri Jahromi, S., Pourshafie, M.R., Mirabzadeh, E., Tavasoli, A., Katiraee, F., Mostafavi, E. and Abbasian, S., 2015. Punica granatum peel extract toxicity in mice. *Jundishapur Journal of Natural Pharmaceutical Products*, *10*(4).

Bateson, W., 1965. *Experiments in plant hybridisation*. Oliver & Boyd.

Batista, A.L.A., Lins, R.D.A.U., de Souza Coelho, R., do Nascimento Barbosa, D., Belém, N.M. and Celestino, F.J.A., 2014. Clinical efficacy analysis of the mouth rinsing with pomegranate and chamomile plant extracts in the gingival bleeding reduction. *Complementary therapies in clinical practice*, *20*(1), pp.93-98.

Battino, M., Bullon, P., Wilson, M. and Newman, H., 1999. Oxidative injury and inflammatory periodontal diseases: the challenge of anti-oxidants to free radicals and reactive oxygen species. *Critical Reviews in Oral Biology & Medicine*, *10*(4), pp.458-476.

Battold, P. M., & Narayanan, A. S. 1998. Biology of the periodental connective tissues.

Baumgartner, J.C., Watts, C.M. and Xia, T., 2000. Occurrence of Candida albicans in infections of endodontic origin. *Journal of endodontics*, *26*(12), pp.695-698.

Becker, C., Buttler, P. and Gräber, H.G., 2002. Influence of anti-CD49f and anti-CD29 monoclonal antibodies on mitotic activity of epithelial cells (HaCaT) and gingival fibroblasts in vitro. *European journal of oral sciences*, *110*(2), pp.137-143.

Beikler, T., Peters, U., Prior, K., Eisenacher, M. and Flemmig, T.F., 2008. Gene expression in periodontal tissues following treatment. *BMC Medical Genomics*, *1*(1), pp.1-9.

Bekir, J., Mars, M., Souchard, J.P. and Bouajila, J., 2013. Assessment of antioxidant, anti-inflammatory, anti-cholinesterase and cytotoxic activities of pomegranate (Punica granatum) leaves. *Food and chemical toxicology*, *55*, pp.470-475.

Bekir, J., Mars, M., Vicendo, P., Fterrich, A. and Bouajila, J., 2013. Chemical composition and antioxidant, anti-inflammatory, and antiproliferation activities of pomegranate (Punica granatum) flowers. Journal of medicinal food, 16(6), pp.544-550.

Beldon, P., 2010. Basic science of wound healing. *Surgery (Oxford)*, *28*(9), pp.409-412.

Belenguer-Guallar, I., Jiménez-Soriano, Y. and Claramunt-Lozano, A., 2014. Treatment of recurrent aphthous stomatitis. A literature review. *Journal of clinical and experimental dentistry*, *6*(2), p.e168.

Belkaid, Y. and Harrison, O.J., 2017. Homeostatic immunity and the microbiota. *Immunity*, *46*(4), pp.562-576.

Ben-Sasson, S.Z., Hu-Li, J., Quiel, J., Cauchetaux, S., Ratner, M., Shapira, I., Dinarello, C.A. and Paul, W.E., 2009. IL-1 acts directly on CD4 T cells to enhance their antigen-driven expansion and differentiation. *Proceedings of the National Academy of Sciences*, *106*(17), pp.7119-7124.

Ben-Simhon, Z., Judeinstein, S., Nadler-Hassar, T., Trainin, T., Bar-Ya'akov, I., Borochov-Neori, H. and Holland, D., 2011. A pomegranate (Punica granatum L.) WD40-repeat gene is a functional homologue of Arabidopsis TTG1 and is involved in the regulation of anthocyanin biosynthesis during pomegranate fruit development. *Planta*, *234*(5), pp.865-881.

BenSaad, L.A., Kim, K.H., Quah, C.C., Kim, W.R. and Shahimi, M., 2017. Anti-inflammatory potential of ellagic acid, gallic acid and punicalagin A&B isolated from Punica granatum. *BMC complementary and alternative medicine*, *17*(1), pp.1-10.

Berg, J.M. and Shi, Y., 1996. The galvanization of biology: a growing appreciation for the roles of zinc. *Science*, *271*(5252), pp.1081-1085.

Bergmeier, L.A. ed., 2018. *Oral mucosa in health and disease: a concise handbook*. Springer.

Bernbom, N., Jørgensen, R.L., Ng, Y.Y., Meyer, R.L., Kingshott, P., Vejborg, R.M., Klemm, P., Besenbacher, F. and Gram, L., 2006. Bacterial adhesion to stainless steel is reduced by aqueous fish extract coatings. *Biofilms*, *3*(1), p.25.

Bernstein, I.A., Chakrabarti, S.G., Kumaroo, K.K. and Sibrack, L.A., 1970. Synthesis of protein in the mammalian epidermis. *Journal of Investigative Dermatology*, *55*(5), pp.291-302.

Beyenal, H., Donovan, C., Lewandowski, Z. and Harkin, G., 2004. Three-dimensional biofilm structure quantification. *Journal of microbiological methods*, *59*(3), pp.395-413.

Bhattacharyya, A., Chattopadhyay, R., Mitra, S. and Crowe, S.E., 2014. Oxidative stress: an essential factor in the pathogenesis of gastrointestinal mucosal diseases. *Physiological reviews*, *94*(2), pp.329-354.

Bink, A., Vandenbosch, D., Coenye, T., Nelis, H., Cammue, B.P. and Thevissen, K., 2011. Superoxide dismutases are involved in Candida albicans biofilm persistence against miconazole. *Antimicrobial agents and chemotherapy*, *55*(9), pp.4033-4037.

Binney, A., Addy, M., McKeown, S. and Everatt, L., 1995. The effect of a commercially available triclosan-containing toothpaste compared to a sodium-fluoride-containing toothpaste and a chlorhexidine rinse on 4-day plaque regrowth. *Journal of clinical periodontology*, *22*(11), pp.830-834.

Biswas, T.K. and Mukherjee, B., 2003. Plant medicines of Indian origin for wound healing activity: a review. *The international journal of lower extremity wounds*, *2*(1), pp.25-39.

Black, G.V., 1899. Susceptibility and immunity to dental caries. *Dent. Cosmos*, *41*, pp.826-830.

Bloomquist, C., Lundebrek, R., McClintock, K., Resch, D., Dunny, G., Reilly, B. and Liljemark, W., 1994, January. AN EXTRACELLULAR BACTERIAL FACTOR WHICH STARTS BACTERIAL-DNA REPLICATION. In *JOURNAL OF DENTAL RESEARCH* (Vol. 73, pp. 302-302). 1619 DUKE ST, ALEXANDRIA, VA 22314: AMER ASSOC DENTAL RESEARCH.

Bloomquist, C.G., Reilly, B.E. and Liljemark, W.F., 1996. Adherence, accumulation, and cell division of a natural adherent bacterial population. *Journal of bacteriology*, *178*(4), pp.1172-1177.

Bobyrev, V.N., Rozkolupa, N.V. and Skripnikova, T.P., 1994. Experimental and clinical bases for the use of antioxidants as agents for treating and preventing periodontitis. *Stomatologiia*, *73*(3), pp.11-18.

Bodiba, D.C., Prasad, P., Srivastava, A., Crampton, B. and Lall, N.S., 2018. Antibacterial activity of Azadirachta indica, Pongamia pinnata, Psidium guajava, and Mangifera indica and their mechanism of action against Streptococcus mutans. *Pharmacognosy magazine*, *14*(53), p.76.

Bohmont, C., Aaronson, L.M., Mann, K. and Pardini, R.S., 1987. Inhibition of mitochondrial NADH oxidase, succinoxidase, and ATPase by naturally occurring flavonoids. *Journal of natural products*, *50*(3), pp.427-433.

Boks, N.P., Kaper, H.J., Norde, W., van der Mei, H.C. and Busscher, H.J., 2009. Mobile and immobile adhesion of staphylococcal strains to hydrophilic and hydrophobic surfaces. *Journal of colloid and interface science*, *331*(1), pp.60-64.

Bonini, M.G., Rota, C., Tomasi, A. and Mason, R.P., 2006. The oxidation of 2′, 7′-dichlorofluorescin to reactive oxygen species: a self-fulfilling prophesy?. *Free Radical Biology and Medicine*, *40*(6), pp.968-975.

Bortner, C.D., Oldenburg, N.B. and Cidlowski, J.A., 1995. The role of DNA fragmentation in apoptosis. *Trends in cell biology*, *5*(1), pp.21-26.

Boss, P.K., Davies, C. and Robinson, S.P., 1996. Expression of anthocyanin biosynthesis pathway genes in red and white grapes. *Plant molecular biology*, *32*(3), pp.565-569.

Bosshardt, D. and Schroeder, H.E., 1990. Evidence for rapid multipolar and slow unipolar production of human cellular and acellular cementum matrix with intrinsic fibers. *Journal of clinical periodontology*, *17*(9), pp.663-668.

Bosshardt, D.D., 2005. Are cementoblasts a subpopulation of osteoblasts or a unique phenotype?. *Journal of dental research*, *84*(5), pp.390-406.

Bostan, L.E., Taylor, Z.A., Carré, M.J., MacNeil, S., Franklin, S.E. and Lewis, R., 2016. A comparison of friction behaviour for ex vivo human, tissue engineered and synthetic skin. *Tribology International*, *103*, pp.487-495.

Bostanci, N. and Belibasakis, G.N., 2012. Porphyromonas gingivalis: an invasive and evasive opportunistic oral pathogen. *FEMS microbiology letters*, *333*(1), pp.1-9.

Boukamp, P., Petrussevska, R.T., Breitkreutz, D., Hornung, J., Markham, A. and Fusenig, N.E., 1988. Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. *Journal of Cell Biology*, *106*(3), pp.761-771.

Boukef, K., Souissi, H. R., & Balansard, G., 1982. Contribution to the study on plants used in traditional medicine in Tunisia. *Plant Med Phytother*, *16*(4), 260-279.

Boukortt, K.N., Saidi-Ouahrani, N., Boukerzaza, B., Ouhaibi-Djellouli, H., Hachmaoui, K., Benaissa, F.Z., Taleb, L., Drabla-Ouahrani, H., Deba, T., Ouledhamou, S.A. and Mehtar, N., 2015. Association analysis of the IL-1 gene cluster polymorphisms with aggressive and chronic periodontitis in the Algerian population. *Archives of oral biology*, *60*(10), pp.1463-1470.

Bowen, W.H. and Koo, H.J.C.R., 2011. Biology of Streptococcus mutans-derived glucosyltransferases: role in extracellular matrix formation of cariogenic biofilms. *Caries research*, *45*(1), pp.69-86.

Bowen, W.H., Burne, R.A., Wu, H. and Koo, H., 2018. Oral biofilms: pathogens, matrix, and polymicrobial interactions in microenvironments. *Trends in microbiology*, *26*(3), pp.229-242.

Bozkurt, F.Y., Ay, Z.Y., Berker, E., Tepe, E. and Akkuş, S., 2006. Anti-inflammatory cytokines in gingival crevicular fluid in patients with periodontitis and rheumatoid arthritis: a preliminary report. *Cytokine*, *35*(3-4), pp.180-185.

Brading, M.G. and Marsh, P.D., 2003. The oral environment: the challenge for antimicrobials in oral care products. *International dental journal*, *53*(6), pp.353-362.

Brady, L.J., Maddocks, S.E., Larson, M.R., Forsgren, N., Persson, K., Deivanayagam, C.C. and Jenkinson, H.F., 2010. The changing faces of Streptococcus antigen I/II polypeptide family adhesins. *Molecular microbiology*, *77*(2), pp.276-286.

Braga, L.C., Shupp, J.W., Cummings, C., Jett, M., Takahashi, J.A., Carmo, L.S., Chartone-Souza, E. and Nascimento, A.M.A., 2005. Pomegranate extract inhibits Staphylococcus aureus growth and subsequent enterotoxin production. *Journal of ethnopharmacology*, *96*(1-2), pp.335-339.

Braga, R. R. (2019). Calcium phosphates as ion-releasing fillers in restorative resin-based materials. *Dental Materials*, *35*(1), 3-14.

Branda, S.S., Vik, Å., Friedman, L. and Kolter, R., 2005. Biofilms: the matrix revisited. *Trends in microbiology*, *13*(1), pp.20-26.

Brandtzaeg, P. and Johansen, F.E., 2005. Mucosal B cells: phenotypic characteristics, transcriptional regulation, and homing properties. *Immunological reviews*, *206*(1), pp.32-63.

Brazil, G.M., Kenefick, L., Callanan, M., Haro, A., De Lorenzo, V., Dowling, D.N. and O'gara, F., 1995. Construction of a rhizosphere pseudomonad with potential to degrade polychlorinated biphenyls and detection of bph gene expression in the rhizosphere. *Applied and Environmental Microbiology*, *61*(5), pp.1946-1952.

Brenner, D., Blaser, H. and Mak, T.W., 2015. Regulation of tumour necrosis factor signalling: live or let die. *Nature Reviews Immunology*, *15*(6), pp.362-374.

Brighenti, V., Groothuis, S.F., Prencipe, F.P., Amir, R., Benvenuti, S. and Pellati, F., 2017. Metabolite fingerprinting of Punica granatum L.(pomegranate) polyphenols by means of high-performance liquid chromatography with diode array and electrospray ionization-mass spectrometry detection. *Journal of Chromatography a*, *1480*, pp.20-31.

Brooks, W., Demuth, D.R., Gil, S. and Lamont, R.J., 1997. Identification of a Streptococcus gordonii SspB domain that mediates adhesion to Porphyromonas gingivalis. *Infection and immunity*, *65*(9), pp.3753-3758.

Brouillard, R., Mazza, G., Saad, Z., Albrecht-Gary, A.M. and Cheminat, A., 1989. The co-pigmentation reaction of anthocyanins: a microprobe for the structural study of aqueous solutions. *Journal of the American Chemical Society*, *111*(7), pp.2604-2610.

Brown, M.R., Allison, D.G. and Gilbert, P., 1988. Resistance of bacterial biofilms to antibiotics a growth-rate related effect?. *Journal of Antimicrobial Chemotherapy*, *22*(6), pp.777-780.

Bruni, S., Guglielmi, V. and Pozzi, F., 2010. Surface-enhanced Raman spectroscopy (SERS) on silver colloids for the identification of ancient textile dyes: Tyrian purple and madder. *Journal of Raman Spectroscopy: An International Journal for Original Work in all Aspects of Raman Spectroscopy, Including Higher Order Processes, and also Brillouin and Rayleigh Scattering*, *41*(2), pp.175-180.

Buckley, C.D., Pilling, D., Lord, J.M., Akbar, A.N., Scheel-Toellner, D. and Salmon, M., 2001. Fibroblasts regulate the switch from acute resolving to chronic persistent inflammation. *Trends in immunology*, *22*(4), pp.199-204.

Burger, O., Weiss, E., Sharon, N., Tabak, M., Neeman, I. and Ofek, I., 2002. Inhibition of Helicobacter pylori adhesion to human gastric mucus by a high-molecular-weight constituent of cranberry juice. *Critical reviews in food science and nutrition*, *42*(S3), pp.279-284.

Burne, R.A., Chen, Y.Y.M. and Penders, J.E., 1997. Analysis of gene expression in Streptococcus mutans in biofilms in vitro. *Advances in dental research*, *11*(1), pp.100-109.

Butler, J.S. and Loh, S.N., 2007. Zn2+-dependent misfolding of the p53 DNA binding domain. *Biochemistry*, *46*(10), pp.2630-2639.

Butt, T. and Satti, L., 2011. Efficacy of zinc as an antibacterial agent against enteric bacterial pathogens. *J Ayub Med Coll Abbottabad*, *23*(2).

Cáceres, A., Girón, L. M., Alvarado, S. R., & Torres, M. F., 1987. Screening of antimicrobial activity of plants popularly used in Guatemala for the treatment of dermatomucosal diseases. *Journal of ethnopharmacology*, *20*(3), 223-237.

Çam, M. and Hışıl, Y., 2010. Pressurised water extraction of polyphenols from pomegranate peels. *Food chemistry*, *123*(3), pp.878-885.

Campisi, G., Panzarella, V., Matranga, D., Calvino, F., Pizzo, G., Muzio, L.L. and Porter, S., 2008. Risk factors of oral candidosis: a twofold approach of study by fuzzy logic and traditional statistic. *Archives of oral biology*, *53*(4), pp.388-397.

Campos, M.S., Marchini, L., Bernardes, L.A.S., Paulino, L.C. and Nobrega, F.G., 2008. Biofilm microbial communities of denture stomatitis. *Oral microbiology and immunology*, *23*(5), pp.419-424.

Camps, J. and Pashley, D., 2003. In vivo sensitivity of human root dentin to air blast and scratching. *Journal of periodontology*, *74*(11), pp.1589-1594.

Canabarro, A., Valle, C., Farias, M.R., Santos, F.B., Lazera, M. and Wanke, B., 2013. Association of subgingival colonization of Candida albicans and other yeasts with severity of chronic periodontitis. *Journal of periodontal research*, *48*(4), pp.428-432.

Cannell, R.J. ed., 1998. *Natural products isolation* (Vol. 4). Springer Science & Business Media.
Cao, G., Sofic, E. and Prior, R.L., 1997. Antioxidant and prooxidant behavior of flavonoids: structure-activity relationships. *Free radical biology and medicine*, *22*(5), pp.749-760.

Carbonell-Capella, J.M., Buniowska, M., Barba, F.J., Esteve, M.J. and Frígola, A., 2014. Analytical methods for determining bioavailability and bioaccessibility of bioactive compounds from fruits and vegetables: A review. *Comprehensive Reviews in Food Science and Food Safety*, *13*(2), pp.155-171.

Carlsson, J., 1997. Bacterial metabolism in dental biofilms. *Advances in Dental Research*, *11*(1), pp.75-80.

Carmeliet, P., 2003. Angiogenesis in health and disease. *Nature medicine*, *9*(6), pp.653-660.

Carter, K., Landini, G. and Walmsley, A.D., 2001. Plaque removal characteristics of electric toothbrushes using an in vitro plaque model. *Journal of clinical periodontology*, *28*(11), pp.1045-1049.

Casey, A.L., Mermel, L.A., Nightingale, P. and Elliott, T.S., 2008. Antimicrobial central venous catheters in adults: a systematic review and meta-analysis. *The Lancet infectious diseases*, *8*(12), pp.763-776.

Castro, L. and Freeman, B.A., 2001. Reactive oxygen species in human health and disease. *Nutrition (Burbank, Los Angeles County, Calif.)*, *17*(2), pp.161-165.

Caufield, P.W., Dasanayake, A.P., Li, Y., Pan, Y., Hsu, J. and Hardin, J.M., 2000. Natural history of Streptococcus sanguinis in the oral cavity of infants: evidence for a discrete window of infectivity. *Infection and immunity*, *68*(7), pp.4018-4023.

Ccahuana-Vásquez, R.A. and Cury, J.A., 2010. S. mutans biofilm model to evaluate antimicrobial substances and enamel demineralization. *Brazilian oral research*, *24*(2), pp.135-141.

Cegelski, L., Marshall, G.R., Eldridge, G.R. and Hultgren, S.J., 2008. The biology and future prospects of antivirulence therapies. *Nature Reviews Microbiology*, *6*(1), pp.17-27.

Celiksoy, V. and Heard, C.M., 2021. Antimicrobial Potential of Pomegranate Extracts. In *Pomegranate*. IntechOpen.

Celiksoy, V., Moses, R.L., Sloan, A.J., Moseley, R. and Heard, C.M., 2021. Synergistic In Vitro Antimicrobial Activity of Pomegranate Rind Extract and Zinc (II) against Micrococcus luteus under Planktonic and Biofilm Conditions. *Pharmaceutics*, *13*(6), p.851.

Cerdá, B., Cerón, J.J., Tomás-Barberán, F.A. and Espín, J.C., 2003. Repeated oral administration of high doses of the pomegranate ellagitannin punicalagin to rats for 37 days is not toxic. *Journal of agricultural and food chemistry*, *51*(11), pp.3493-3501.

César de Souza Vasconcelos, L., Sampaio, M.C.C., Sampaio, F.C. and Higino, J.S., 2003. Use of Punica granatum as an antifungal agent against candidosis associated with denture stomatitis. *Mycoses*, *46*(5-6), pp.192-196.

Chace, K.V., Carubelli, R. and Nordquist, R.E., 1991. The role of nonenzymatic glycosylation, transition metals, and free radicals in the formation of collagen aggregates. *Archives of biochemistry and biophysics*, *288*(2), pp.473-480.

ChaichiSemsari, M., MaheriSis, N., Sadaghian, M., Eshratkhah, B. and Hassanpour, S., 2011. Effects of administration of industrial tannins on nutrient excretion parameters during naturally acquired mixed nematode infections in Moghani sheep. *Journal of American Science*, *7*(6), pp.245-248.

Chandra, R., & Jadhav, V. T., 2009. Pomegranate research and development in India and future thrusts. *Preparation for the Sources of the 2nd Green Revolution in Indian Agriculture*, *2*, 39-44.

Chandra, R., & Tarachand, D., 2010. Pomegranate culture in Deccan plateau of India. *Fruit, Vegetable and Cereal Science and Biotechnology*, *4*(Special Issue 2), 113-119.

Chang, S.P., Shen, S.C., Lee, W.R., Yang, L.L. and Chen, Y.C., 2011. Imatinib mesylate induction of ROS-dependent apoptosis in melanoma B16F0 cells. *Journal of dermatological science*, *62*(3), pp.183-191.

Chapple, I.L. and Matthews, J.B., 2007. The role of reactive oxygen and antioxidant species in periodontal tissue destruction. *Periodontology 2000*, *43*(1), pp.160-232.

Chapple, I.L.C., 1997. Reactive oxygen species and antioxidants in inflammatory diseases. *Journal of clinical periodontology*, *24*(5), pp.287-296.

Chauhan, D.N., Singh, P.R., Shah, K. and Chauhan, N.S., 2020. Natural oral care in dental therapy: Current and future prospects. *Natural Oral Care in Dental Therapy*, pp.1-29.

Chauhan, S., Upadhyay, M.K., Rishi, N. and Rishi, S., 2011. Phytofabrication of silver nanoparticles using pomegranate fruit seeds. *International journal of Nanomaterials and Biostructures*, *1*(2), pp.17-21.

Chebaibi, A. and Filali, F.R., 2013. Bactericidal activity and phytochemical screening of Moroccan pomegranate (Punica granatum Linn.) peel aqueous extracts. *Journal of Medicinal Plants Research*, *7*(14), pp.887-891.

Chen, L. and Wen, Y.M., 2011. The role of bacterial biofilm in persistent infections and control strategies. *International journal of oral science*, *3*(2), pp.66-73.

Chen, L., Arbieva, Z.H., Guo, S., Marucha, P.T., Mustoe, T.A. and DiPietro, L.A., 2010. Positional differences in the wound transcriptome of skin and oral mucosa. *BMC genomics*, *11*(1), pp.1-15.

Chen, M., Yu, Q. and Sun, H., 2013. Novel strategies for the prevention and treatment of biofilm related infections. *International journal of molecular sciences*, *14*(9), pp.18488-18501.

Chen, P.B., Davern, L.B., Katz, J., Eldridge, J.H. and Michalek, S.M., 1996. Host responses induced by co-infection with Porphyromonas gingivalis and Actinobacillus actinomycetemcomitans in a murine model. *Oral microbiology and immunology*, *11*(4), pp.274-281.

Chen, Q., Li, Y. and Chen, Z., 2012. Separation, identification, and quantification of active constituents in Fructus Psoraleae by high-performance liquid chromatography with UV, ion trap mass spectrometry, and electrochemical detection. *Journal of pharmaceutical analysis*, *2*(2), pp.143-151.

Chen, X., Daliri, E.B.M., Kim, N., Kim, J.R., Yoo, D. and Oh, D.H., 2020. Microbial etiology and prevention of dental caries: exploiting natural products to inhibit cariogenic biofilms. *Pathogens*, *9*(7), p.569.

Cheng, G., Wozniak, K., Wallig, M.A., Fidel Jr, P.L., Trupin, S.R. and Hoyer, L.L., 2005. Comparison between Candida albicans agglutinin-like sequence gene expression patterns in human clinical specimens and models of vaginal candidiasis. *Infection and immunity*, *73*(3), pp.1656-1663.

Chidambara Murthy, K.N., Jayaprakasha, G.K. and Singh, R.P., 2002. Studies on antioxidant activity of pomegranate (Punica granatum) peel extract using in vivo models. *Journal of Agricultural and Food Chemistry*, *50*(17), pp.4791-4795.

Chidambara Murthy, K.N., Reddy, V.K., Veigas, J.M. and Murthy, U.D., 2004. Study on wound healing activity of Punica granatum peel. *Journal of Medicinal Food*, *7*(2), pp.256-259.

Chinsembu, K.C., 2016. Plants and other natural products used in the management of oral infections and improvement of oral health. *Acta tropica*, *154*, pp.6-18.

Chiorcea-Paquim, A.M., Enache, T.A., De Souza Gil, E. and Oliveira-Brett, A.M., 2020. Natural phenolic antioxidants electrochemistry: Towards a new food science methodology. *Comprehensive Reviews in Food Science and Food Safety*, *19*(4), pp.1680-1726.

Chiron, S., Tomczak, C., Duperray, A., Lainé, J., Bonne, G., Eder, A., Hansen, A., Eschenhagen, T., Verdier, C. and Coirault, C., 2012. Complex interactions between human myoblasts and the surrounding 3D fibrin-based matrix. *PloS one*, *7*(4), p.e36173.

Cho, J. and Lee, D.G., 2011. The antimicrobial peptide arenicin-1 promotes generation of reactive oxygen species and induction of apoptosis. *Biochimica et Biophysica Acta (BBA)-General Subjects*, *1810*(12), pp.1246-1251.

Cho, J.H., Kim, J.K., Lee, H.Y. and Yoon, J.H., 2013. Surgical anatomy of human soft palate. *The Laryngoscope*, *123*(11), pp.2900-2904.

Ciofu, O., Mandsberg, L.F., Wang, H. and Høiby, N., 2012. Phenotypes selected during chronic lung infection in cystic fibrosis patients: implications for the treatment of Pseudomonas aeruginosa biofilm infections. *FEMS Immunology & Medical Microbiology*, *65*(2), pp.215-225.

Číž, M., Čížová, H., Denev, P., Kratchanova, M., Slavov, A. and Lojek, A., 2010. Different methods for control and comparison of the antioxidant properties of vegetables. *Food Control*, *21*(4), pp.518-523.

Clark, M.B. and Clark, D.A., 2018. Oral development and pathology. *Ochsner Journal*, *18*(4), pp.339-344.

Clark, W.B., Beem, J.E., Nesbitt, W.E., Cisar, J.O., Tseng, C.C. and Levine, M.J., 1989. Pellicle receptors for Actinomyces viscosus type 1 fimbriae in vitro. *Infection and immunity*, *57*(10), pp.3003-3008.

Clayden, E.C., 1952. A discussion on the preparation of bone sections by the paraffin wax method with special reference to the control of decalcification. *Journal of Medical Laboratory Technology*, *10*, p.103.

Cleland Jr, W.P., 2001. Opportunities and obstacles in veterinary dental drug delivery. *Advanced drug delivery reviews*, *50*(3), pp.261-275.

Clinical and Laboratory Standards Institute, 2012. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. *Approved standard M07-A9*, *32*.

Clinical and Laboratory Standards Institute. Methods for antimicrobial susceptibility testing of anaerobic bacteria; Approved standard, 8th ed. M11-A8. Wayne, PA: Clinical and Laboratory Standards Institute, 2012.

Collins, L.M.C. and Dawes, C., 1987. The surface area of the adult human mouth and thickness of the salivary film covering the teeth and oral mucosa. *Journal of dental research*, *66*(8), pp.1300-1302.

Cook, G.S., Costerton, J.W. and Lamont, R.J., 1998. Biofilm formation by Porphyromonas gingivalis and Streptococcus gordonii. *Journal of periodontal research*, *33*(6), pp.323-327.

Corbin, A., Pitts, B., Parker, A. and Stewart, P.S., 2011. Antimicrobial penetration and efficacy in an in vitro oral biofilm model. *Antimicrobial agents and chemotherapy*, *55*(7), pp.3338-3344.

Cordier, W. and Steenkamp, V., 2015. Evaluation of four assays to determine cytotoxicity of selected crude medicinal plant extracts in vitro. *Journal of Pharmaceutical Research International*, pp.16-21.
2
Costello, E.K., Lauber, C.L., Hamady, M., Fierer, N., Gordon, J.I. and Knight, R., 2009. Bacterial community variation in human body habitats across space and time. *science*, *326*(5960), pp.1694-1697.

Costerton, J.W., 1999. Introduction to biofilm. *International journal of antimicrobial agents*, *11*(3-4), pp.217-221.

Costerton, J.W., Lewandowski, Z., Caldwell, D.E., Korber, D.R. and Lappin-Scott, H.M., 1995. Microbial biofilms. *Annual review of microbiology*, *49*(1), pp.711-745.

Costerton, J.W., Lewandowski, Z., DeBeer, D., Caldwell, D., Korber, D. and James, G., 1994. Biofilms, the customized microniche. *Journal of bacteriology*, *176*(8), pp.2137-2142.

Costerton, J.W., Stewart, P.S. and Greenberg, E.P., 1999. Bacterial biofilms: a common cause of persistent infections. *Science*, *284*(5418), pp.1318-1322.

Costerton, W., Veeh, R., Shirtliff, M., Pasmore, M., Post, C. and Ehrlich, G., 2003. The application of biofilm science to the study and control of chronic bacterial infections. *The Journal of clinical investigation*, *112*(10), pp.1466-1477.

Coussens, L.M. and Werb, Z., 2002. Inflammation and cancer. *Nature*, *420*(6917), pp.860-867.

Couve, E., 1986. Ultrastructural changes during the life cycle of human odontoblasts. *Archives of oral biology*, *31*(10), pp.643-651.

Coventry, J., Griffiths, G., Scully, C. and Tonetti, M., 2000. Periodontal disease. *Bmj*, *321*(7252), pp.36-39.

Cowan, M.M., 1999. Plant products as antimicrobial agents. *Clinical microbiology reviews*, *12*(4), pp.564-582.

Cozens, D. and Read, R.C., 2012. Anti-adhesion methods as novel therapeutics for bacterial infections. *Expert review of anti-infective therapy*, *10*(12), pp.1457-1468.

Cruchley, A.T. and Bergmeier, L.A., 2018. Structure and functions of the oral mucosa. In *Oral Mucosa in Health and Disease* (pp. 1-18). Springer, Cham.

Cui, M.C., Zhang, J.Y., Chen, S.J., Jiang, H.L., Zhou, H.B. and Ling, Q.Z., 2016. Identification of alkaloids and flavonoids in all parts of Fritillaria thunbergii using LC-LTQ-Orbitrap MSn. *Zhongguo Zhong yao za zhi= Zhongguo zhongyao zazhi= China journal of Chinese materia medica*, *41*(11), pp.2124-2130.

Cummins, D., 1992. Mechanisms of action of clinically proven anti-plaque agents. *Clinical and Biological Aspects of Dentifrices. pp205-228. New York: Oxford Medical Publications*.

Cushnie, T.T. and Lamb, A.J., 2011. Recent advances in understanding the antibacterial properties of flavonoids. *International journal of antimicrobial agents*, *38*(2), pp.99-107.

Czaplicki, S., 2013. Chromatography in bioactivity analysis of compounds. *Column chromatography*, pp.99-122.

da Silva, J.A.T., Rana, T.S., Narzary, D., Verma, N., Meshram, D.T. and Ranade, S.A., 2013. Pomegranate biology and biotechnology: A review. *Scientia Horticulturae*, *160*, pp.85-107.

Dabholkar, C.S., Shah, M., Kathariya, R., Bajaj, M. and Doshi, Y., 2016. Comparative evaluation of antimicrobial activity of pomegranate-containing mouthwash against Oral-biofilm forming organisms: an Invitro microbial study. *Journal of clinical and diagnostic research: JCDR*, *10*(3), p.ZC65.

Daep, C.A., James, D.M., Lamont, R.J. and Demuth, D.R., 2006. Structural characterization of peptide-mediated inhibition of Porphyromonas gingivalis biofilm formation. *Infection and immunity*, *74*(10), pp.5756-5762.

Daep, C.A., Lamont, R.J. and Demuth, D.R., 2008. Interaction of Porphyromonas gingivalis with oral streptococci requires a motif that resembles the eukaryotic nuclear receptor box protein-protein interaction domain. *Infection and immunity*, *76*(7), pp.3273-3280.

Daep, C.A., Novak, E.A., Lamont, R.J. and Demuth, D.R., 2011. Structural dissection and in vivo effectiveness of a peptide inhibitor of Porphyromonas gingivalis adherence to Streptococcus gordonii. *Infection and immunity*, *79*(1), pp.67-74.

Dai, Z., Nair, V., Khan, M. and Ciolino, H.P., 2010. Pomegranate extract inhibits the proliferation and viability of MMTV-Wnt-1 mouse mammary cancer stem cells in vitro. *Oncology reports*, *24*(4), pp.1087-1091.

Dalle, F., Wächtler, B., L'Ollivier, C., Holland, G., Bannert, N., Wilson, D., Labruère, C., Bonnin, A. and Hube, B., 2010. Cellular interactions of Candida albicans with human oral epithelial cells and enterocytes. *Cellular microbiology*, *12*(2), pp.248-271.

Darveau, R.P., 2010. Periodontitis: a polymicrobial disruption of host homeostasis. *Nature Reviews Microbiology*, *8*(7), pp.481-490.

Das, A.K., Mandal, S.C., Banerjee, S.K., Sinha, S., Das, J., Saha, B.P. and Pal, M., 1999. Studies on antidiarrhoeal activity of Punica granatum seed extract in rats. *Journal of ethnopharmacology*, *68*(1-3), pp.205-208.

Davey, M.E. and Duncan, M.J., 2006. Enhanced biofilm formation and loss of capsule synthesis: deletion of a putative glycosyltransferase in Porphyromonas gingivalis. *Journal of bacteriology*, *188*(15), pp.5510-5523.

Davies, D., 2003. Understanding biofilm resistance to antibacterial agents. *Nature reviews Drug discovery*, *2*(2), pp.114-122.

Davies, G.E., Francis, J., Martin, A.R., Rose, F.L. and Swain, G., 1954. 1: 6-di-4′-chlorophenyldiguanidohexane ("hibitane"*). Laboratory investigation of a new antibacterial agent of high potency. *British journal of pharmacology and chemotherapy*, *9*(2), pp.192-196.

Davies, M.J., Richardson, P.D., Woolf, N., Katz, D.R. and Mann, J., 1993. Risk of thrombosis in human atherosclerotic plaques: role of extracellular lipid, macrophage, and smooth muscle cell content. *Heart*, *69*(5), pp.377-381.

Davies, R.M., Ellwood, R.P. and Davies, G.M., 2003. The rational use of fluoride toothpaste. *International journal of dental hygiene*, *1*(1), pp.3-8.

Davis, S.C. and Perez, R., 2009. Cosmeceuticals and natural products: wound healing. *Clinics in dermatology*, *27*(5), pp.502-506.

Dawson, D.V., Drake, D.R., Hill, J.R., Brogden, K.A., Fischer, C.L. and Wertz, P.W., 2013. Organization, barrier function and antimicrobial lipids of the oral mucosa. *International journal of cosmetic science*, *35*(3), pp.220-223.

De Brum, T.F., Zadra, M., Piana, M., Boligon, A.A., Fröhlich, J.K., De Freitas, R.B., Stefanello, S.T., Froeder, A.L.F., Belke, B.V., Nunes, L.T. and da Silva Jesus, R., 2013. HPLC analysis of phenolics compounds and antioxidant capacity of leaves of Vitex megapotamica (Sprengel) Moldenke. *Molecules*, *18*(7), pp.8342-8357.

De Camargo Pereira, G., Guimarães, G.N., Planello, A.C., Santamaria, M.P., de Souza, A.P., Line, S.R. and Marques, M.R., 2013. Porphyromonas gingivalis LPS stimulation downregulates DNMT1, DNMT3a, and JMJD3 gene expression levels in human HaCaT keratinocytes. *Clinical oral investigations*, *17*(4), pp.1279-1285.

De Carvalho, F.G., Silva, D.S., Hebling, J., Spolidorio, L.C. and Spolidorio, D.M.P., 2006. Presence of mutans streptococci and Candida spp. in dental plaque/dentine of carious teeth and early childhood caries. *Archives of oral biology*, *51*(11), pp.1024-1028.

De Luca, V. and St Pierre, B., 2000. The cell and developmental biology of alkaloid biosynthesis. *Trends in plant science*, *5*(4), pp.168-173.

De Oliveira, J.R., de Castro, V.C., Vilela, P.D.G.F., Camargo, S.E.A., Carvalho, C.A.T., Jorge, A.O.C. and de Oliveira, L.D., 2013. Cytotoxicity of Brazilian plant extracts against oral microorganisms of interest to dentistry. *BMC complementary and alternative medicine*, *13*(1), pp.1-7.

De Rijke, E., Zappey, H., Ariese, F., Gooijer, C. and Udo, A., 2003. Liquid chromatography with atmospheric pressure chemical ionization and electrospray ionization mass spectrometry of flavonoids with triple-quadrupole and ion-trap instruments. *Journal of Chromatography A*, *984*(1), pp.45-58.

Décaillet, F., Giannopoulou, C., Cionca, N., Almaghlouth, A.A. and Mombelli, A., 2012. Microbial profiles of patients seeking treatment for periodontitis: Influence of origin, smoking and age?. *Schweizer Monatsschrift für Zahnmedizin*, *122*(3), pp.198-204.

Del Rio, D., Rodriguez-Mateos, A., Spencer, J.P., Tognolini, M., Borges, G. and Crozier, A., 2013. Dietary (poly) phenolics in human health: structures, bioavailability, and evidence of protective effects against chronic diseases. *Antioxidants & redox signaling*, *18*(14), pp.1818-1892.

Delattin, N., Cammue, B.P. and Thevissen, K., 2014. Reactive oxygen species-inducing antifungal agents and their activity against fungal biofilms. *Future medicinal chemistry*, *6*(1), pp.77-90.

Deming, R.L., Allred, A.L., Dahl, A.R., Herlinger, A.W. and Kestner, M.O., 1976. Tripositive mercury. Low temperature electrochemical oxidation of 1, 4, 8, 11-tetraazacyclotetradecanemercury (II) tetrafluoroborate. *Journal of the American Chemical Society*, *98*(14), pp.4132-4137.

Demuth, D.R. and Irvine, D.C., 2002. Structural and functional variation within the alanine-rich repetitive domain of streptococcal antigen I/II. *Infection and immunity*, *70*(11), pp.6389-6398.

Demuth, D.R., Duan, Y., Brooks, W., Holmes, A.R., McNab, R. and Jenkinson, H.F., 1996. Tandem genes encode cell-surface polypeptides SspA and SspB which mediate adhesion of the oral bacterium Streptococcus gordonii to human and bacterial receptors. *Molecular microbiology*, *20*(2), pp.403-413.

Derakhshan, Z., Ferrante, M., Tadi, M., Ansari, F., Heydari, A., Hosseini, M.S., Conti, G.O. and Sadrabad, E.K., 2018. Antioxidant activity and total phenolic content of ethanolic extract of pomegranate peels, juice and seeds. *Food and chemical toxicology*, *114*, pp.108-111.

Deshpande, P., Ralston, D.R. and MacNeil, S., 2013. The use of allodermis prepared from Euro skin bank to prepare autologous tissue engineered skin for clinical use. *Burns*, *39*(6), pp.1170-1177.

Desmouliere, A., Redard, M., Darby, I. and Gabbiani, G., 1995. Apoptosis mediates the decrease in cellularity during the transition between granulation tissue and scar. *The American journal of pathology*, *146*(1), p.56.

Desta, B., 1995. Ethiopian traditional herbal drugs. Part I: Studies on the toxicity and therapeutic activity of local taenicidal medications. *Journal of Ethnopharmacology*, *45*(1), pp.27-33.

Deters, A.M., Meyer, U. and Stintzing, F.C., 2012. Time-dependent bioactivity of preparations from cactus pear (Opuntia ficus indica) and ice plant (Mesembryanthemum crystallinum) on human skin fibroblasts and keratinocytes. *Journal of ethnopharmacology*, *142*(2), pp.438-444.

Devanshu, S., Rahul, M., Annu, G., Kishan, S. and Anroop, N., 2010. Quantitative bioanalysis by LC-MS/MS: a review. *Journal of pharmaceutical and biomedical sciences*, *7*(7).

Devatkal, S.K., Jaiswal, P., Jha, S.N., Bharadwaj, R. and Viswas, K.N., 2013. Antibacterial activity of aqueous extract of pomegranate peel against Pseudomonas stutzeri isolated from poultry meat. *Journal of food science and technology*, *50*(3), pp.555-560.

Devillers, J., Steiman, R. and Seigle-Murandi, F., 1989. The usefulness of the agar-well diffusion method for assessing chemical toxicity to bacteria and fungi. *Chemosphere*, *19*(10-11), pp.1693-1700.

Di Stefano, V., Pitonzo, R., Novara, M.E., Bongiorno, D., Indelicato, S., Gentile, C., Avellone, G., Bognanni, R., Scandurra, S. and Melilli, M.G., 2019. Antioxidant activity and phenolic composition in pomegranate (Punica granatum L.) genotypes from south Italy by UHPLC–Orbitrap-MS approach. *Journal of the Science of Food and Agriculture*, *99*(3), pp.1038-1045.

Dickinson, B.C. and Chang, C.J., 2011. Chemistry and biology of reactive oxygen species in signaling or stress responses. *Nature chemical biology*, *7*(8), p.504.

Dickinson, G.M. and Bisno, A.L., 1993. Infections associated with prosthetic devices: clinical considerations. *The International journal of artificial organs*, *16*(11), pp.749-754.

Diegelmann, R.F. and Evans, M.C., 2004. Wound healing: an overview of acute, fibrotic and delayed healing. *Front biosci*, *9*(1), pp.283-289.

Dietrich, N., Tan, C.H., Cubillas, C., Earley, B.J. and Kornfeld, K., 2016. Insights into zinc and cadmium biology in the nematode Caenorhabditis elegans. *Archives of biochemistry and biophysics*, *611*, pp.120-133.

Dinarello, C.A., 2000. Proinflammatory cytokines. *Chest*, *118*(2), pp.503-508.

Dixon, R.A., 2001. Natural products and plant disease resistance. *Nature*, *411*(6839), pp.843-847.

Donkin, S.G., Ohlson, D.L. and Teaf, C.M., 2000. Properties and effects of metals. *Principles of toxicology: environmental and industrial applications*, pp.325-344.

Donlan, R.M. and Costerton, J.W., 2002. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clinical microbiology reviews*, *15*(2), pp.167-193.

Dovi, J.V., He, L.K. and DiPietro, L.A., 2003. Accelerated wound closure in neutrophil-depleted mice. *Journal of leukocyte biology*, *73*(4), pp.448-455.

Dragar, P. and Moravcova, J., 2004. Recent advances in analysis of Chinese medical plants and traditional medicines. *Journal of Chromatography B*, *812*(1-2), pp.3-21.

Driffield, K., Miller, K., Bostock, J.M., O'neill, A.J. and Chopra, I., 2008. Increased mutability of Pseudomonas aeruginosa in biofilms. *Journal of Antimicrobial Chemotherapy*, *61*(5), pp.1053-1056.

Dû, L.D. and Kolenbrander, P.E., 2000. Identification of saliva-regulated genes of Streptococcus gordonii DL1 by differential display using random arbitrarily primed PCR. *Infection and Immunity*, *68*(8), pp.4834-4837.

Duman, A.D., Ozgen, M., Dayisoylu, K.S., Erbil, N. and Durgac, C., 2009. Antimicrobial activity of six pomegranate (Punica granatum L.) varieties and their relation to some of their pomological and phytonutrient characteristics. *Molecules*, *14*(5), pp.1808-1817.

Dumin, J.A., Dickeson, S.K., Stricker, T.P., Bhattacharyya-Pakrasi, M., Roby, J.D., Santoro, S.A. and Parks, W.C., 2001. Pro-collagenase-1 (matrix metalloproteinase-1) binds the α2β1 integrin upon release from keratinocytes migrating on type I collagen. *Journal of Biological Chemistry*, *276*(31), pp.29368-29374.

Dunne Jr, W.M., 2002. Bacterial adhesion: seen any good biofilms lately?. *Clinical microbiology reviews*, *15*(2), pp.155-166.

Dupont, B., Graybill, J.R., Armstrong, D., Laroche, R., Touze, J.E. and Wheat, L.J., 1992. Fungal infections in AIDS patients. *Journal of Medical and Veterinary Mycology*, *30*(sup1), pp.19-28.

Duque, C., Stipp, R.N., Wang, B., Smith, D.J., Höfling, J.F., Kuramitsu, H.K., Duncan, M.J. and Mattos-Graner, R.O., 2011. Downregulation of GbpB, a component of the VicRK regulon, affects biofilm formation and cell surface characteristics of Streptococcus mutans. *Infection and immunity*, *79*(2), pp.786-796.

Dutzan, N., Abusleme, L., Bridgeman, H., Greenwell-Wild, T., Zangerle-Murray, T., Fife, M.E., Bouladoux, N., Linley, H., Brenchley, L., Wemyss, K. and Calderon, G., 2017. On-going mechanical damage from mastication drives homeostatic Th17 cell responses at the oral barrier. *Immunity*, *46*(1), pp.133-147.

El Nemr, A., 2009. Potential of pomegranate husk carbon for Cr (VI) removal from wastewater: Kinetic and isotherm studies. *Journal of Hazardous Materials*, *161*(1), pp.132-141.

El-Ashtoukhy, E.S., Amin, N.K. and Abdelwahab, O., 2008. Removal of lead (II) and copper (II) from aqueous solution using pomegranate peel as a new adsorbent. *Desalination*, *223*(1-3), pp.162-173.

El-Sherbini, G.M., Ibrahim, K.M., El Sherbiny, E.T., Abdel-Hady, N.M. and Morsy, T.A., 2010. Efficacy of Punica granatum extract on in-vitro and in-vivo control of Trichomonas vaginalis. *Journal of the Egyptian Society of Parasitology*, *40*(1), pp.229-244.

Elfalleh, W., Hannachi, H., Tlili, N., Yahia, Y., Nasri, N. and Ferchichi, A., 2012. Total phenolic contents and antioxidant activities of pomegranate peel, seed, leaf and flower. *Journal of Medicinal Plants Research*, *6*(32), pp.4724-4730.

Elfalleh, W., Tlili, N., Nasri, N., Yahia, Y., Hannachi, H., Chaira, N., Ying, M. and Ferchichi, A., 2011. Antioxidant capacities of phenolic compounds and tocopherols from Tunisian pomegranate (Punica granatum) fruits. *Journal of food science*, *76*(5), pp.C707-C713.

Eli, L., Bar-Tat, Y. and Kostovetzki, I., 2001. At first glance: social meanings of dental appearance. *Journal of public health dentistry*, *61*(3), pp.150-154.

Ellis, I.R. and Schor, S.L., 1996. Differential effects of TGF-β1 on hyaluronan synthesis by fetal and adult skin fibroblasts: Implications for cell migration and wound healing. *Experimental cell research*, *228*(2), pp.326-333.

Eltay, E.G., Gismalla, B.G., Mukhtar, M.M. and Awadelkarim, M.O., 2021. Punica granatum peel extract as adjunct irrigation to nonsurgical treatment of chronic gingivitis. *Complementary Therapies in Clinical Practice*, *43*, p.101383.

Endo, E.H., Ueda-Nakamura, T. and Nakamura, C.V., 2012. Activity of spray-dried microparticles containing pomegranate peel extract against Candida albicans. *Molecules*, *17*(9), pp.10094-10107.

Epstein, J.B., 1990. Antifungal therapy in oropharyngeal mycotic infections. *Oral surgery, oral medicine, oral pathology*, *69*(1), pp.32-41.

Eskan, M.A., Jotwani, R., Abe, T., Chmelar, J., Lim, J.H., Liang, S., Ciero, P.A., Krauss, J.L., Li, F., Rauner, M. and Hofbauer, L.C., 2012. The leukocyte integrin antagonist Del-1 inhibits IL-17-mediated inflammatory bone loss. *Nature immunology*, *13*(5), pp.465-473.

Espeland, E.M. and Wetzel, R.G., 2001. Complexation, stabilization, and UV photolysis of extracellular and surface-bound glucosidase and alkaline phosphatase: implications for biofilm microbiota. *Microbial ecology*, *42*(4), pp.572-585.

Everette, J.D., Bryant, Q.M., Green, A.M., Abbey, Y.A., Wangila, G.W. and Walker, R.B., 2010. Thorough study of reactivity of various compound classes toward the Folin− Ciocalteu reagent. *Journal of agricultural and food chemistry*, *58*(14), pp.8139-8144.

Facchini, P.J., Bird, D.A. and St-Pierre, B., 2004. Can Arabidopsis make complex alkaloids?. *Trends in plant science*, *9*(3), pp.116-122.

Fahmy, Z.H., El-Shennawy, A.M., El-Komy, W., Ali, E. and Hamid, S.A., 2009. Potential antiparasitic activity of pomegranate extracts against schistosomules and mature worms of Schistosoma mansoni: in vitro and in vivo study. *Australian Journal of Basic and Applied Sciences*, *3*(4), pp.4634-4643.

Fakhruddin, K.S., Ngo, H.C. and Samaranayake, L.P., 2019. Cariogenic microbiome and microbiota of the early primary dentition: A contemporary overview. *Oral diseases*, *25*(4), pp.982-995.

Falsetta, M.L., Klein, M.I., Colonne, P.M., Scott-Anne, K., Gregoire, S., Pai, C.H., Gonzalez-Begne, M., Watson, G., Krysan, D.J., Bowen, W.H. and Koo, H., 2014. Symbiotic relationship between Streptococcus mutans and Candida albicans synergizes virulence of plaque biofilms in vivo. *Infection and immunity*, *82*(5), pp.1968-1981.

Fan, W., Chi, Y. and Zhang, S., 2008. The use of a tea polyphenol dip to extend the shelf life of silver carp (Hypophthalmicthys molitrix) during storage in ice. *Food chemistry*, *108*(1), pp.148-153.

Fathima, A. and Rao, J.R., 2016. Selective toxicity of Catechin—a natural flavonoid towards bacteria. *Applied microbiology and biotechnology*, *100*(14), pp.6395-6402.

Fattouch, S., Caboni, P., Coroneo, V., Tuberoso, C.I., Angioni, A., Dessi, S., Marzouki, N. and Cabras, P., 2007. Antimicrobial activity of Tunisian quince (Cydonia oblonga Miller) pulp and peel polyphenolic extracts. *Journal of Agricultural and Food Chemistry*, *55*(3), pp.963-969.

Fawole, O.A., Makunga, N.P. and Opara, U.L., 2012. Antibacterial, antioxidant and tyrosinase-inhibition activities of pomegranate fruit peel methanolic extract. *BMC complementary and alternative medicine*, *12*(1), pp.1-11.

Fejerskov, O., Nyvad, B. and Kidd, E. eds., 2015. *Dental caries: the disease and its clinical management*. John Wiley & Sons.

Feller, L., Altini, M., Khammissa, R.A.G., Chandran, R., Bouckaert, M. and Lemmer, J., 2013. Oral mucosal immunity. *Oral surgery, oral medicine, oral pathology and oral radiology*, *116*(5), pp.576-583.

Fernandes, L., Pereira, J.A., Lopéz-Cortés, I., Salazar, D.M., Ramalhosa, E. and Casal, S., 2015. Fatty acid, vitamin E and sterols composition of seed oils from nine different pomegranate (Punica granatum L.) cultivars grown in Spain. *Journal of Food Composition and Analysis*, *39*, pp.13-22.

Ferrazzano, G.F., Scioscia, E., Sateriale, D., Pastore, G., Colicchio, R., Pagliuca, C., Cantile, T., Alcidi, B., Coda, M., Ingenito, A. and Scaglione, E., 2017. In vitro antibacterial activity of pomegranate juice and peel extracts on cariogenic bacteria. *BioMed research international*, *2017*.

Feuille, F., Ebersole, J.L., Kesavalu, L., Stepfen, M.J. and Holt, S.C., 1996. Mixed infection with Porphyromonas gingivalis and Fusobacterium nucleatum in a murine lesion model: potential synergistic effects on virulence. *Infection and immunity*, *64*(6), pp.2094-2100.

Fidel Jr, P.L., Vazquez, J.A. and Sobel, J.D., 1999. Candida glabrata: review of epidemiology, pathogenesis, and clinical disease with comparison to C. albicans. *Clinical microbiology reviews*, *12*(1), pp.80-96.

Field, T.R., White, A., Elborn, J.S. and Tunney, M.M., 2005. Effect of oxygen limitation on the in vitro antimicrobial susceptibility of clinical isolates of Pseudomonas aeruginosa grown planktonically and as biofilms. *European Journal of Clinical Microbiology and Infectious Diseases*, *24*(10), pp.677-687.

Fiorellini, J.P., Ishikawa, S.O. and Kim, D.M., 2006. Gingival inflammation. Clinical periodontology.

Fiorellini, J.P., Kim, D.M. and Ishikawa, S.O., 2006. Clinical features of gingivitis. *Carranza's clinical periodontology. Philadelphia: Saunders, Elsevier*, pp.362-372.

Firatli, E., Unal, T., Onan, U. and Sandalli, P., 1994. Antioxidative activities of some chemotherapeutics. A possible mechanism in reducing gingival inflammation. *Journal of clinical periodontology*, *21*(10), pp.680-683.

Fischer, U.A., Carle, R. and Kammerer, D.R., 2011. Identification and quantification of phenolic compounds from pomegranate (Punica granatum L.) peel, mesocarp, aril and differently produced juices by HPLC-DAD–ESI/MSn. *Food chemistry*, *127*(2), pp.807-821.

Fischer, U.A., Jaksch, A.V., Carle, R. and Kammerer, D.R., 2012. Determination of lignans in edible and nonedible parts of pomegranate (Punica granatum L.) and products derived therefrom, particularly focusing

on the quantitation of isolariciresinol using HPLC-DAD-ESI/MS n. *Journal of agricultural and food chemistry*, *60*(1), pp.283-292.

Fleck, A., Cabral, P.F., Vieira, F.F., Pinheiro, D.A., Pereira, C.R., Santos, W.C. and Machado, T.B., 2016. Punica granatum L. hydrogel for wound care treatment: From case study to phytomedicine standardization. *Molecules*, *21*(8), p.1059.

Flemming, H.C. and Wingender, J., 2001. Relevance of microbial extracellular polymeric substances (EPSs)-Part I: Structural and ecological aspects. *Water science and technology*, *43*(6), pp.1-8.

Fletcher, B.S., Kujubu, D.A., Perrin, D.M. and Herschman, H.R., 1992. Structure of the mitogen-inducible TIS10 gene and demonstration that the TIS10-encoded protein is a functional prostaglandin G/H synthase. *Journal of Biological Chemistry*, *267*(7), pp.4338-4344.

Fletcher, M., 1996. Bacterial attachment in aquatic environments: a diversity of surfaces and adhesion strategies. *Bacterial adhesion: molecular and ecological diversity*, *19*, pp.1-24.

Fonseca, J.E., Santos, M.J., Canhao, H. and Choy, E., 2009. Interleukin-6 as a key player in systemic inflammation and joint destruction. *Autoimmunity reviews*, *8*(7), pp.538-542.

Foss SR, Nakamura CV, Ueda-Nakamura T, Cortez DA, Endo EH, Dias Filho BP. Antifungal activity of pomegranate peel extract and isolated compound punicalagin against dermatophytes. Annals of clinical microbiology and antimicrobials. 2014 Dec 1;13(1):32.

Foster, J.S. and Kolenbrander, P.E., 2004. Development of a multispecies oral bacterial community in a saliva-conditioned flow cell. *Applied and environmental microbiology*, *70*(7), pp.4340-4348.

Fracassetti, D., Costa, C., Moulay, L. and Tomás-Barberán, F.A., 2013. Ellagic acid derivatives, ellagitannins, proanthocyanidins and other phenolics, vitamin C and antioxidant capacity of two powder products from camu-camu fruit (Myrciaria dubia). *Food chemistry*, *139*(1-4), pp.578-588.

Frantz, C., Stewart, K.M. and Weaver, V.M., 2010. The extracellular matrix at a glance. *Journal of cell science*, *123*(24), pp.4195-4200.

Fraser, V.J., Jones, M., Dunkel, J., Storfer, S., Medoff, G. and Dunagan, W.C., 1992. Candidemia in a tertiary care hospital: epidemiology, risk factors, and predictors of mortality. *Clinical Infectious Diseases*, *15*(3), pp.414-421.

Frison, E. A., & Servinsky, J., 1995. *Directory of European institutions holding crop genetic resources collections: Vol. 2. Index*.

Fujita, T., Yoshimoto, T., Kajiya, M., Ouhara, K., Matsuda, S., Takemura, T., Akutagawa, K., Takeda, K., Mizuno, N. and Kurihara, H., 2018. Regulation of defensive function on gingival epithelial cells can prevent periodontal disease. *Japanese dental science review*, *54*(2), pp.66-75.

Fukumoto, L.R. and Mazza, G., 2000. Assessing antioxidant and prooxidant activities of phenolic compounds. *Journal of agricultural and food chemistry*, *48*(8), pp.3597-3604.

Furiga, A., Lonvaud-Funel, A. and Badet, C., 2009. In vitro study of antioxidant capacity and antibacterial activity on oral anaerobes of a grape seed extract. *Food Chemistry*, *113*(4), pp.1037-1040.

Furiga, A., Lonvaud-Funel, A., Dorignac, G. and Badet, C., 2008. In vitro anti-bacterial and anti-adherence effects of natural polyphenolic compounds on oral bacteria. *Journal of applied microbiology*, *105*(5), pp.1470-1476.

Gabler, W.L., Bullock, W.W. and Creamer, H.R., 1987. The influence of chlorhexidine on superoxide generation by induced human neutrophils. *Journal of periodontal research*, *22*(6), pp.445-450.

Galati, G. and O'brien, P.J., 2004. Potential toxicity of flavonoids and other dietary phenolics: significance for their chemopreventive and anticancer properties. *Free Radical Biology and Medicine*, *37*(3), pp.287-303.

Galvão, L.C.D.C., Furletti, V.F., Bersan, S.M.F., da Cunha, M.G., Ruiz, A.L.T.G., Carvalho, J.E.D., Sartoratto, A., Rehder, V.L.G., Figueira, G.M., Teixeira Duarte, M.C. and Ikegaki, M., 2012. Antimicrobial activity of essential oils against Streptococcus mutans and their antiproliferative effects. *Evidence-Based Complementary and Alternative Medicine*, *2012*.

Ganeshkumar, N., Song, M.E.J.A. and McBride, B.C., 1988. Cloning of a Streptococcus sanguis adhesin which mediates binding to saliva-coated hydroxyapatite. *Infection and immunity*, *56*(5), pp.1150-1157.

Ganss, C., Klimek, J., Brune, V. and Schürmann, A., 2004. Effects of two fluoridation measures on erosion progression in human enamel and dentine in situ. *Caries research*, *38*(6), pp.561-566.

Ganzera, M., 2008. Quality control of herbal medicines by capillary electrophoresis: Potential, requirements and applications. *Electrophoresis*, *29*(17), pp.3489-3503.

Garcés-Ortíz, M., Ledesma-Montes, C. and Reyes-Gasga, J., 2015. Scanning electron microscopic study on the fibrillar structures within dentinal tubules of human dentin. *Journal of endodontics*, *41*(9), pp.1510-1514.

Garcia-Villalba, R., Espín, J.C., Aaby, K., Alasalvar, C., Heinonen, M., Jacobs, G., Voorspoels, S., Koivumaki, T., Kroon, P.A., Pelvan, E. and Saha, S., 2015. Validated method for the characterization and quantification of extractable and nonextractable ellagitannins after acid hydrolysis in pomegranate fruits, juices, and extracts. *Journal of agricultural and food chemistry*, *63*(29), pp.6555-6566.

Garcia, M.C., Lee, J.T., Ramsook, C.B., Alsteens, D., Dufrêne, Y.F. and Lipke, P.N., 2011. A role for amyloid in cell aggregation and biofilm formation. *PloS one*, *6*(3), p.e17632.

Garlet, G.P., 2010. Destructive and protective roles of cytokines in periodontitis: a re-appraisal from host defense and tissue destruction viewpoints. *Journal of dental research*, *89*(12), pp.1349-1363.

Garlet, G.P., Cardoso, C.R.B., Campanelli, A.P., Ferreira, B.R., Avila-Campos, M.J., Cunha, F.Q. and Silva, J.S., 2007. The dual role of p55 tumour necrosis factor-α receptor in Actinobacillus actinomycetemcomitans-induced experimental periodontitis: host protection and tissue destruction. *Clinical & Experimental Immunology*, *147*(1), pp.128-138.

Garlet, G.P., Martins Jr, W., Ferreira, B.R., Milanezi, C.M. and Silva, J.S., 2003. Patterns of chemokines and chemokine receptors expression in different forms of human periodontal disease. *Journal of periodontal research*, *38*(2), pp.210-217.

Garlick, J.A., Parks, W.C., Welgus, H.G. and Taichman, L.B., 1996. Re-epithelialization of human oral keratinocytes in vitro. *Journal of dental research*, *75*(3), pp.912-918.

Gautam, R. and Jachak, S.M., 2009. Recent developments in anti-inflammatory natural products. *Medicinal research reviews*, *29*(5), pp.767-820.

Geesey, G.G., Richardson, W.T., Yeomans, H.G., Irvin, R.T. and Costerton, J.W., 1977. Microscopic examination of natural sessile bacterial populations from an alpine stream. *Canadian Journal of Microbiology*, *23*(12), pp.1733-1736.

Gemmell, E. and Seymour, G.J., 2004. Immunoregulatory control of Th1/Th2 cytokine profiles in periodontal disease. *Periodontology 2000*, *35*(1), pp.21-41.

Gemmell, E., Marshall, R.I. and Seymour, G.J., 1997. Cytokines and prostaglandins in immune homeostasis and tissue destruction in periodontal disease. *Periodontology 2000*, *14*(1), pp.112-143.

Gemmell, E., Yamazaki, K. and Seymour, G.J., 2002. Destructive periodontitis lesions are determined by the nature of the lymphocytic response. *Critical Reviews in Oral Biology & Medicine*, *13*(1), pp.17-34.

Ghalayani, P., Zolfaghary, B., Farhad, A.R., Tavangar, A. and Soleymani, B., 2013. The efficacy of Punica granatum extract in the management of recurrent aphthous stomatitis. *Journal of research in pharmacy practice*, *2*(2), p.88.

Ghannoum, M.A., Jurevic, R.J., Mukherjee, P.K., Cui, F., Sikaroodi, M., Naqvi, A. and Gillevet, P.M., 2010. Characterization of the oral fungal microbiome (mycobiome) in healthy individuals. *PLoS pathogens*, *6*(1), p.e1000713.

Ghasemian, A., Mehrabian, S. and Majd, A., 2006. Peel extracts of two Iranian cultivars of pomegranate (Punica granatum) have antioxidant and antimutagenic activities. *Pak J Biol Sci*, *7*, pp.1402-405.

Ghisalberti, E.L., 1979. Propolis: a review. *Bee world*, *60*(2), pp.59-84.

Ghosh, P.K. and Gaba, A., 2013. Phyto-extracts in wound healing. *Journal of Pharmacy & Pharmaceutical Sciences*, *16*(5), pp.760-820.

Giannobile, W.V., Beikler, T., Kinney, J.S., Ramseier, C.A., Morelli, T. and Wong, D.T., 2009. Saliva as a diagnostic tool for periodontal disease: current state and future directions. *Periodontology 2000*, *50*, p.52.

Gibbons, R.J., Hay, D.I. and Schlesinger, D.H., 1991. Delineation of a segment of adsorbed salivary acidic proline-rich proteins which promotes adhesion of Streptococcus gordonii to apatitic surfaces. *Infection and immunity*, *59*(9), pp.2948-2954.

Gibbs, C.D., Atherton, S.E., Huntington, E., Lynch, R.J.M. and Duckworth, R.M., 1995. Effect of low levels of fluoride on calcium uptake by demineralized human enamel. *Archives of Oral Biology*, *40*(9), pp.879-881.

Gil, M.I., Artes, F. and Tomas-Barberan, F.A., 1996a. Minimal processing and modified atmosphere packaging effects on pigmentation of pomegranate seeds. *Journal of Food Science*, *61*(1), pp.161-164.

Gil, M.I., García-Viguera, C., Artés, F. and Tomás-Barberán, F.A., 1995. Changes in pomegranate juice pigmentation during ripening. *Journal of the Science of Food and Agriculture*, *68*(1), pp.77-81.

Gil, M.I., Martınez, J.A. and Artés, F., 1996b. Minimally processed pomegranate seeds. *LWT-Food Science and Technology*, *29*(8), pp.708-713.

Gil, M.I., Tomás-Barberán, F.A., Hess-Pierce, B., Holcroft, D.M. and Kader, A.A., 2000. Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing. *Journal of Agricultural and Food chemistry*, *48*(10), pp.4581-4589.

Gilbert, R.J. and Williams, P.E., 1987. The oral retention and antiplaque efficacy of triclosan in human volunteers. *British journal of clinical pharmacology*, *23*(5), pp.579-583.

Gjermo, P. 1989. Chlorhexidine and related compounds. *J. dent. Res.*, *68*, 1602-1608.

Glazer, I., Masaphy, S., Marciano, P., Bar-Ilan, I., Holland, D., Kerem, Z. and Amir, R., 2012. Partial identification of antifungal compounds from Punica granatum peel extracts. *Journal of agricultural and food chemistry*, *60*(19), pp.4841-4848.

Glim, J.E., van Egmond, M., Niessen, F.B., Everts, V. and Beelen, R.H., 2013. Detrimental dermal wound healing: what can we learn from the oral mucosa?. *Wound Repair and Regeneration*, *21*(5), pp.648-660.

Glimcher, L.H., 2007. Trawling for treasure: tales of T-bet. *Nature immunology*, *8*(5), pp.448-450.

Gokhale, N., Patwardhan, A. and Cowan, J.A., 2007. Metalloaminoglycosides: chemistry and biological relevance. In *Aminoglycoside Antibiotics: From Chemical Biology to Drug Discovery* (pp. 235-254). John Wiley.

Goldberg, M. and Smith, A.J., 2004. Cells and extracellular matrices of dentin and pulp: a biological basis for repair and tissue engineering. *Critical Reviews in Oral Biology & Medicine*, *15*(1), pp.13-27.

Gómez-Caravaca, A.M., Verardo, V., Toselli, M., Segura-Carretero, A., Fernández-Gutiérrez, A. and Caboni, M.F., 2013. Determination of the major phenolic compounds in pomegranate juices by HPLC–DAD–ESI-MS. *Journal of agricultural and food chemistry*, *61*(22), pp.5328-5337.

Gomis-Rüth, F.X., 2003. Structural aspects of the metzincin clan of metalloendopeptidases. *Molecular biotechnology*, *24*(2), pp.157-202.

Gonthier, M.P., Remesy, C., Scalbert, A., Cheynier, V., Souquet, J.M., Poutanen, K. and Aura, A.M., 2006. Microbial metabolism of caffeic acid and its esters chlorogenic and caftaric acids by human faecal microbiota in vitro. *Biomedicine & pharmacotherapy*, *60*(9), pp.536-540.

Goodman, A.E., Marshall, K.C. and Hermansson, M., 1994. Gene transfer among bacteria under conditions of nutrient depletion in simulated and natural aquatic environments. *FEMS microbiology ecology*, *15*(1-2), pp.55-60.

Górska, R., Gregorek, H., Kowalski, J., Laskus-Perendyk, A., Syczewska, M. and Madaliński, K., 2003. Relationship between clinical parameters and cytokine profiles in inflamed gingival tissue and serum samples from patients with chronic periodontitis. *Journal of clinical periodontology*, *30*(12), pp.1046-1052.

Gorur, A., Lyle, D.M., Schaudinn, C. and Costerton, J.W., 2009. Biofilm removal with a dental water jet. *Compendium of continuing education in dentistry (Jamesburg, NJ: 1995)*, *30*, pp.1-6.

Goudouri, O.M., Kontonasaki, E., Lohbauer, U. and Boccaccini, A.R., 2014. Antibacterial properties of metal and metalloid ions in chronic periodontitis and peri-implantitis therapy. *Acta biomaterialia*, *10*(8), pp.3795-3810.

Gould, S.W., Fielder, M.D., Kelly, A.F. and Naughton, D.P., 2009. Anti-microbial activities of pomegranate rind extracts: enhancement by cupric sulphate against clinical isolates of S. aureus, MRSA and PVL positive CA-MSSA. *BMC Complementary and Alternative Medicine*, *9*(1), pp.1-6.

Gould, S.W.J., Fielder, M.D., Kelly, A.F., Sankary, W.E. and Naughton, D.P., 2009. Antimicrobial pomegranate rind extracts: enhancement by Cu (II) and vitamin C combinations against clinical isolates of Pseudomonas aeruginosa. *British journal of biomedical science*, *66*(3), pp.129-132.

Goultschin, J. and Levy, H., 1986. Inhibition of Superoxide generation by human polymorphonuclear leukocytes with chlorhexidine: its possible relation to periodontal disease. *Journal of periodontology*, *57*(7), pp.422-425.

Gözlekçi, Ş., Saraçoğlu, O., Onursal, E. and Özgen, M., 2011. Total phenolic distribution of juice, peel, and seed extracts of four pomegranate cultivars. *Pharmacognosy magazine*, *7*(26), p.161.

Grasso, D., Smets, B.F., Strevett, K.A., Machinist, B.D., Van Oss, C.J., Giese, R.F. and Wu, W., 1996. Impact of physiological state on surface thermodynamics and adhesion of Pseudomonas aeruginosa. *Environmental Science & Technology*, *30*(12), pp.3604-3608.

Graves, D.T. and Cochran, D., 2003. The contribution of interleukin-1 and tumor necrosis factor to periodontal tissue destruction. *Journal of periodontology*, *74*(3), pp.391-401.

Graves, D.T., Fine, D., Teng, Y.T.A., Van Dyke, T.E. and Hajishengallis, G., 2008. The use of rodent models to investigate host–bacteria interactions related to periodontal diseases. *Journal of clinical periodontology*, *35*(2), pp.89-105.

Gray, M.J., Chang, D., Zhang, Y., Liu, J. and Bensoussan, A., 2010. Development of liquid chromatography/mass spectrometry methods for the quantitative analysis of herbal medicine in biological fluids: a review. *Biomedical Chromatography*, *24*(1), pp.91-103.

Graziani, F., Karapetsa, D., Alonso, B. and Herrera, D., 2017. Nonsurgical and surgical treatment of periodontitis: how many options for one disease?. *Periodontology 2000*, *75*(1), pp.152-188.

Greenwald, R.A. and Moy, W.W., 1979. Inhibition of collagen gelation by action of the superoxide radical. *Arthritis & Rheumatism: Official Journal of the American College of Rheumatology*, *22*(3), pp.251-259.

Greiling, D. and Clark, R.A., 1997. Fibronectin provides a conduit for fibroblast transmigration from collagenous stroma into fibrin clot provisional matrix. *Journal of cell science*, *110*(7), pp.861-870.

Gren, M.S. and Mirastschijski, U., 2004. The release of zinc ions from and cytocompatibility of two zinc oxide dressings. *Journal of wound care*, *13*(9), pp.367-369.

Grimaldi, A., Parada, C. and Chai, Y., 2015. A comprehensive study of soft palate development in mice. *PloS one*, *10*(12), p.e0145018.

Gronthos, S., Brahim, J., Li, W., Fisher, L.W., Cherman, N., Boyde, A., DenBesten, P., Robey, P.G. and Shi, S., 2002. Stem cell properties of human dental pulp stem cells. *Journal of dental research*, *81*(8), pp.531-535.

Gross, E.L., Leys, E.J., Gasparovich, S.R., Firestone, N.D., Schwartzbaum, J.A., Janies, D.A., Asnani, K. and Griffen, A.L., 2010. Bacterial 16S sequence analysis of severe caries in young permanent teeth. *Journal of clinical microbiology*, *48*(11), pp.4121-4128.

Gross, M.J. and Logan, B.E., 1995. Influence of different chemical treatments on transport of Alcaligenes paradoxus in porous media. *Applied and environmental microbiology*, *61*(5), pp.1750-1756.

Grossman, E., Reiter, G., Sturzenberger, O.P., De La Rosa, M., Dickinson, T.D., Flrretti, G.A., Ludlam, G.E. and Meckel, A.H., 1986. Six-month study of the effects of a chlorhexidine mouthrinse on gingivitis in adults. *Journal of Periodontal Research*, *21*, pp.33-43.

Grosvenor, P.W., Supriono, A. and Gray, D.O., 1995. Medicinal plants from Riau Province, Sumatra, Indonesia. Part 2: antibacterial and antifungal activity. *Journal of ethnopharmacology*, *45*(2), pp.97-111.

Gruber, R., 2019. Osteoimmunology: Inflammatory osteolysis and regeneration of the alveolar bone. *Journal of Clinical Periodontology*, *46*, pp.52-69.

Guarino, L., Miller, T., Baazara, M., & Obadi, N., 1990. Socotra: the Island of Bliss revisited. *Diversity*, *6*(3-4), 28-31.

Guentsch, A., Kramesberger, M., Sroka, A., Pfister, W., Potempa, J. and Eick, S., 2011. Comparison of gingival crevicular fluid sampling methods in patients with severe chronic periodontitis. *Journal of periodontology*, *82*(7), pp.1051-1060.

Guida, R.A., 1988. Candidiasis of the oropharynx and esophagus. *Ear, nose, & throat journal*, *67*(11), pp.832-840.

Gulube, Z. and Patel, M., 2016. Effect of Punica granatum on the virulence factors of cariogenic bacteria Streptococcus mutans. *Microbial pathogenesis*, *98*, pp.45-49.

Gunsolley, J.C., 2006. A meta-analysis of six-month studies of antiplaque and antigingivitis agents. *The journal of the American dental association*, *137*(12), pp.1649-1657.

Guo, C., Yang, J., Wei, J., Li, Y., Xu, J. and Jiang, Y., 2003. Antioxidant activities of peel, pulp and seed fractions of common fruits as determined by FRAP assay. *Nutrition research*, *23*(12), pp.1719-1726.

Guo, S.A. and DiPietro, L.A., 2010. Factors affecting wound healing. *Journal of dental research*, *89*(3), pp.219-229.

Gurtner, G.C., Werner, S., Barrandon, Y. and Longaker, M.T., 2008. Wound repair and regeneration. *Nature*, *453*(7193), pp.314-321.

Gutkowski, K., Chwist, A. and Hartleb, M., 2011. Liver injury induced by high-dose methylprednisolone therapy: a case report and brief review of the literature. *Hepatitis Monthly*, *11*(8), p.656.

Haase, H. and Rink, L., 2009. Functional significance of zinc-related signaling pathways in immune cells. *Annual review of nutrition*, *29*, pp.133-152.

Haekkinen, L.A.R.I., UITTO, V.J. and Larjava, H., 2000. Cell biology of gingival wound healing. *Periodontology 2000*, *24*(1), pp.127-152.

Haffajee, A.D. and Socransky, S.S., 2009. Relation of body mass index, periodontitis and Tannerella forsythia. *Journal of clinical periodontology*, *36*(2), pp.89-99.

Haffajee, A.D., 1994. Microbial etiological agents of destructive periodontal diseases. *Periodontology 2000*, *5*, pp.78-111.

Hagerman, A.E. and Butler, L.G., 1981. The specificity of proanthocyanidin-protein interactions. *Journal of Biological Chemistry*, *256*(9), pp.4494-4497.

Haidari, M., Ali, M., Casscells III, S.W. and Madjid, M., 2009. Pomegranate (Punica granatum) purified polyphenol extract inhibits influenza virus and has a synergistic effect with oseltamivir. *Phytomedicine*, *16*(12), pp.1127-1136.

Hajishengallis, G. and Lamont, R.J., 2012. Beyond the red complex and into more complexity: the polymicrobial synergy and dysbiosis (PSD) model of periodontal disease etiology. *Molecular oral microbiology*, *27*(6), pp.409-419.

Hajishengallis, G., 2014. Immunomicrobial pathogenesis of periodontitis: keystones, pathobionts, and host response. *Trends in immunology*, *35*(1), pp.3-11.

Hajishengallis, G., Darveau, R.P. and Curtis, M.A., 2012. The keystone-pathogen hypothesis. *Nature Reviews Microbiology*, *10*(10), pp.717-725.

Häkkinen, L., Hildebrand, H.C., Berndt, A., Kosmehl, H. and Larjava, H., 2000. Immunolocalization of tenascin-C, α9 integrin subunit, and αvβ6 integrin during wound healing in human oral mucosa. *Journal of Histochemistry & Cytochemistry*, *48*(7), pp.985-998.

Hall-Stoodley, L. and Stoodley, P., 2009. Evolving concepts in biofilm infections. *Cellular microbiology*, *11*(7), pp.1034-1043.

Hamada, S. and Slade, H.D., 1980. Biology, immunology, and cariogenicity of Streptococcus mutans. *Microbiological reviews*, *44*(2), p.331.

Hamada, S. and Torii, M., 1978. Effect of sucrose in culture media on the location of glucosyltransferase of Streptococcus mutans and cell adherence to glass surfaces. *Infection and Immunity*, *20*(3), pp.592-599.

Hamada, S., Koga, T. and Ooshima, T., 1984. Virulence factors of Streptococcus mutans and dental caries prevention. *Journal of dental research*, *63*(3), pp.407-411.

Hamdan, I.I., 2003. Comparative in vitro investigations of the interaction between some macrolides and Cu (II), Zn (II) and Fe (II). *Die Pharmazie-An International Journal of Pharmaceutical Sciences*, *58*(3), pp.223-224.

Han, B., Fang, W.H., Zhao, S., Yang, Z. and Hoang, B.X., 2020. Zinc sulfide nanoparticles improve skin regeneration. *Nanomedicine: Nanotechnology, Biology and Medicine*, *29*, p.102263.

Hanada, T. and Yoshimura, A., 2002. Regulation of cytokine signaling and inflammation. *Cytokine & growth factor reviews*, *13*(4-5), pp.413-421.

Hancock, J.F., 1992. Plant evolution and the origin of crop species. CABI. *Harlan JR*.

Hannas, A.R., Pereira, J.C., Granjeiro, J.M. and Tjäderhane, L., 2007. The role of matrix metalloproteinases in the oral environment. *Acta Odontologica Scandinavica*, *65*(1), pp.1-13.

Hao, Q. and Maret, W., 2005. Imbalance between pro-oxidant and pro-antioxidant functions of zinc in disease. *Journal of Alzheimer's Disease*, *8*(2), pp.161-170.

Hara, A.T., Lippert, F. and Zero, D.T., 2013. Interplay between experimental dental pellicles and stannous-containing toothpaste on dental erosion-abrasion. *Caries research*, *47*(4), pp.325-329.

Harborne, A.J., 1998. *Phytochemical methods a guide to modern techniques of plant analysis*. springer science & business media.

Harborne, J.B., 2013. The flavonoids: advances in research since 1980.

Harlan, J.R., 1992. *Crops and man* (No. Ed. 2). American Society of Agronomy.

Harrell, J.C. and Stein, S.H., 1995. Prostaglandin E2 regulates gingival mononuclear cell immunoglobulin production. *Journal of periodontology*, *66*(3), pp.222-227.

Hashimoto, M., Ogawa, S., Asai, Y., Takai, Y. and Ogawa, T., 2003. Binding of Porphyromonas gingivalis fimbriae to Treponema denticola dentilisin. *FEMS microbiology letters*, *226*(2), pp.267-271.

Haslam, E., 1996. Natural polyphenols (vegetable tannins) as drugs: possible modes of action. *Journal of natural products*, *59*(2), pp.205-215.

Haslam, E., 1998. *Practical polyphenolics: from structure to molecular recognition and physiological action*. Cambridge University Press.

Hassanpour, H., Yousef, H., Jafar, H. and Mohammad, A., 2011. Antioxidant capacity and phytochemical properties of cornelian cherry (Cornus mas L.) genotypes in Iran. *Scientia Horticulturae*, *129*(3), pp.459-463.

Hassanpour, S., MaheriSis, N. and Eshratkhah, B., 2011. Plants and secondary metabolites (Tannins): A Review.

Hassanpour, S., Sadaghian, M., MaheriSis, N., Eshratkhah, B. and ChaichiSemsari, M., 2011. Effect of condensed tannin on controlling faecal protein excretion in nematode-infected sheep: in vivo study. *Journal of American Science*, *7*(5), pp.896-900.

Hayouni, E.A., Miled, K., Boubaker, S., Bellasfar, Z., Abedrabba, M., Iwaski, H., Oku, H., Matsui, T., Limam, F. and Hamdi, M., 2011. Hydroalcoholic extract based-ointment from Punica granatum L. peels with enhanced in vivo healing potential on dermal wounds. *Phytomedicine*, *18*(11), pp.976-984.

Heber, D., Seeram, N.P., Wyatt, H., Henning, S.M., Zhang, Y., Ogden, L.G., Dreher, M. and Hill, J.O., 2007. Safety and antioxidant activity of a pomegranate ellagitannin-enriched polyphenol dietary supplement in overweight individuals with increased waist size. *Journal of agricultural and food chemistry*, *55*(24), pp.10050-10054.

Heddle, C., Nobbs, A.H., Jakubovics, N.S., Gal, M., Mansell, J.P., Dymock, D. and Jenkinson, H.F., 2003. Host collagen signal induces antigen I/II adhesin and invasin gene expression in oral Streptococcus gordonii. *Molecular microbiology*, *50*(2), pp.597-607.

Helander, I.M., Alakomi, H.L., Latva-Kala, K., Mattila-Sandholm, T., Pol, I., Smid, E.J., Gorris, L.G. and von Wright, A., 1998. Characterization of the action of selected essential oil components on Gram-negative bacteria. *Journal of agricultural and food chemistry*, *46*(9), pp.3590-3595.

Helwany, M. and Rathee, M., 2021. Anatomy, head and neck, palate. *StatPearls [Internet]*.

Henrici, A.T., 1933. Studies of freshwater bacteria: I. A direct microscopic technique. *Journal of bacteriology*, *25*(3), pp.277-287.

Hernández-Corroto, E., Marina, M.L. and García, M.C., 2019. Extraction and identification by high resolution mass spectrometry of bioactive substances in different extracts obtained from pomegranate peel. *Journal of Chromatography A*, *1594*, pp.82-92.

Hernandez, F., Melgarejo, P., Tomas-Barberan, F.A. and Artes, F., 1999. Evolution of juice anthocyanins during ripening of new selected pomegranate (Punica granatum) clones. *European Food Research and Technology*, *210*(1), pp.39-42.

Heydorn, A., Nielsen, A.T., Hentzer, M., Sternberg, C., Givskov, M., Ersbøll, B.K. and Molin, S., 2000. Quantification of biofilm structures by the novel computer program COMSTAT. *Microbiology*, *146*(10), pp.2395-2407.

Hill, M.J. and Marsh, P.D., 1989. *Human Microbial Ecology*. CRC Press.

Hinz, B., Phan, S.H., Thannickal, V.J., Prunotto, M., Desmoulière, A., Varga, J., De Wever, O., Mareel, M. and Gabbiani, G., 2012. Recent developments in myofibroblast biology: paradigms for connective tissue remodeling. *The American journal of pathology*, *180*(4), pp.1340-1355.

Hobby, G.L., Meyer, K. and Chaffee, E., 1942. Observations on the Mechanism of Action of Penicillin. *Proceedings of the Society for Experimental Biology and Medicine*, *50*(2), pp.281-285.

Hochstein, A.O. and Bhatia, A., 2014. Collagen: its role in wound healing. *Wound Manage*, *4*(1), pp.104-109.

Hockenhull, J.C., Dwan, K.M., Smith, G.W., Gamble, C.L., Boland, A., Walley, T.J. and Dickson, R.C., 2009. The clinical effectiveness of central venous catheters treated with anti-infective agents in preventing catheter-related bloodstream infections: a systematic review. *Critical care medicine*, *37*(2), pp.702-712.

Hodnick, W. F., MllosavljeviĆ, E. B., Nelson, J. H., & Pardini, R. S. (1988). Electrochemistry of flavonoids: relationships between redox potentials, inhibition of mitochondrial respiration, and production of oxygen radicals by flavonoids. *Biochemical pharmacology*, *37*(13), 2607-2611.

Hofer, U. (2019). The cost of antimicrobial resistance. *Nature Reviews Microbiology*, *17*(1), 3-3.

Holland, D., & Bar-Ya'akov, I., 2008. The pomegranate: new interest in an ancient fruit. *Chronica Horticulturae*, *48*(3), 12-15.

Holland, D. and Bar-Ya'akov, I., 2014. Pomegranate: aspects concerning dynamics of health beneficial phytochemicals and therapeutic properties with respect to the tree cultivar and the environment. In *Medicinal and aromatic plants of the Middle-East* (pp. 225-239). Springer, Dordrecht.

Holland, D., Hatib, K. and Bar-Ya'akov, I., 2009. 2 Pomegranate: botany, Horticulture, Breeding. *Horticultural reviews*, *35*(2), pp.127-191.

Hollebeeck, S., Winand, J., Hérent, M.F., During, A., Leclercq, J., Larondelle, Y. and Schneider, Y.J., 2012. Anti-inflammatory effects of pomegranate (Punica granatum L.) husk ellagitannins in Caco-2 cells, an in vitro model of human intestine. *Food & function*, *3*(8), pp.875-885.

Holton, T.A. and Cornish, E.C., 1995. Genetics and biochemistry of anthocyanin biosynthesis. *The Plant Cell*, *7*(7), p.1071.

Hooks, T., Niu, G., Masabni, J., Sun, Y. and Ganjegunte, G., 2021. Performance and phytochemical content of 22 pomegranate (Punica granatum) varieties. *HortScience*, *1*(aop), pp.1-9.

Houghton, P.J., Hylands, P.J., Mensah, A.Y., Hensel, A. and Deters, A.M., 2005. In vitro tests and ethnopharmacological investigations: wound healing as an example. *Journal of ethnopharmacology*, *100*(1-2), pp.100-107.

Hourfar, J., Kanavakis, G., Bister, D., Schätzle, M., Awad, L., Nienkemper, M., Goldbecher, C. and Ludwig, B., 2015. Three dimensional anatomical exploration of the anterior hard palate at the level of the third ruga for the placement of mini-implants–a cone-beam CT study. *European journal of orthodontics*, *37*(6), pp.589-595.

Houston, D., 2011. *Towards a nanomedicine-based broad-spectrum topical virucidal therapeutic system* (Doctoral dissertation, Cardiff University).

Houston, D.M., Bugert, J., Denyer, S.P. and Heard, C.M., 2017a. Anti-inflammatory activity of Punica granatum L.(Pomegranate) rind extracts applied topically to ex vivo skin. *European Journal of Pharmaceutics and Biopharmaceutics*, *112*, pp.30-37.

Houston, D.M., Bugert, J.J., Denyer, S.P. and Heard, C.M., 2017b. Potentiated virucidal activity of pomegranate rind extract (PRE) and punicalagin against Herpes simplex virus (HSV) when co-administered with zinc (II) ions, and antiviral activity of PRE against HSV and aciclovir-resistant HSV. *PloS one*, *12*(6), p.e0179291.

Houston, D.M., Robins, B., Bugert, J.J., Denyer, S.P. and Heard, C.M., 2017c. In vitro permeation and biological activity of punicalagin and zinc (II) across skin and mucous membranes prone to Herpes simplex virus infection. *European Journal of Pharmaceutical Sciences*, *96*, pp.99-106.

Howell, A.B. and D'Souza, D.H., 2013. The pomegranate: effects on bacteria and viruses that influence human health. *Evidence-Based Complementary and Alternative Medicine*, *2013*.

Huang, D., Ou, B. and Prior, R.L., 2005. The chemistry behind antioxidant capacity assays. *Journal of agricultural and food chemistry*, *53*(6), pp.1841-1856.

Huang, G.J., Haake, S.K., Kim, J.W. and Park, N.H., 1998. Differential expression of interleukin-8 and intercellular adhesion molecule-1 by human gingival epithelial cells in response to Actinobacillus actinomycetemcomitans or Porphyromonas gingivalis infection. *Oral microbiology and immunology*, *13*(5), pp.301-309.

Huang, T.H.W., Peng, G., Kota, B.P., Li, G.Q., Yamahara, J., Roufogalis, B.D. and Li, Y., 2005. Pomegranate flower improves cardiac lipid metabolism in a diabetic rat model: role of lowering circulating lipids. *British journal of pharmacology*, *145*(6), pp.767-774.

Huang, X., Xie, M., Xie, Y., Mei, F., Lu, X., Li, X. and Chen, L., 2020. The roles of osteocytes in alveolar bone destruction in periodontitis. *Journal of Translational Medicine*, *18*(1), pp.1-15.

Hughes, F.J., 2015. Periodontium and periodontal disease. In *Stem cell biology and tissue engineering in dental sciences* (pp. 433-444). Academic Press.

Hugo, W.B. and Longworth, A.R., 1965. Cytological aspects of the mode of action of chlorhexidine diacetate. *Journal of Pharmacy and Pharmacology*, *17*(1), pp.28-32.

Huja, S.S., Fernandez, S.A., Hill, K.J. and Li, Y., 2006. Remodeling dynamics in the alveolar process in skeletally mature dogs. *The Anatomical Record Part A: Discoveries in Molecular, Cellular, and Evolutionary Biology: An Official Publication of the American Association of Anatomists*, *288*(12), pp.1243-1249.

Hukkanen, A.T., Kokko, H.I., Buchala, A.J., McDougall, G.J., Stewart, D., Kärenlampi, S.O. and Karjalainen, R.O., 2007. Benzothiadiazole induces the accumulation of phenolics and improves resistance to powdery mildew in strawberries. *Journal of agricultural and food chemistry*, *55*(5), pp.1862-1870.

Hwang, B., Hwang, J.S., Lee, J., Kim, J.K., Kim, S.R., Kim, Y. and Lee, D.G., 2011. Induction of yeast apoptosis by an antimicrobial peptide, Papiliocin. *Biochemical and biophysical research communications*, *408*(1), pp.89-93.

Hwang, C.S., Rhie, G.E., Kim, S.T., Kim, Y.R., Huh, W.K., Baek, Y.U. and Kang, S.O., 1999. Copper-and zinc-containing superoxide dismutase and its gene from Candida albicans. *Biochimica et Biophysica Acta (BBA)-General Subjects*, *1427*(2), pp.245-255.

Iglesias-Bartolome, R., Uchiyama, A., Molinolo, A.A., Abusleme, L., Brooks, S.R., Callejas-Valera, J.L., Edwards, D., Doci, C., Asselin-Labat, M.L., Onaitis, M.W. and Moutsopoulos, N.M., 2018. Transcriptional signature primes human oral mucosa for rapid wound healing. *Science translational medicine*, *10*(451).

Ikigai, H., Nakae, T., Hara, Y. and Shimamura, T., 1993. Bactericidal catechins damage the lipid bilayer. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, *1147*(1), pp.132-136.

Ingram, G.S., Horay, C.P. and Stead, W.J., 1992. Interaction of zinc with dental mineral. *Caries research*, *26*(4), pp.248-253.

Ismail, T., Sestili, P. and Akhtar, S., 2012. Pomegranate peel and fruit extracts: a review of potential anti-inflammatory and anti-infective effects. *Journal of ethnopharmacology*, *143*(2), pp.397-405.

Ito, H., Li, P., Koreishi, M., Nagatomo, A., Nishida, N. and Yoshida, T., 2014. Ellagitannin oligomers and a neolignan from pomegranate arils and their inhibitory effects on the formation of advanced glycation end products. *Food chemistry*, *152*, pp.323-330.

Iyer, V.R., Eisen, M.B., Ross, D.T., Schuler, G., Moore, T., Lee, J.C., Trent, J.M., Staudt, L.M., Hudson, J., Boguski, M.S. and Lashkari, D., 1999. The transcriptional program in the response of human fibroblasts to serum. *science*, *283*(5398), pp.83-87.

Izano, E.A., Shah, S.M. and Kaplan, J.B., 2009. Intercellular adhesion and biocide resistance in nontypeable Haemophilus influenzae biofilms. *Microbial pathogenesis*, *46*(4), pp.207-213.

Izano, E.A., Wang, H., Ragunath, C., Ramasubbu, N. and Kaplan, J.B., 2007. Detachment and killing of Aggregatibacter actinomycetemcomitans biofilms by dispersin B and SDS. *Journal of dental research*, *86*(7), pp.618-622.

Jachak, S.M., 2007. PGE synthase inhibitors as an alternative to COX-2 inhibitors. *Current opinion in investigational drugs (London, England: 2000)*, *8*(5), pp.411-415.

Jacqueline, C., Caillon, J., Le Mabecque, V., Miègeville, A.F., Donnio, P.Y., Bugnon, D. and Potel, G., 2003. In vitro activity of linezolid alone and in combination with gentamicin, vancomycin or rifampicin against methicillin-resistant Staphylococcus aureus by time–kill curve methods. *Journal of Antimicrobial Chemotherapy*, *51*(4), pp.857-864.

Jadhav, S., Shah, R., Bhave, M. and Palombo, E.A., 2013. Inhibitory activity of yarrow essential oil on Listeria planktonic cells and biofilms. *Food Control*, *29*(1), pp.125-130.

Jain, S. P., & Puri, H. S., 1984. Ethnomedicinal plants of jaunsar-bawar hills, uttar pradesh, india. *Journal of Ethnopharmacology*, *12*(2), 213-222.

Jakubovics, N.S., Strömberg, N., Van Dolleweerd, C.J., Kelly, C.G. and Jenkinson, H.F., 2005. Differential binding specificities of oral streptococcal antigen I/II family adhesins for human or bacterial ligands. *Molecular microbiology*, *55*(5), pp.1591-1605.

Janecki, A. and Kolodziej, H., 2010. Anti-adhesive activities of flavan-3-ols and proanthocyanidins in the interaction of group A-streptococci and human epithelial cells. *Molecules*, *15*(10), pp.7139-7152.

Järvinen, H., Tenovuo, J. and Huovinen, P.J.A.A., 1993. In vitro susceptibility of Streptococcus mutans to chlorhexidine and six other antimicrobial agents. *Antimicrobial Agents and Chemotherapy*, *37*(5), pp.1158-1159.

Jassim, S A A, G S A B Stewart, and S P Denyer., 1995. Antiviral and antifungal composition and method. World Patent 22254.

Jefferson, K.K., Goldmann, D.A. and Pier, G.B., 2005. Use of confocal microscopy to analyze the rate of vancomycin penetration through Staphylococcus aureus biofilms. *Antimicrobial agents and chemotherapy*, *49*(6), pp.2467-2473.

Jenkinson, H.F. and Lamont, R.J., 1997. Streptococcal adhesion and colonization. *Critical Reviews in Oral Biology & Medicine*, *8*(2), pp.175-200.

Jenkinson, H.F. and Lamont, R.J., 2005. Oral microbial communities in sickness and in health. *Trends in microbiology*, *13*(12), pp.589-595.

Jesse Joel, T., Suluvoy, J.K. and Varghese, J., 2019. Evaluation of the secondary metabolites of the waste pomegranate rind and its cytotoxicity against oral cancer (KB 3-1). *Journal of Pure and Applied Microbiology*, *13*(3), pp.1667-1672.

Jiang, Z.L., Cui, Y.Q., Gao, R., Li, Y., Fu, Z.C., Zhang, B. and Guan, C.C., 2013. Study of TNF-α, IL-1β and LPS levels in the gingival crevicular fluid of a rat model of diabetes mellitus and periodontitis. *Disease markers*, *34*(5), pp.295-304.

John, S., Briatka, T. and Rudolf, E., 2011. Diverse sensitivity of cells representing various stages of colon carcinogenesis to increased extracellular zinc: implications for zinc chemoprevention. *Oncology reports*, *25*(3), pp.769-780.

Jones, P.W. and Williams, D.R., 2004. The use and role of zinc and its compounds in wound healing. *Metal ions in biological systems*, *41*, pp.139-184.

Jurenka, J., 2008. Therapeutic applications of pomegranate (Punica granatum L.): a review. *Alternative medicine review*, *13*(2).

Kakehashi, A., Schepens, C.L., de Sousa-Neto, A., Jalkh, A.E. and Trempe, C.L., 1993. Biomicroscopic findings of posterior vitreoschisis.

Kalinowska, M., Mazur, L., Jabłońska-Trypuć, A. and Lewandowski, W., 2018. A new calcium 2, 5-dihydroxybenzoate: Synthesis, characterization and antioxidant studies and stress mediated cytotoxity in MCF-7 cells. *Journal of Saudi Chemical Society*, *22*(6), pp.742-756.

Kaloriti, D., Jacobsen, M., Yin, Z., Patterson, M., Tillmann, A., Smith, D.A., Cook, E., You, T., Grimm, M.J., Bohovych, I. and Grebogi, C., 2014. Mechanisms underlying the exquisite sensitivity of Candida albicans to combinatorial cationic and oxidative stress that enhances the potent fungicidal activity of phagocytes. *MBio*, *5*(4), pp.e01334-14.

Kampfer, J., Göhring, T.N., Attin, T. and Zehnder, M., 2007. Leakage of food-borne Enterococcus faecalis through temporary fillings in a simulated oral environment. *International endodontic journal*, *40*(6), pp.471-477.

Kanatt, S.R., Chander, R. and Sharma, A., 2010. Antioxidant and antimicrobial activity of pomegranate peel extract improves the shelf life of chicken products. *International journal of food science & technology*, *45*(2), pp.216-222.

Kaneria, M.J., Bapodara, M.B. and Chanda, S.V., 2012. Effect of extraction techniques and solvents on antioxidant activity of pomegranate (Punica granatum L.) leaf and stem. *Food Analytical Methods*, *5*(3), pp.396-404.

Kao, H.K., Chen, B., Murphy, G.F., Li, Q., Orgill, D.P. and Guo, L., 2011. Peripheral blood fibrocytes: enhancement of wound healing by cell proliferation, re-epithelialization, contraction, and angiogenesis. *Annals of surgery*, *254*(6), pp.1066-1074.

Kaplan, J.Á., 2010. Biofilm dispersal: mechanisms, clinical implications, and potential therapeutic uses. *Journal of dental research*, *89*(3), pp.205-218.

Karamanev, D.G., Chavarie, C. and Samson, R., 1998. Soil immobilization: new concept for biotreatment of soil contaminants. *Biotechnology and bioengineering*, *57*(4), pp.471-476.

Kataoka, M., Kido, J.I., Shinohara, Y. and Nagata, T., 2005. Drug-induced gingival overgrowth—a review. *Biological and Pharmaceutical Bulletin*, *28*(10), pp.1817-1821.

Kato, M. T., Lancia, M., Sales-Peres, S. H. C., & Buzalaf, M. A. R. (2010). Preventive effect of commercial desensitizing toothpastes on bovine enamel erosion in vitro. *Caries Research*, *44*(2), 85-89.

Kato, J.Y., Matsuoka, M., Polyak, K., Massague, J. and Sherr, C.J., 1994. Cyclic AMP-induced G1 phase arrest mediated by an inhibitor (p27Kip1) of cyclin-dependent kinase 4 activation. *Cell*, *79*(3), pp.487-496.

Katsikis, P.D., Chu, C.Q., Brennan, F.M., Maini, R.N. and Feldmann, M., 1994. Immunoregulatory role of interleukin 10 in rheumatoid arthritis. *The Journal of experimental medicine*, *179*(5), pp.1517-1527.

Kaufman, M. and Wiesman, Z., 2007. Pomegranate oil analysis with emphasis on MALDI-TOF/MS triacylglycerol fingerprinting. *Journal of agricultural and food chemistry*, *55*(25), pp.10405-10413.

Kelly, C.G. and Younson, J.S., 2000. Anti-adhesive strategies in the prevention of infectious disease at mucosal surfaces. Expert opinion on investigational drugs, 9(8), pp.1711-1721.

Kelly, S.L., Lamb, D.C., Kelly, D.E., Manning, N.J., Loeffler, J., Hebart, H., Schumacher, U. and Einsele, H., 1997. Resistance to fluconazole and cross-resistance to amphotericin B in Candida albicans from AIDS patients caused by defective sterol Δ5, 6-desaturation. *FEBS letters*, *400*(1), pp.80-82.

Kesavalu, L., Holt, S.C. and Ebersole, J.L., 1998. Virulence of a polymicrobic complex, Treponema denticola and Porphyromonas gingivalis, in a murine model. *Oral microbiology and immunology*, *13*(6), pp.373-377.

Khalil, A.A., Khan, M.R. and Shabbir, M.A., 2018. In vitro antioxidant activity and punicalagin content quantification of pomegranate peel obtained as agro-waste after juice extraction. *Pakistan Journal of Agricultural Sciences*, *55*(1).

Khalili, B., Rafieian, M., Hejazi, S.H., Yusefi, H.A., Yektaian, N. and SHIRANI, B.L., 2011. Effect of Achillea millefolium, Artemisia absinthium & Juglans regia leaves extracts on Trichomonas vaginalis, in vitra.

Khan, A.U., Islam, B., Khan, S.N. and Akram, M., 2011. A proteomic approach for exploring biofilm in Streptococcus mutans. *Bioinformation*, *5*(10), p.440.

Khan, J.A. and Hanee, S., 2011. Antibacterial properties of Punica granatum peels. *International Journal of Applied Biology and Pharmaceutical Technology*, *2*(3), pp.23-27.

Khan, N., Syed, D.N., Pal, H.C., Mukhtar, H. and Afaq, F., 2012. Pomegranate fruit extract inhibits UVB-induced inflammation and proliferation by modulating NF-κB and MAPK signaling pathways in mouse skin. *Photochemistry and photobiology*, *88*(5), pp.1126-1134.

Khan, N., Syed, D.N., Pal, H.C., Mukhtar, H. and Afaq, F., 2012. Pomegranate fruit extract inhibits UVB-induced inflammation and proliferation by modulating NF-κB and MAPK signaling pathways in mouse skin. *Photochemistry and photobiology*, *88*(5), pp.1126-1134.

Khan, S.A., 2009. The role of pomegranate (Punica granatum L.) in colon cancer. *Pakistan journal of pharmaceutical sciences*, *22*(3).

Kim, D., Liu, Y., Benhamou, R.I., Sanchez, H., Simón-Soro, Á., Li, Y., Hwang, G., Fridman, M., Andes, D.R. and Koo, H., 2018. Bacterial-derived exopolysaccharides enhance antifungal drug tolerance in a cross-kingdom oral biofilm. *The ISME journal*, *12*(6), pp.1427-1442.

Kinane, D.F. and Lappin, D.F., 2002. Immune processes in periodontal disease: a review. *Annals of Periodontology*, *7*(1), pp.62-71.

Kinane, D.F., Stathopoulou, P.G. and Papapanou, P.N., 2017. Periodontal diseases. *Nature Reviews Disease Primers*, *3*(1), pp.1-14.

Klančnik, A., Šimunović, K., Sterniša, M., Ramić, D., Možina, S.S. and Bucar, F., 2021. Anti-adhesion activity of phytochemicals to prevent Campylobacter jejuni biofilm formation on abiotic surfaces. *Phytochemistry Reviews*, *20*(1), pp.55-84.

Klančnik, A., Vučković, D., Plankl, M., Abram, M. and Smole Možina, S., 2013. In vivo modulation of Campylobacter jejuni virulence in response to environmental stress. *Foodborne pathogens and disease*, *10*(6), pp.566-572.

Klass, B.R., Branford, O.A., Grobbelaar, A.O. and Rolfe, K.J., 2010. The effect of epigallocatechin-3-gallate, a constituent of green tea, on transforming growth factor-β1–stimulated wound contraction. *Wound Repair and Regeneration*, *18*(1), pp.80-88.

Klemm, P., Vejborg, R.M. and Hancock, V., 2010. Prevention of bacterial adhesion. *Applied microbiology and biotechnology*, *88*(2), pp.451-459.

Knoll-Köhler, E. and Stiebel, J., 2002. Amine fluoride gel affects the viability and the generation of superoxide anions in human polymorphonuclear leukocytes: an in vitro study. *European Journal of Oral Sciences*, *110*(4), pp.296-301.

Knutsson, G., Jontell, M. and Bergenholtz, G., 1994. Determination of plasma proteins in dentinal fluid from cavities prepared in healthy young human teeth. *Archives of oral biology*, *39*(3), pp.185-190.

Kobayashi, D., Kondo, K., Uehara, N., Otokozawa, S., Tsuji, N., Yagihashi, A. and Watanabe, N., 2002. Endogenous reactive oxygen species is an important mediator of miconazole antifungal effect. *Antimicrobial Agents and Chemotherapy*, *46*(10), pp.3113-3117.

Kogan, S., Sood, A. and Garnick, M., 2017. Zinc and Wound Healing: A Review of Zinc Physiology and Clinical Applications. *Wounds: a compendium of clinical research and practice*, *29*(4), pp.102-106.

Kohen, R. and Nyska, A., 2002. Invited review: Oxidation of biological systems: oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification. *Toxicologic pathology*, *30*(6), pp.620-650.

Kolaczkowska, E. and Kubes, P., 2013. Neutrophil recruitment and function in health and inflammation. *Nature reviews immunology*, *13*(3), pp.159-175.

Kolenbrander, P.E. and Andersen, R.N., 1986. Multigeneric aggregations among oral bacteria: a network of independent cell-to-cell interactions. *Journal of bacteriology*, *168*(2), pp.851-859.

Kolenbrander, P.E. and Andersen, R.N., 1990a. Characterization of Streptococcus gordonii (S. sanguis) PK488 adhesin-mediated coaggregation with Actinomyces naeslundii PK606. *Infection and immunity*, *58*(9), pp.3064-3072.

Kolenbrander, P.E. and London, J., 1993. Adhere today, here tomorrow: oral bacterial adherence. *Journal of bacteriology*, *175*(11), pp.3247-3252.

Kolenbrander, P.E., 1988. Intergeneric coaggregation among human oral bacteria and ecology of dental plaque. *Annual Reviews in Microbiology*, *42*(1), pp.627-656.

Kolenbrander, P.E., Andersen, R.N. and Holdeman, L.V., 1985. Coaggregation of oral Bacteroides species with other bacteria: central role in coaggregation bridges and competitions. *Infection and Immunity*, *48*(3), pp.741-746.

Kolenbrander, P.E., Andersen, R.N. and Moore, L.V., 1989. Coaggregation of Fusobacterium nucleatum, Selenomonas flueggei, Selenomonas infelix, Selenomonas noxia, and Selenomonas sputigena with strains from 11 genera of oral bacteria. *Infection and immunity*, *57*(10), pp.3194-3203.

Kolenbrander, P.E., Andersen, R.N. and Moore, L.V., 1990b. Intrageneric coaggregation among strains of human oral bacteria: potential role in primary colonization of the tooth surface. *Applied and environmental microbiology*, *56*(12), pp.3890-3894.

Kolter, R., Siegele, D.A. and Tormo, A., 1993. The stationary phase of the bacterial life cycle. *Annual review of microbiology*, *47*(1), pp.855-874.

Kong, W., Jin, C., Xiao, X., Zhao, Y., Liu, W., Li, Z. and Zhang, P., 2010. Determination of multicomponent contents in Calculus bovis by ultra-performance liquid chromatography–evaporative light scattering detection and its application for quality control. *Journal of separation science*, *33*(10), pp.1518-1527.

Koo, H. and Bowen, W.H., 2014. Candida albicans and Streptococcus mutans: a potential synergistic alliance to cause virulent tooth decay in children. *Future microbiology*, *9*(12), pp.1295-1297.

Korzeniewski, C. and Callewaert, D.M., 1983. An enzyme-release assay for natural cytotoxicity. *Journal of immunological methods*, *64*(3), pp.313-320.

Koychev, S., Dommisch, H., Chen, H. and Pischon, N., 2017. Antimicrobial effects of mastic extract against oral and periodontal pathogens. *Journal of periodontology*, *88*(5), pp.511-517.

Kraan, H., Vrieling, H., Czerkinsky, C., Jiskoot, W., Kersten, G. and Amorij, J.P., 2014. Buccal and sublingual vaccine delivery. *Journal of controlled release*, *190*, pp.580-592.

Krachler, A.M. and Orth, K., 2013. Targeting the bacteria–host interface: strategies in anti-adhesion therapy. *Virulence*, *4*(4), pp.284-294.

Kraszni, M., Marosi, A. and Larive, C.K., 2013. NMR assignments and the acid–base characterization of the pomegranate ellagitannin punicalagin in the acidic pH-range. *Analytical and bioanalytical chemistry*, *405*(17), pp.5807-5816.

Krzyściak, W., Jurczak, A., Kościelniak, D., Bystrowska, B., & Skalniak, A. (2014). The virulence of Streptococcus mutans and the ability to form biofilms. *European Journal of Clinical Microbiology & Infectious Diseases*, *33*(4), 499-515.

Kroeze, K.L., Boink, M.A., Sampat-Sardjoepersad, S.C., Waaijman, T., Scheper, R.J. and Gibbs, S., 2012. Autocrine regulation of re-epithelialization after wounding by chemokine receptors CCR1, CCR10, CXCR1, CXCR2, and CXCR3. *Journal of Investigative Dermatology*, *132*(1), pp.216-225.

Krumova, K. and Cosa, G., 2016. Overview of reactive oxygen species.

Kuboniwa, M., Tribble, G.D., James, C.E., Kilic, A.O., Tao, L., Herzberg, M.C., Shizukuishi, S. and Lamont, R.J., 2006. Streptococcus gordonii utilizes several distinct gene functions to recruit Porphyromonas gingivalis into a mixed community. *Molecular microbiology*, *60*(1), pp.121-139.

Kulkarni, S.S., Gokhale, A.V., Bodake, U.M. and Pathade, G.R., 2011. Cotton Dyeing with Natural Dye Extracted from Pomegranate (Punica granatum) Peel. *Universal Journal of Environmental Research & Technology*, *1*(2).

Kullberg, B.J. and AM, O.L., 2002. Epidemiology of opportunistic invasive mycoses. *European journal of medical research*, *7*(5), pp.183-191.

Kurgan, S. and Kantarci, A., 2018. Molecular basis for immunohistochemical and inflammatory changes during progression of gingivitis to periodontitis. *Periodontology 2000*, *76*(1), pp.51-67.

La Rue, J. H., 1980. Growing pomegranates in California, Univ. *Calif. Div. Ag. Sci. leaflet*, 24-59.

Labrecque, J., Bodet, C., Chandad, F. and Grenier, D., 2006. Effects of a high-molecular-weight cranberry fraction on growth, biofilm formation and adherence of Porphyromonas gingivalis. *Journal of Antimicrobial Chemotherapy*, *58*(2), pp.439-443.

Lacruz, R.S., Habelitz, S., Wright, J.T. and Paine, M.L., 2017. Dental enamel formation and implications for oral health and disease. *Physiological Reviews*, *97*(3), pp.939-993.

LaFleur, M.D., Kumamoto, C.A. and Lewis, K., 2006. Candida albicans biofilms produce antifungal-tolerant persister cells. *Antimicrobial agents and chemotherapy*, *50*(11), pp.3839-3846.

Lamarre, C., LeMay, J.D., Deslauriers, N. and Bourbonnais, Y., 2001. Candida albicans expresses an unusual cytoplasmic manganese-containing superoxide dismutase (SOD3 gene product) upon the entry and during the stationary phase. *Journal of Biological Chemistry*, *276*(47), pp.43784-43791.

Lamme, E.N., Van Leeuwen, R.T., Brandsma, K., Van Marle, J. and Middelkoop, E., 2000. Higher numbers of autologous fibroblasts in an artificial dermal substitute improve tissue regeneration and modulate scar tissue formation. *The Journal of Pathology: A Journal of the Pathological Society of Great Britain and Ireland*, *190*(5), pp.595-603.

Lamont, R.J. and Rosan, B., 1990. Adherence of mutans streptococci to other oral bacteria. *Infection and immunity*, *58*(6), pp.1738-1743.

Lamont, R.J., El-Sabaeny, A., Park, Y., Cook, G.S., Costerton, J.W. and Demuth, D.R., 2002. Role of the Streptococcus gordonii SspB protein in the development of Porphyromonas gingivalis biofilms on streptococcal substrates. *Microbiology*, *148*(6), pp.1627-1636.

Lamont, R.J., Gil, S., Demuth, D.R., Malamud, D. and Rosan, B., 1994. Molecules of Streptococcus gordonii that bind to Porphyromonas gingivalis. *Microbiology*, *140*(4), pp.867-872.

Lamont, R.J., Hersey, S.G. and Rosan, B., 1992. Characterization of the adherence of Porphyromonas gingivalis to oral streptococci. *Oral microbiology and immunology*, *7*(4), pp.193-197.

Lamont, R.J., Rosan, B.U.R.T.O.N., Murphy, G.M. and Baker, C.T., 1988. Streptococcus sanguis surface antigens and their interactions with saliva. *Infection and immunity*, *56*(1), pp.64-70.

Lane, J.A., Mehra, R.K., Carrington, S.D. and Hickey, R.M., 2010. The food glycome: a source of protection against pathogen colonization in the gastrointestinal tract. *International journal of food microbiology*, *142*(1-2), pp.1-13.

Lang, N.P., Hotz, P., Graf, H., Geering, A.H., Saxer, U.P., Sturzenberger, O.P. and Meckel, A.H., 1982. Effects of supervised chlorhexidine mouthrinses in children: a longitudinal clinical trial. *Journal of periodontal research*, *17*(1), pp.101-111.

Lang, N.P., Schätzle, M.A. and Löe, H., 2009. Gingivitis as a risk factor in periodontal disease. *Journal of clinical periodontology*, *36*, pp.3-8.

Langille, S.E., Geesey, G.G. and Weiner, R.M., 2000. Polysaccharide-specific probes inhibit adhesion of Hyphomonas rosenbergii strain VP-6 to hydrophilic surfaces. *Journal of Industrial Microbiology and Biotechnology*, *25*(2), pp.81-85.

Lansdown, A.B., Mirastschijski, U., Stubbs, N., Scanlon, E. and Ågren, M.S., 2007. Zinc in wound healing: theoretical, experimental, and clinical aspects. *Wound repair and regeneration*, *15*(1), pp.2-16.

Lansdown, A.B.G., Sampson, B. and Rowe, A., 1999. Sequential changes in trace metal, metallothionein and calmodulin concentrations in healing skin wounds. *The Journal of Anatomy*, *195*(3), pp.375-386.

Lansky, E.P. and Newman, R.A., 2007. Punica granatum (pomegranate) and its potential for prevention and treatment of inflammation and cancer. *Journal of ethnopharmacology*, *109*(2), pp.177-206.

Lantzouraki, D.Z., Sinanoglou, V.J., Tsiaka, T., Proestos, C. and Zoumpoulakis, P., 2015. Total phenolic content, antioxidant capacity and phytochemical profiling of grape and pomegranate wines. *RSC advances*, *5*(123), pp.101683-101692.

Larjava, H., Koivisto, L., Häkkinen, L. and Heino, J., 2011. Epithelial integrins with special reference to oral epithelia. *Journal of dental research*, *90*(12), pp.1367-1376.

Lau, A., Sorrell, T.C., Chen, S., Stanley, K., Iredell, J. and Halliday, C., 2008. Multiplex tandem PCR: a novel platform for rapid detection and identification of fungal pathogens from blood culture specimens. *Journal of Clinical Microbiology*, *46*(9), pp.3021-3027.

Lawrence, T., 2009. The nuclear factor NF-κB pathway in inflammation. *Cold Spring Harbor perspectives in biology*, *1*(6), p.a001651.

Lawrence, W.J.C., 1950. 1. Genetic control of biochemical synthesis as exemplified by plant" genetics-flower colours. In *Biochemical Society Symposia No. 4. Biochemical aspects of genetics.* (pp. 3-9). Cambridge University Press.

Lee, H.G. and Eun, H.C., 1999. Differences between fibroblasts cultured from oral mucosa and normal skin: implication to wound healing. *Journal of dermatological science*, *21*(3), pp.176-182.

Lee, J. and Lee, D.G., 2015. Novel antifungal mechanism of resveratrol: apoptosis inducer in Candida albicans. *Current microbiology*, *70*(3), pp.383-389.

Lee, S.R., 2018. Critical role of zinc as either an antioxidant or a prooxidant in cellular systems. *Oxidative medicine and cellular longevity*, *2018*.

Lei, Y., Tang, Z., Liao, R. and Guo, B., 2011. Hydrolysable tannin as environmentally friendly reducer and stabilizer for graphene oxide. *Green chemistry*, *13*(7), pp.1655-1658.

Leid, J.G., Shirtliff, M.E., Costerton, J.W. and Stoodley, A.P., 2002. Human leukocytes adhere to, penetrate, and respond to Staphylococcus aureus biofilms. *Infection and immunity*, *70*(11), pp.6339-6345.

Leverett, D.H., Adair, S.M., Vaughan, B.W., Proskin, H.M. and Moss, M.E., 1997. Randomized clinical trial of the effect of prenatal fluoride supplements in preventing dental caries. *Caries research*, *31*(3), pp.174-179.

Levin, B.R. and Rozen, D.E., 2006. Non-inherited antibiotic resistance. *Nature Reviews Microbiology*, *4*(7), pp.556-562.

Levin, C. and Maibach, H.I., 2002. Topical corticosteroid-induced adrenocortical insufficiency. *American journal of clinical dermatology*, *3*(3), pp.141-147.

Levin, G.M., 1994. Pomegranate (Punica granatum) plant genetic resources in Turkmenistan. *Bulletin des Ressources Phytogenetiques (IPGRI/FAO); Noticiario de Recursos Fitogeneticos (IPGRI/FAO)*.

Levin, G.M., 2006. *Pomegranate* (pp. 1-130). Tempe: Third Millennium Publishing.

Lewis, I.A., Schommer, S.C., Hodis, B., Robb, K.A., Tonelli, M., Westler, W.M., Sussman, M.R. and Markley, J.L., 2007. Method for determining molar concentrations of metabolites in complex solutions from two-dimensional 1H− 13C NMR spectra. *Analytical chemistry*, *79*(24), pp.9385-9390.

Lewis, K., 2001. Riddle of biofilm resistance. *Antimicrobial agents and chemotherapy*, *45*(4), pp.999-1007.

Lewis, K., 2005. Persister cells and the riddle of biofilm survival. *Biochemistry (Moscow)*, *70*(2), pp.267-274.

Lewis, K., 2010. Persister cells. *Annual review of microbiology*, *64*, pp.357-372.

Lewis, R.A., 1990. Interactions of eicosanoids and cytokines in immune regulation. *Advances in prostaglandin, thromboxane, and leukotriene research*, *20*, pp.170-178.

Li, T., Khah, M.K., Slavnic, S., Johansson, I. and Strömberg, N., 2001. Different type 1 fimbrial genes and tropisms of commensal and potentially pathogenic Actinomyces spp. with different salivary acidic proline-rich protein and statherin ligand specificities. *Infection and immunity*, *69*(12), pp.7224-7233.

Li, X., Wasila, H., Liu, L., Yuan, T., Gao, Z., Zhao, B. and Ahmad, I., 2015. Physicochemical characteristics, polyphenol compositions and antioxidant potential of pomegranate juices from 10 Chinese cultivars and the environmental factors analysis. *Food chemistry*, *175*, pp.575-584.

Li, Y., Guo, C., Yang, J., Wei, J., Xu, J. and Cheng, S., 2006. Evaluation of antioxidant properties of pomegranate peel extract in comparison with pomegranate pulp extract. *Food chemistry*, *96*(2), pp.254-260.

Li, Y., Ling, J. and Jiang, Q., 2021. Inflammasomes in alveolar bone loss. *Frontiers in Immunology*, *12*.

Li, Y., Wang, H., Si, N., Ren, W., Han, L., Xin, S., Zuo, R., Wei, X., Yang, J., Zhao, H. and Bian, B., 2015. Metabolic profiling analysis of berberine, palmatine, jatrorrhizine, coptisine and epiberberine in zebrafish by ultra-high performance liquid chromatography coupled with LTQ Orbitrap mass spectrometer. *Xenobiotica*, *45*(4), pp.302-311.

Li, Z. and Gu, L., 2011. Effects of mass ratio, pH, temperature, and reaction time on fabrication of partially purified pomegranate ellagitannin− gelatin nanoparticles. *Journal of agricultural and food chemistry*, *59*(8), pp.4225-4231.

Li, Z., Percival, S.S., Bonard, S. and Gu, L., 2011. Fabrication of nanoparticles using partially purified pomegranate ellagitannins and gelatin and their apoptotic effects. *Molecular nutrition & food research*, *55*(7), pp.1096-1103.

Liang, C.C., Park, A.Y. and Guan, J.L., 2007. In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. *Nature protocols*, *2*(2), pp.329-333.

Liang, C.C., Park, A.Y. and Guan, J.L., 2007. In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. *Nature protocols*, *2*(2), pp.329-333.

Liang, Y.Z., Xie, P. and Chan, K., 2004. Quality control of herbal medicines. *Journal of chromatography B*, *812*(1-2), pp.53-70.
Libermann, T.A. and Zerbini, L.F., 2006. Targeting transcription factors for cancer gene therapy. *Current gene therapy*, *6*(1), pp.17-33.

Liljemark, W.F., Bloomquist, C.G., Reilly, B.E., Bernards, C.J., Townsend, D.W., Pennock, A.T. and LeMoine, J.L., 1997. Growth dynamics in a natural biofilm and its impact on oral disease management. *Advances in dental research*, *11*(1), pp.14-23.

Lin, L.T., Chen, T.Y., Chung, C.Y., Noyce, R.S., Grindley, T.B., McCormick, C., Lin, T.C., Wang, G.H., Lin, C.C. and Richardson, C.D., 2011. Hydrolyzable tannins (chebulagic acid and punicalagin) target viral glycoprotein-glycosaminoglycan interactions to inhibit herpes simplex virus 1 entry and cell-to-cell spread. *Journal of virology*, *85*(9), pp.4386-4398.

Lin, P.H., Sermersheim, M., Li, H., Lee, P.H., Steinberg, S.M. and Ma, J., 2018. Zinc in wound healing modulation. *Nutrients*, *10*(1), p.16.

Liu, S., Yi, L.Z. and Liang, Y.Z., 2008. Traditional Chinese medicine and separation science. *Journal of Separation Science*, *31*(11), pp.2113-2137.

Liu, Y. and Seeram, N.P., 2018. Liquid chromatography coupled with time-of-flight tandem mass spectrometry for comprehensive phenolic characterization of pomegranate fruit and flower extracts used as ingredients in botanical dietary supplements. *Journal of separation science*, *41*(15), pp.3022-3033.

Liu, Y., Zhang, Y., Wang, L., Guo, Y. and Xiao, S., 2013. Prevalence of Porphyromonas gingivalis four rag locus genotypes in patients of orthodontic gingivitis and periodontitis. *PloS one*, *8*(4), p.e61028.

Löe, H., Theilade, E. and Jensen, S.B., 1965. Experimental gingivitis in man. *The Journal of periodontology*, *36*(3), pp.177-187.

Loesche, W., 2007. Dental caries and periodontitis: contrasting two infections that have medical implications. *Infectious disease clinics of North America*, *21*(2), pp.471-502.

Loesche, W.J. and Nafe, D., 1973. Reduction of supragingival plaque accumulations in institutionalized Down's syndrome patients by periodic treatment with topical kanamycin. *Archives of oral biology*, *18*(9), pp.1131-1143.

LOESCHE, W.J., 1976. Chemotherapy of dental plaque infections. *Oral Sci Rev*, *9*, pp.65-107.

Loesche, W.J., 1986. Role of Streptococcus mutans in human dental decay. *Microbiological reviews*, *50*(4), pp.353-380.

Loesche, W.J., Bradbury, D.R. and Woolfolk, M.P., 1977. Reduction of dental decay in rampant caries individuals following short-term kanamycin treatment. *Journal of dental research*, *56*(3), pp.254-265.

Loimaranta, V., Jakubovics, N.S., Hytonen, J., Finne, J., Jenkinson, H.F. and Stromberg, N., 2005. Fluid- or surface-phase human salivary scavenger protein gp340 exposes different bacterial recognition properties. *Infection and immunity*, *73*(4), pp.2245-2252.

Lokmic, Z., Musyoka, J., Hewitson, T.D. and Darby, I.A., 2012. Hypoxia and hypoxia signaling in tissue repair and fibrosis. *International review of cell and molecular biology*, *296*, pp.139-185.

Lomovskaya, O. and Watkins, W., 2001. Inhibition of efflux pumps as a novel approach to combat drug resistance in bacteria. *Journal of molecular microbiology and biotechnology*, *3*(2), pp.225-236.

Löning, T.H., Albers, H.K., Lisboa, B.P., Burkhardt, A. and Caselitz, J., 1980. Prostaglandin E and the local immune response in chronic periodontal disease: immunohistochemical and radioimmunological observations. *Journal of periodontal research*, *15*(5), pp.525-535.

Loos, B.G., 2005. Systemic markers of inflammation in periodontitis. *Journal of periodontology*, *76*, pp.2106-2115.

Lorimier, S., Bouthors, S., Droulle, C., Maquin, D.L., Maquart, F.X., Gillery, P., Emonard, H. and Hornebeck, W., 1997. The rate of fibrinolysis is increased by free retraction of human gingival fibroblast populated fibrin lattices. *The international journal of biochemistry & cell biology*, *29*(1), pp.181-189.

Lorimier, S., Gillery, P., Hornebeck, W., Chastang, F., Laurent-Maquin, D., Bouthors, S., Droulle, C., Potron, G. and Maquart, F.X., 1996. Tissue origin and extracellular matrix control neutral proteinase activity in human fibroblast three-dimensional cultures. *Journal of cellular physiology*, *168*(1), pp.188-198.

Lorimier, S., Hornebeck, W., Godeau, G., Pellat, B., Gillery, P., Maquart, E.X. and Laurent-Maquin, D., 1998. Morphometric studies of collagen and fibrin lattices contracted by human gingival fibroblasts; comparison with dermal fibroblasts. *Journal of dental research*, *77*(9), pp.1717-1729.

Love, R.M. and Jenkinson, H.F., 2002. Invasion of dentinal tubules by oral bacteria. *Critical reviews in oral biology & medicine*, *13*(2), pp.171-183.

Love, R.M., McMillan, M.D., Park, Y. and Jenkinson, H.F., 2000. Coinvasion of dentinal tubules by Porphyromonas gingivalis and Streptococcus gordonii depends upon binding specificity of streptococcal antigen I/II adhesin. *Infection and immunity*, *68*(3), pp.1359-1365.

Lu, J., Ding, K. and Yuan, Q., 2008. Determination of punicalagin isomers in pomegranate husk. *Chromatographia*, *68*(3), pp.303-306.

Lu, Q., Haragopal, H., Slepchenko, K.G., Stork, C. and Li, Y.V., 2016. Intracellular zinc distribution in mitochondria, ER and the Golgi apparatus. *International journal of physiology, pathophysiology and pharmacology*, *8*(1), p.35.

Lu, Y., Su, C. and Liu, H., 2014. Candida albicans hyphal initiation and elongation. *Trends in microbiology*, *22*(12), pp.707-714.

Lucci, P., Saurina, J. and Núñez, O., 2017. Trends in LC-MS and LC-HRMS analysis and characterization of polyphenols in food. *TrAC Trends in Analytical Chemistry*, *88*, pp.1-24.

Lukiswanto, B.S., Miranti, A., Sudjarwo, S.A., Primarizky, H. and Yuniarti, W.M., 2019. Evaluation of wound healing potential of pomegranate (Punica granatum) whole fruit extract on skin burn wound in rats (Rattus norvegicus). *Journal of advanced veterinary and animal research*, *6*(2), p.202.

Lundqvist, C., Baranov, V., Teglund, S., Hammarström, S. and Hammarström, M.L., 1994. Cytokine profile and ultrastructure of intraepithelial gamma delta T cells in chronically inflamed human gingiva suggest a cytotoxic effector function. *The Journal of Immunology*, *153*(5), pp.2302-2312.

Ma, G.Z., Wang, C.M., Li, L., Ding, N. and Gao, X.L., 2015. Effect of pomegranate peel polyphenols on human prostate cancer PC-3 cells in vivo. *Food science and biotechnology*, *24*(5), pp.1887-1892.

Ma, Y., Chen, M., Jones, J.E., Ritts, A.C., Yu, Q. and Sun, H., 2012. Inhibition of Staphylococcus epidermidis biofilm by trimethylsilane plasma coating. *Antimicrobial agents and chemotherapy*, *56*(11), pp.5923-5937.

Machado, T.B., Pinto, A.V., Pinto, M.C.F.R., Leal, I.C.R., Silva, M.G., Amaral, A.C.F., Kuster, R.M. and Netto-dosSantos, K.R., 2003. In vitro activity of Brazilian medicinal plants, naturally occurring naphthoquinones and their analogues, against methicillin-resistant Staphylococcus aureus. *International journal of antimicrobial agents*, *21*(3), pp.279-284.

Madureira, D.F., Lima, I.L.D.A., Costa, G.C., Lages, E.M.B., Martins, C.C. and Da Silva, T.A., 2018. Tumor necrosis factor-alpha in gingival crevicular fluid as a diagnostic marker for periodontal diseases: a systematic review. *Journal of Evidence Based Dental Practice*, *18*(4), pp.315-331.

Maeda, K., Nagata, H., Nonaka, A., Kataoka, K., Tanaka, M. and Shizukuishi, S., 2004. Oral streptococcal glyceraldehyde-3-phosphate dehydrogenase mediates interaction with Porphyromonas gingivalis fimbriae. *Microbes and infection*, *6*(13), pp.1163-1170.

Maeda, K., Nagata, H., Yamamoto, Y., Tanaka, M., Tanaka, J., Minamino, N. and Shizukuishi, S., 2004. Glyceraldehyde-3-phosphate dehydrogenase of Streptococcus oralis functions as a coadhesin for Porphyromonas gingivalis major fimbriae. *Infection and Immunity*, *72*(3), pp.1341-1348.

Maeda, K., Tribble, G.D., Tucker, C.M., Anaya, C., Shizukuishi, S., Lewis, J.P., Demuth, D.R. and Lamont, R.J., 2008. A Porphyromonas gingivalis tyrosine phosphatase is a multifunctional regulator of virulence attributes. *Molecular microbiology*, *69*(5), pp.1153-1164.

Mah, T.F., 2012. Biofilm-specific antibiotic resistance. *Future microbiology*, *7*(9), pp.1061-1072.

Mah, T.F.C. and O'Toole, G.A., 2001. Mechanisms of biofilm resistance to antimicrobial agents. *Trends in microbiology*, *9*(1), pp.34-39.

Mahady, G.B., 2005. Medicinal plants for the prevention and treatment of bacterial infections. *Current pharmaceutical design*, *11*(19), pp.2405-2427.

Mahdihassan, S., 1984. Outline of the beginnings of alchemy and its antecedents. *The American journal of Chinese medicine*, *12*(01n04), 32-42.

Mak, K., Manji, A., Gallant-Behm, C., Wiebe, C., Hart, D.A., Larjava, H. and Häkkinen, L., 2009. Scarless healing of oral mucosa is characterized by faster resolution of inflammation and control of myofibroblast action compared to skin wounds in the red Duroc pig model. *Journal of dermatological science*, *56*(3), pp.168-180.

Malviya, S., Jha, A. and Hettiarachchy, N., 2014. Antioxidant and antibacterial potential of pomegranate peel extracts. *Journal of food science and technology*, *51*(12), pp.4132-4137.

Mandel, I.D., 1987. The functions of saliva. *Journal of dental research*, *66*(1_suppl), pp.623-627.

Mansour, E., Khaled, A.B., Lachiheb, B., Abid, M., Bachar, K. and Ferchichi, A., 2018. Phenolic Compounds, Antioxidant, and Antibacterial Activities of Peel Extract from Tunisian Pomegranat e.

Maresso, A.W. and Schneewind, O., 2008. Sortase as a target of anti-infective therapy. *Pharmacological reviews*, *60*(1), pp.128-141.

Margadant, C., Charafeddine, R.A. and Sonnenberg, A., 2010. Unique and redundant functions of integrins in the epidermis. *The FASEB Journal*, *24*(11), pp.4133-4152.

Mariani, E., Mangialasche, F., Feliziani, F.T., Cecchetti, R., Malavolta, M., Bastiani, P., Baglioni, M., Dedoussis, G., Fulop, T., Herbein, G. and Jajte, J., 2008. Effects of zinc supplementation on antioxidant enzyme activities in healthy old subjects. *Experimental gerontology*, *43*(5), pp.445-451.

Mariotti, A., 1999. Dental plaque-induced gingival diseases. *Annals of periodontology*, *4*(1), pp.7-17.
Marsh, P. and Martin, M., 1992. Acquisition, adherence, distribution and functions of the oral microflora. In *Oral microbiology* (pp. 56-97). Springer, Boston, MA.

Marsh, P.D., 1994. Microbial ecology of dental plaque and its significance in health and disease. *Advances in dental research*, *8*(2), pp.263-271.

Marsh, P.D., 2003. Are dental diseases examples of ecological catastrophes?. *Microbiology*, *149*(2), pp.279-294.

Marsh, P.D., 2010. Controlling the oral biofilm with antimicrobials. *Journal of dentistry*, *38*, pp.S11-S15.

Marsh, P.D., Lewis, M.A., Williams, D. and Martin, M.V., 2009. *Oral microbiology e-book*. Elsevier health sciences.

Marshall Jr, G.W., Marshall, S.J., Kinney, J.H. and Balooch, M., 1997. The dentin substrate: structure and

Marshall, K.C., 1992. Biofilms: an overview of bacterial adhesion, activity, and control at surfaces. *Am. Soc. Microbiol. News.*, *58*, pp.202-207.

Marsh, P. D., McDermid, A. S., McKee, A. S., & Baskerville, A. (1994). The effect of growth rate and haemin on the virulence and proteolytic activity of Porphyromonas gingivalis W50. *Microbiology*, *140*(4), 861-865.

Marshall, K.C., STOUT, R. and Mitchell, R., 1971. Mechanism of the initial events in the sorption of marine bacteria to surfaces. *Microbiology*, *68*(3), pp.337-348.

Marston, H. D., Dixon, D. M., Knisely, J. M., Palmore, T. N., & Fauci, A. S. (2016). Antimicrobial resistance. *Jama*, *316*(11), 1193-1204.

Martinotti, S. and Ranzato, E., 2019. Scratch wound healing assay. In *Epidermal cells* (pp. 225-229). Humana, New York, NY.

Marx, R.E., Cillo Jr, J.E. and Ulloa, J.J., 2007. Oral bisphosphonate-induced osteonecrosis: risk factors, prediction of risk using serum CTX testing, prevention, and treatment. *Journal of Oral and Maxillofacial Surgery*, *65*(12), pp.2397-2410.

Masár, M., Hradski, J., Schmid, M.G. and Szucs, R., 2020. Advantages and Pitfalls of Capillary Electrophoresis of Pharmaceutical Compounds and Their Enantiomers in Complex Samples: Comparison of Hydrodynamically Opened and Closed Systems. *International Journal of Molecular Sciences*, *21*(18), p.6852.

Mason, M.R., Nagaraja, H.N., Camerlengo, T., Joshi, V. and Kumar, P.S., 2013. Deep sequencing identifies ethnicity-specific bacterial signatures in the oral microbiome. *PloS one*, *8*(10), p.e77287.

Mastrogiovanni, F., Mukhopadhya, A., Lacetera, N., Ryan, M.T., Romani, A., Bernini, R. and Sweeney, T., 2019. Anti-inflammatory effects of pomegranate peel extracts on in vitro human intestinal caco-2 cells and ex vivo porcine colonic tissue explants. *Nutrients*, *11*(3), p.548.

Mathabe, M.C., Nikolova, R.V., Lall, N. and Nyazema, N.Z., 2006. Antibacterial activities of medicinal plants used for the treatment of diarrhoea in Limpopo Province, South Africa. *Journal of ethnopharmacology*, *105*(1-2), pp.286-293.

Mattos-Graner, R.O., Smith, D.J., King, W.F. and Mayer, M.P.A., 2000. Water-insoluble glucan synthesis by mutans streptococcal strains correlates with caries incidence in 12-to 30-month-old children. *Journal of dental research*, *79*(6), pp.1371-1377.

Mattos-Guaraldi, A.L., Formiga, L.C.D. and Pereira, G.A., 2000. Cell surface components and adhesionin Corynebacterium diphtheriae. *Microbes and infection*, *2*(12), pp.1507-1512.

Mayer, F.L., Wilson, D. and Hube, B., 2013. Candida albicans pathogenicity mechanisms. *Virulence*, *4*(2), pp.119-128.

Mayyas, A., Abu-Sini, M., Amr, R., Akasheh, R.T., Zalloum, W., Khdair, A., Hamad, I., Aburjai, T., Darwish, R.M. and Abu-Qatouseh, L., 2021. Novel in vitro and in vivo anti-Helicobacter pylori effects of pomegranate peel ethanol extract. *Veterinary World*, *14*(1), p.120.

McBain, A.J., Bartolo, R.G., Catrenich, C.E., Charbonneau, D., Ledder, R.G. and Gilbert, P., 2003. Effects of triclosan-containing rinse on the dynamics and antimicrobial susceptibility of in vitro plaque ecosystems. *Antimicrobial agents and chemotherapy*, *47*(11), pp.3531-3538.

McCarrell, E.M., Gould, S.W., Fielder, M.D., Kelly, A.F., El Sankary, W. and Naughton, D.P., 2008. Antimicrobial activities of pomegranate rind extracts: enhancement by addition of metal salts and vitamin C. *BMC Complementary and Alternative Medicine*, *8*(1), pp.1-7.

McConnell, S.A., Gubbins, P.O. and Anaissie, E.J., 2003. Do antimicrobial-impregnated central venous catheters prevent catheter-related bloodstream infection?. *Clinical infectious diseases*, *37*(1), pp.65-72.

McEldowney, S. and Fletcher, M., 1986. Variability of the influence of physicochemical factors affecting bacterial adhesion to polystyrene substrata. *Applied and Environmental Microbiology*, *52*(3), pp.460-465.

McKeown, S.T.W., Barnes, J.J., Hyland, P.L., Lundy, F.T., Fray, M.J. and Irwin, C.R., 2007. Matrix metalloproteinase-3 differences in oral and skin fibroblasts. *Journal of dental research*, *86*(5), pp.457-462.

McMurry, L.M., Oethinger, M. and Levy, S.B., 1998. Triclosan targets lipid synthesis. *Nature*, *394*(6693), pp.531-532.

McNeill, K. and Hamilton, I.R., 2003. Acid tolerance response of biofilm cells of Streptococcus mutans. *FEMS microbiology letters*, *221*(1), pp.25-30.

Meade, E.A., Smith, W.L. and Dewitt, D.L., 1993. Differential inhibition of prostaglandin endoperoxide synthase (cyclooxygenase) isozymes by aspirin and other non-steroidal anti-inflammatory drugs. *Journal of Biological Chemistry*, *268*(9), pp.6610-6614.

Meerts, I.A.T.M., Verspeek-Rip, C.M., Buskens, C.A.F., Keizer, H.G., Bassaganya-Riera, J., Jouni, Z.E., Van Huygevoort, A.H.B.M., Van Otterdijk, F.M. and Van de Waart, E.J., 2009. Toxicological evaluation of pomegranate seed oil. *Food and Chemical Toxicology*, *47*(6), pp.1085-1092.

Mehta, V.V., Rajesh, G., Rao, A., Shenoy, R. and BH, M.P., 2014. Antimicrobial efficacy of Punica granatum mesocarp, Nelumbo nucifera leaf, Psidium guajava leaf and Coffea Canephora extract on common oral pathogens: an in-vitro study. *Journal of clinical and diagnostic research: JCDR*, *8*(7), p.ZC65.

Meléndez, P.A. and Capriles, V.A., 2006. Antibacterial properties of tropical plants from Puerto Rico. *Phytomedicine*, *13*(4), pp.272-276.

Melgarejo Moreno, P. and Martinez Valero, R., 1992. Pomegranate tree.

Melgarejo, P., Salazar, D.M. and Artes, F., 2000. Organic acids and sugars composition of harvested pomegranate fruits. *European Food Research and Technology*, *211*(3), pp.185-190.

Mena, P., Calani, L., Dall'Asta, C., Galaverna, G., García-Viguera, C., Bruni, R., Crozier, A. and Del Rio, D., 2012. Rapid and comprehensive evaluation of (poly) phenolic compounds in pomegranate (Punica granatum L.) juice by UHPLC-MSn. *Molecules*, *17*(12), pp.14821-14840.

Mendieta, C.F., Reeve, C.M. and Romero, J.C., 1985. Biosynthesis of prostaglandins in gingiva of patients with chronic periodontitis. *Journal of periodontology*, *56*(1), pp.44-47.

Menezes, S.M., Cordeiro, L.N. and Viana, G.S., 2006. Punica granatum (pomegranate) extract is active against dental plaque. *Journal of herbal pharmacotherapy*, *6*(2), pp.79-92.

Mercado, F.B., Marshall, R.I., Klestov, A.C. and Bartold, P.M., 2001. Relationship between rheumatoid arthritis and periodontitis. *Journal of periodontology*, *72*(6), pp.779-787.

Metwalli, K.H., Khan, S.A., Krom, B.P. and Jabra-Rizk, M.A., 2013. Streptococcus mutans, Candida albicans, and the human mouth: a sticky situation. *PLoS pathogens*, *9*(10), p.e1003616.

Miles, A.A., Misra, S.S. and Irwin, J.O., 1938. The estimation of the bactericidal power of the blood. *Epidemiology & Infection*, *38*(6), pp.732-749.

Miller, D.R., Lamster, I.B. and Chasens, A.I., 1984. Role of the polymorphonuclear leukocyte in periodontal health and disease. *Journal of clinical periodontology*, *11*(1), pp.1-15.

Miller, M.D. and Krangel, M.S., 1992. Biology and biochemistry of the chemokines: a family of chemotactic and inflammatory cytokines. *Critical reviews in immunology*, *12*(1-2), pp.17-46.

Miller, W.D., 1890. The microorganisms of the human mouth. *The local and general disease which are caused by them*, pp.274-341.

Millsop, J.W. and Fazel, N., 2016. Oral candidiasis. *Clinics in dermatology*, *34*(4), pp.487-494.

Minhas, S., Sajjad, A., Kashif, M., Taj, F., Al Waddani, H. and Khurshid, Z., 2019. Oral ulcers presentation in systemic diseases: An update. *Open access Macedonian journal of medical sciences*, *7*(19), p.3341.

Miörner, H., Johansson, G. and Kronvall, G., 1983. Lipoteichoic acid is the major cell wall component responsible for surface hydrophobicity of group A streptococci. *Infection and immunity*, *39*(1), pp.336-343.

Mitsiadis, T.A., Feki, A., Papaccio, G. and Catón, J., 2011. Dental pulp stem cells, niches, and notch signaling in tooth injury. *Advances in dental research*, *23*(3), pp.275-279.

Mo, J., Panichayupakaranant, P., Kaewnopparat, N., Nitiruangjaras, A. and Reanmongkol, W., 2014a. Wound healing activities of standardized pomegranate rind extract and its major antioxidant ellagic acid in rat dermal wounds. *Journal of natural medicines*, *68*(2), pp.377-386.

Mo, J., Panichayupakaranant, P., Kaewnopparat, N., Songkro, S. and Reanmongkol, W., 2014b. Topical anti-inflammatory potential of standardized pomegranate rind extract and ellagic acid in contact dermatitis. *Phytotherapy research*, *28*(4), pp.629-632.

Mocchegiani, E., Costarelli, L., Giacconi, R., Piacenza, F., Basso, A. and Malavolta, M., 2011. Zinc, metallothioneins and immunosenescence: effect of zinc supply as nutrigenomic approach. *Biogerontology*, *12*(5), pp.455-465.

Moon, H.W. and Bunn, T.O., 1993. Vaccines for preventing enterotoxigenic Escherichia coli infections in farm animals. *Vaccine*, *11*(2), pp.213-220.

Moore, S., Calder, K.A., Miller, N.J. and Rice-Evans, C.A., 1994. Antioxidant activity of saliva and periodontal disease. *Free radical research*, *21*(6), pp.417-425.

Moran, J., Addy, M., Newcombe, R.G. and Marlow, I., 2001. A study to assess the plaque inhibitory action of a newly formulated triclosan toothpaste. *Journal of clinical periodontology*, *28*(1), pp.86-89.

Morasso, M.I. and Tomic-Canic, M., 2005. Epidermal stem cells: the cradle of epidermal determination, differentiation and wound healing. *Biology of the Cell*, *97*(3), pp.173-183.

Moriguchi, T., Omura, M., Matsuta, N., & Kozaki, I., 1987. In vitro adventitious shoot formation from anthers of pomegranate. *HortScience*, *22*(5), 947-948.

Mortazavi, H., Safi, Y., Baharvand, M. and Rahmani, S., 2016. Diagnostic features of common oral ulcerative lesions: an updated decision tree. *International journal of dentistry*, *2016*.

Moses, R.L., Boyle, G.M., Howard-Jones, R.A., Errington, R.J., Johns, J.P., Gordon, V., Reddell, P., Steadman, R. and Moseley, R., 2020. Novel epoxy-tiglianes stimulate skin keratinocyte wound healing responses and re-epithelialization via protein kinase C activation. *Biochemical Pharmacology*, *178*, p.114048.

Moue, M., Tohno, M., Shimazu, T., Kido, T., Aso, H., Saito, T. and Kitazawa, H., 2008. Toll-like receptor 4 and cytokine expression involved in functional immune response in an originally established porcine intestinal epitheliocyte cell line. *Biochimica et Biophysica Acta (BBA)-General Subjects*, *1780*(2), pp.134-144.

Moutsopoulos, N.M. and Konkel, J.E., 2018. Tissue-specific immunity at the oral mucosal barrier. *Trends in immunology*, *39*(4), pp.276-287.

Mphahlele, R.R., Fawole, O.A., Makunga, N.P. and Opara, U.L., 2017. Functional properties of pomegranate fruit parts: Influence of packaging systems and storage time. *Journal of Food Measurement and Characterization*, *11*(4), pp.2233-2246.

Mphahlele, R.R., Fawole, O.A., Makunga, N.P. and Opara, U.L., 2017. Functional properties of pomegranate fruit parts: Influence of packaging systems and storage time. *Journal of Food Measurement and Characterization*, *11*(4), pp.2233-2246.

Mueller-Harvey, I., 2001. Analysis of hydrolysable tannins. *Animal feed science and technology*, *91*(1-2), pp.3-20.

Mullins, M.G., Bouquet, A. and Williams, L.E., 1992. *Biology of the grapevine*. Cambridge University Press.

Murciano, C., Moyes, D.L., Runglall, M., Tobouti, P., Islam, A., Hoyer, L.L. and Naglik, J.R., 2012. Evaluation of the role of Candida albicans agglutinin-like sequence (Als) proteins in human oral epithelial cell interactions. *PLoS One*, *7*(3), p.e33362.

Murzakhmetova, M., Moldakarimov, S., Tancheva, L., Abarova, S. and Serkedjieva, J., 2008. Antioxidant and prooxidant properties of a polyphenol-rich extract from Geranium sanguineum L. in vitro and in vivo. *Phytotherapy Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives*, *22*(6), pp.746-751.

Myers, S.R., Leigh, I.M. and Navsaria, H., 2007. Epidermal repair results from activation of follicular and epidermal progenitor keratinocytes mediated by a growth factor cascade. *Wound repair and regeneration*, *15*(5), pp.693-701.

Nachlas, M.M., Margulies, S.I., Goldberg, J.D. and Seligman, A.M., 1960. The determination of lactic dehydrogenase with a tetrazolium salt. *Analytical biochemistry*, *1*(4-5), pp.317-326.

Nagar, R., 1990. Syntheses, characterization, and microbial activity of some transition metal complexes involving potentially active O and N donor heterocyclic ligands. *Journal of inorganic biochemistry*, *40*(4), pp.349-356.

Nagaraju, N., & Rao, K. N.,1990. A survey of plant crude drugs of Rayalaseema, Andhra Pradesh, India. *Journal of Ethnopharmacology*, *29*(2), 137-158.

Nagase, H., Visse, R. and Murphy, G., 2006. Structure and function of matrix metalloproteinases and TIMPs. *Cardiovascular research*, *69*(3), pp.562-573.

Naglik, J.R., Moyes, D.L., Wächtler, B. and Hube, B., 2011. Candida albicans interactions with epithelial cells and mucosal immunity. *Microbes and Infection*, *13*(12-13), pp.963-976.

Nair, P.N.R., 2006. On the causes of persistent apical periodontitis: a review. *International endodontic journal*, *39*(4), pp.249-281.

Nakamura, M., 2018. Histological and immunological characteristics of the junctional epithelium. *Japanese Dental Science Review*, *54*(2), pp.59-65.

Nanci, A., 2017. *Ten Cate's Oral Histology-e-book: development, structure, and function*. Elsevier Health Sciences.

Naqvi, S., Khan, M. S. Y., & Vohora, S. B., 1991. Anti-bacterial, anti-fungal and anthelmintic investigations on Indian medicinal plants. *Fitoterapia*, *62*(3), 221-228.

Nassar, M., Tabib, Y., Capucha, T., Mizraji, G., Nir, T., Pevsner-Fischer, M., Zilberman-Schapira, G., Heyman, O., Nussbaum, G., Bercovier, H. and Wilensky, A., 2017. GAS6 is a key homeostatic immunological regulator of host–commensal interactions in the oral mucosa. *Proceedings of the National Academy of Sciences*, *114*(3), pp.E337-E346.

National Committee for Clinical Laboratory Standards and Barry, A.L., 1999. *Methods for determining bactericidal activity of antimicrobial agents: approved guideline* (Vol. 19, No. 18). Wayne, PA: National Committee for Clinical Laboratory Standards.

National Library of Medicine (2021) *PubChem.* Available at: https://pubchem.ncbi.nlm.nih.gov/ (Accessed: 11 May 2021)

Nayak, P.K., Busani, T., Elamurugu, E., Barquinha, P., Martins, R., Hong, Y. and Fortunato, E., 2010. Zinc concentration dependence study of solution processed amorphous indium gallium zinc oxide thin film transistors using high-k dielectric. *Applied Physics Letters*, *97*(18), p.183504.

Nayak, S.B., Rodrigues, V., Maharaj, S. and Bhogadi, V.S., 2013. Wound healing activity of the fruit skin of Punica granatum. *Journal of medicinal food*, *16*(9), pp.857-861.

Nazir, M.A., 2017. Prevalence of periodontal disease, its association with systemic diseases and prevention. *International journal of health sciences*, *11*(2), p.72.

Negi, P.S. and Jayaprakasha, G.K., 2003. Antioxidant and antibacterial activities of Punica granatum peel extracts. *Journal of food science*, *68*(4), pp.1473-1477.

Negi, P.S., Jayaprakasha, G.K. and Jena, B.S., 2003. Antioxidant and antimutagenic activities of pomegranate peel extracts. *Food chemistry*, *80*(3), pp.393-397.

Neilands, J., Wickström, C., Kinnby, B., Davies, J. R., Hall, J., Friberg, B., & Svensäter, G. (2015). Bacterial profiles and proteolytic activity in peri-implantitis versus healthy sites. *Anaerobe*, *35*, 28-34.

Nesbitt, W.E., Doyle, R.J. and Taylor, K.G., 1982. Hydrophobic interactions and the adherence of Streptococcus sanguis to hydroxylapatite. *Infection and Immunity*, *38*(2), pp.637-644.

Neu, T.R., 1996. Significance of bacterial surface-active compounds in interaction of bacteria with interfaces. *Microbiological reviews*, *60*(1), p.151.

Neurath, A.R., Strick, N., Li, Y.Y. and Debnath, A.K., 2004. Punica granatum (Pomegranate) juice provides an HIV-1 entry inhibitor and candidate topical microbicide. *BMC infectious diseases*, *4*(1), pp.1-12.

Neutra, M.R. and Kozlowski, P.A., 2006. Mucosal vaccines: the promise and the challenge. *Nature reviews immunology*, *6*(2), pp.148-158.

Niel-Weise, B.S., Stijnen, T. and Van den Broek, P.J., 2007. Anti-infective-treated central venous catheters: a systematic review of randomized controlled trials. *Intensive care medicine*, *33*(12), pp.2058-2068.

Nirwana, I., Rachmadi, P. and Rianti, D., 2017. Potential of pomegranate fruit extract (Punica granatum Linn.) to increase vascular endothelial growth factor and platelet-derived growth factor expressions on the post-tooth extraction wound of Cavia cobaya. *Veterinary world*, *10*(8), p.999.

Nishikawara, F., Nomura, Y., Imai, S., Senda, A. and Hanada, N., 2007. Evaluation of cariogenic bacteria. *European journal of dentistry*, *1*(01), pp.031-039.

Nobbs, A.H., Jenkinson, H.F. and Jakubovics, N.S., 2011. Stick to your gums: mechanisms of oral microbial adherence. *Journal of dental research*, *90*(11), pp.1271-1278.

Nobile, C.J. and Johnson, A.D., 2015. Candida albicans biofilms and human disease. *Annual review of microbiology*, *69*, pp.71-92.

Nobile, C.J., Schneider, H.A., Nett, J.E., Sheppard, D.C., Filler, S.G., Andes, D.R. and Mitchell, A.P., 2008. Complementary adhesin function in C. albicans biofilm formation. *Current biology*, *18*(14), pp.1017-1024.

Noda, Y., Kaneyuki, T., Mori, A. and Packer, L., 2002. Antioxidant activities of pomegranate fruit extract and its anthocyanidins: delphinidin, cyanidin, and pelargonidin. *Journal of agricultural and food chemistry*, *50*(1), pp.166-171.

Novák-Pékli, M., Mesbah, M.E.H. and Pethö, G., 1996. Equilibrium studies on tetracycline—metal ion systems. *Journal of pharmaceutical and biomedical analysis*, *14*(8-10), pp.1025-1029.

Novak, K.F. and Novak, M.J., 2006. Clinical periodontology.

Novak, M.L. and Koh, T.J., 2013. Macrophage phenotypes during tissue repair. *Journal of leukocyte biology*, *93*(6), pp.875-881.

Novak, N., Bieber, T. and Katoh, N., 2001. Engagement of FcεRI on human monocytes induces the production of IL-10 and prevents their differentiation in dendritic cells. *The Journal of Immunology*, *167*(2), pp.797-804.

Nurden, A.T., Freson, K. and Seligsohn, U., 2012. Inherited platelet disorders. *Haemophilia*, *18*, pp.154-160.

Nyvad, B.E.N.T.E. and Kilian, M.O.G.E.N.S., 1987. Microbiology of the early colonization of human enamel and root surfaces in vivo. *Scandinavian journal of dental research*, *95*(5), pp.369-380.

O'Dell, B.L., 1992. Zinc plays both structural and catalytic roles in metalloproteins. *Nutrition reviews*, *50*(2), pp.48-50.

O'May, C. and Tufenkji, N., 2011. The swarming motility of Pseudomonas aeruginosa is blocked by cranberry proanthocyanidins and other tannin-containing materials. *Applied and environmental microbiology*, *77*(9), pp.3061-3067.

O'Toole, G.A. and Kolter, R., 1998a. Flagellar and twitching motility are necessary for Pseudomonas aeruginosa biofilm development. *Molecular microbiology*, *30*(2), pp.295-304.

O'Toole, G.A. and Kolter, R., 1998b. Initiation of biofilm formation in Pseudomonas fluorescens WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. *Molecular microbiology*, *28*(3), pp.449-461.

O'Toole, G.A. and Stewart, P.S., 2005. Biofilms strike back. *Nature biotechnology*, *23*(11), pp.1378-1379.

O'Toole, G.A., Gibbs, K.A., Hager, P.W., Phibbs Jr, P.V. and Kolter, R., 2000. The global carbon metabolism regulator Crc is a component of a signal transduction pathway required for biofilm development by Pseudomonas aeruginosa. *Journal of bacteriology*, *182*(2), pp.425-431.

Odds, F.C., 1988. *Candida and candidosis: a review and bibliography*. Bailliere Tindall.

Odds, F.C., 2003. Synergy, antagonism, and what the chequerboard puts between them. *Journal of Antimicrobial Chemotherapy*, *52*(1), pp.1-1.

Ofek, I. and Doyle, R.J., 1994. Common themes in bacterial adhesion. In *Bacterial Adhesion to Cells and Tissues* (pp. 513-561). Springer, Boston, MA.

Ofek, I. and Doyle, R.J., 2012. *Bacterial adhesion to cells and tissues*. Springer Science & Business Media.

Ofek, I., Hasty, D.L. and Sharon, N., 2003. Anti-adhesion therapy of bacterial diseases: prospects and problems. *FEMS Immunology & Medical Microbiology*, *38*(3), pp.181-191.

Ogawa, R., 2011. Mechanobiology of scarring. *Wound Repair and Regeneration*, *19*, pp.s2-s9.

Okonogi, S., Duangrat, C., Anuchpreeda, S., Tachakittirungrod, S. and Chowwanapoonpohn, S., 2007. Comparison of antioxidant capacities and cytotoxicities of certain fruit peels. *Food chemistry*, *103*(3), pp.839-846.

Oksman-Caldentey, K.M. and Inzé, D., 2004. Plant cell factories in the post-genomic era: new ways to produce designer secondary metabolites. *Trends in plant science*, *9*(9), pp.433-440.

Okuda, K., Hanada, N., Usui, Y., Takeuchi, H., Koba, H., Nakao, R., Watanabe, H. and Senpuku, H., 2010. Inhibition of Streptococcus mutans adherence and biofilm formation using analogues of the SspB peptide. *Archives of oral biology*, *55*(10), pp.754-762.

Olajide, O.A., Kumar, A., Velagapudi, R., Okorji, U.P. and Fiebich, B.L., 2014. Punicalagin inhibits neuroinflammation in LPS-activated rat primary microglia. *Molecular nutrition & food research*, *58*(9), pp.1843-1851.

Olszewska, E. and Woodson, B.T., 2019. Palatal anatomy for sleep apnea surgery. *Laryngoscope investigative otolaryngology*, *4*(1), pp.181-187.

Ono, N.N., Bandaranayake, P.C. and Tian, L., 2012. Establishment of pomegranate (Punica granatum) hairy root cultures for genetic interrogation of the hydrolyzable tannin biosynthetic pathway. *Planta*, *236*(3), pp.931-941.

Orak, H.H., Yagar, H. and Isbilir, S.S., 2012. Comparison of antioxidant activities of juice, peel, and seed of pomegranate (Punica granatum L.) and inter-relationships with total phenolic, Tannin, anthocyanin, and flavonoid contents. *Food Science and Biotechnology*, *21*(2), pp.373-387.

Orgil, O., Schwartz, E., Baruch, L., Matityahu, I., Mahajna, J. and Amir, R., 2014. The antioxidative and anti-proliferative potential of non-edible organs of the pomegranate fruit and tree. *LWT-Food Science and Technology*, *58*(2), pp.571-577.

Ott, M., Gogvadze, V., Orrenius, S. and Zhivotovsky, B., 2007. Mitochondria, oxidative stress and cell death. *Apoptosis*, *12*(5), pp.913-922.

Otto, M., 2008. Staphylococcal biofilms. *Bacterial biofilms*, pp.207-228.

Oudane, B., Boudemagh, D., Bounekhel, M., Sobhi, W., Vidal, M. and Broussy, S., 2018. Isolation, characterization, antioxidant activity, and protein-precipitating capacity of the hydrolyzable tannin punicalagin from pomegranate yellow peel (Punica granatum). *Journal of Molecular Structure*, *1156*, pp.390-396.

Pacios, S., Kang, J., Galicia, J., Gluck, K., Patel, H., Ovaydi-Mandel, A., Petrov, S., Alawi, F. and Graves, D.T., 2012. Diabetes aggravates periodontitis by limiting repair through enhanced inflammation. *The FASEB Journal*, *26*(4), pp.1423-1430.

Page, R.C. and Kornman, K.S., 1997. The pathogenesis of human periodontitis: an introduction. *Periodontology 2000*, *14*(1), pp.9-11.

Pagliarulo, C., De Vito, V., Picariello, G., Colicchio, R., Pastore, G., Salvatore, P. and Volpe, M.G., 2016. Inhibitory effect of pomegranate (Punica granatum L.) polyphenol extracts on the bacterial growth and survival of clinical isolates of pathogenic Staphylococcus aureus and Escherichia coli. *Food chemistry*, *190*, pp.824-831.

Palmer Jr, R.J., Gordon, S.M., Cisar, J.O. and Kolenbrander, P.E., 2003. Coaggregation-mediated interactions of streptococci and actinomyces detected in initial human dental plaque. *Journal of bacteriology*, *185*(11), pp.3400-3409.

Palmer, R.J. and White, D.C., 1997. Developmental biology of biofilms: implications for treatment and control. *Trends in microbiology*, *5*(11), pp.435-440.

Pan, W., Wang, Q. and Chen, Q., 2019. The cytokine network involved in the host immune response to periodontitis. *International journal of oral science*, *11*(3), pp.1-13.

Panagakos, F., & Scannapieco, F. (2011). Periodontal inflammation: from gingivitis to systemic disease?. *Gingival Diseases: Their Aetiology, Prevention and Treatment*, 155-168.

Panda, D. and Manickam, S., 2019. Cavitation technology—The future of greener extraction method: A review on the extraction of natural products and process intensification mechanism and perspectives. *Applied Sciences*, *9*(4), p.766.

Pande, G. and Akoh, C.C., 2009. Antioxidant capacity and lipid characterization of six Georgia-grown pomegranate cultivars. *Journal of agricultural and food chemistry*, *57*(20), pp.9427-9436.

Pang, B., Zhu, Y., Lu, L., Gu, F. and Chen, H., 2016. The applications and features of liquid chromatography-mass spectrometry in the analysis of traditional chinese medicine. *Evidence-Based Complementary and Alternative Medicine*, *2016*.

Papadopoulou, A. and Frazier, R.A., 2004. Characterization of protein–polyphenol interactions. *Trends in food science & technology*, *15*(3-4), pp.186-190.

Paraskevas, S. and Van der Weijden, G.A., 2006. A review of the effects of stannous fluoride on gingivitis. *Journal of clinical periodontology*, *33*(1), pp.1-13.

Parejo, I., Viladomat, F., Bastida, J., Rosas-Romero, A., Saavedra, G., Murcia, M.A., Jiménez, A.M. and Codina, C., 2003. Investigation of Bolivian plant extracts for their radical scavenging activity and antioxidant activity. *Life Sciences*, *73*(13), pp.1667-1681.

Park, Y., Simionato, M.R., Sekiya, K., Murakami, Y., James, D., Chen, W., Hackett, M., Yoshimura, F., Demuth, D.R. and Lamont, R.J., 2005. Short fimbriae of Porphyromonas gingivalis and their role in coadhesion with Streptococcus gordonii. *Infection and immunity*, *73*(7), pp.3983-3989.

Pashley, D.H., Nelson, R. and Pashley, E.L., 1981. In-vivo fluid movement across dentine in the dog. *Archives of oral biology*, *26*(9), pp.707-710.

Patel, C., Dadhaniya, P., Hingorani, L. and Soni, M.G., 2008. Safety assessment of pomegranate fruit extract: acute and subchronic toxicity studies. *Food and Chemical Toxicology*, *46*(8), pp.2728-2735.

Pavithra, D. and Doble, M., 2008. Biofilm formation, bacterial adhesion and host response on polymeric implants—issues and prevention. *Biomedical Materials*, *3*(3), p.034003.

Pérez-Ramírez, I.F., Reynoso-Camacho, R., Saura-Calixto, F. and Pérez-Jiménez, J., 2018. Comprehensive characterization of extractable and nonextractable phenolic compounds by high-performance liquid chromatography–electrospray ionization–quadrupole time-of-flight of a grape/pomegranate pomace dietary supplement. *Journal of agricultural and food chemistry*, *66*(3), pp.661-673.

Petersen, P.E. and Lennon, M.A., 2004. Effective use of fluorides for the prevention of dental caries in the 21st century: the WHO approach. *Community dentistry and oral epidemiology*, *32*(5), pp.319-321.

Petersen, P.E., Bourgeois, D., Ogawa, H., Estupinan-Day, S. and Ndiaye, C., 2005. The global burden of oral diseases and risks to oral health. *Bulletin of the World Health Organization*, *83*, pp.661-669.

Phan, Q.T., Myers, C.L., Fu, Y., Sheppard, D.C., Yeaman, M.R., Welch, W.H., Ibrahim, A.S., Edwards Jr, J.E. and Filler, S.G., 2007. Als3 is a Candida albicans invasin that binds to cadherins and induces endocytosis by host cells. *PLoS Biol*, *5*(3), p.e64.

Phan, T.N. and Marquis, R.E., 2006. Triclosan inhibition of membrane enzymes and glycolysis of Streptococcus mutans in suspensions and biofilms. *Canadian journal of microbiology*, *52*(10), pp.977-983.

Phan, T.N., Buckner, T., Sheng, J., Baldeck, J.D. and Marquis, R.E., 2004. Physiologic actions of zinc related to inhibition of acid and alkali production by oral streptococci in suspensions and biofilms. *Oral microbiology and immunology*, *19*(1), pp.31-38.

Phane Quideau, S., 2009. Chemistry and biology of ellagitannins: an underestimated class of bioactive plant polyphenols. World Scientific.

Phillips, A.J., Sudbery, I. and Ramsdale, M., 2003. Apoptosis induced by environmental stresses and amphotericin B in Candida albicans. *Proceedings of the National Academy of Sciences*, *100*(24), pp.14327-14332.

Pichlmaier, M., Marwitz, V., Kühn, C., Niehaus, M., Klein, G., Bara, C., Haverich, A. and Abraham, W.R., 2008. High prevalence of asymptomatic bacterial colonization of rhythm management devices. *Europace*, *10*(9), pp.1067-1072.

Pirzadeh, M., Caporaso, N., Rauf, A., Shariati, M.A., Yessimbekov, Z., Khan, M.U., Imran, M. and Mubarak, M.S., 2021. Pomegranate as a source of bioactive constituents: A review on their characterization, properties and applications. *Critical reviews in food science and nutrition*, *61*(6), pp.982-999.

Pitts, N.B., Zero, D.T., Marsh, P.D., Ekstrand, K., Weintraub, J.A., Ramos-Gomez, F., Tagami, J., Twetman, S., Tsakos, G. and Ismail, A., 2017. Dental caries. *Nature reviews Disease primers*, *3*(1), pp.1-16.

Pizzi, A., Pasch, H., Rode, K. and Giovando, S., 2009. Polymer structure of commercial hydrolyzable tannins by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry. *Journal of applied polymer science*, *113*(6), pp.3847-3859.

Politis, C., Schoenaers, J., Jacobs, R. and Agbaje, J.O., 2016. Wound healing problems in the mouth. *Frontiers in physiology*, *7*, p.507.

Pöllänen, M.T., Salonen, J.I., Grenier, D. and Uitto, V.J., 2000. Epithelial cell response to challenge of bacterial lipoteichoic acids and lipopolysaccharides in vitro. *Journal of medical microbiology*, *49*(3), pp.245-252.

Pontefract, R.D., 1991. Bacterial adherence: its consequences in food processing. *Canadian Institute of Food Science and Technology Journal*, *24*(3-4), pp.113-117.

Poopedi, E., Marimani, M., AlOmar, S.Y., Aldahmash, B. and Ahmad, A., 2021. Modulation of antioxidant defence system in response to berberine in Candida albicans. *Yeast*, *38*(2), pp.157-169.

Posten, W., Wrone, D.A., Dover, J.S., Arndt, K.A., Silapunt, S. and Alam, M., 2005. Low-level laser therapy for wound healing: mechanism and efficacy. *Dermatologic surgery*, *31*(3), pp.334-340.

Pountos, I., Georgouli, T., Bird, H. and Giannoudis, P.V., 2011. Nonsteroidal anti-inflammatory drugs: prostaglandins, indications, and side effects. *International Journal of Interferon, Cytokine and Mediator Research*, *3*, pp.19-27.

Powell, L.C., Pritchard, M.F., Ferguson, E.L., Powell, K.A., Patel, S.U., Rye, P.D., Sakellakou, S.M., Buurma, N.J., Brilliant, C.D., Copping, J.M. and Menzies, G.E., 2018. Targeted disruption of the extracellular polymeric network of Pseudomonas aeruginosa biofilms by alginate oligosaccharides. *NPJ biofilms and microbiomes*, *4*(1), pp.1-10.

Pozniakovsky, A.I., Knorre, D.A., Markova, O.V., Hyman, A.A., Skulachev, V.P. and Severin, F.F., 2005. Role of mitochondria in the pheromone-and amiodarone-induced programmed death of yeast. *The Journal of cell biology*, *168*(2), pp.257-269.

Prakash, C.V.S. and Prakash, I., 2011. Bioactive chemical constituents from pomegranate (Punica granatum) juice, seed and peel-a review. *Int J Res Chem Environ*, *1*(1), pp.1-18.

Prasad, A., 2013. *Trace elements and iron in human metabolism*. Springer Science & Business Media.

Prasad, A.S., 1995. Zinc: an overview. *Nutrition (Burbank, Los Angeles County, Calif.)*, *11*(1 Suppl), pp.93-99.

Prasad, A.S., 2014. Zinc is an antioxidant and anti-inflammatory agent: its role in human health. *Frontiers in nutrition*, *1*, p.14.

Prasad, D. and Kunnaiah, R., 2014. Punica granatum: A review on its potential role in treating periodontal disease. *Journal of Indian Society of Periodontology*, *18*(4), p.428.

Prashanth, D., Asha, M.K. and Amit, A., 2001. Antibacterial activity of Punica granatum. *Fitoterapia*, *72*(2), pp.171-173.

Pratt, L.A. and Kolter, R., 1999. Genetic analyses of bacterial biofilm formation. *Current opinion in microbiology*, *2*(6), pp.598-603.

Prescott, S.M. and Fitzpatrick, F.A., 2000. Cyclooxygenase-2 and carcinogenesis. *Biochimica et biophysica acta*, *1470*(2), pp.M69-78.

Preshaw, P.M., Taylor, J.J., Jaedicke, K.M., De Jager, M., Bikker, J.W., Selten, W., Bissett, S.M., Whall, K.M., van de Merwe, R., Areibi, A. and Jitprasertwong, P., 2020. Treatment of periodontitis reduces systemic inflammation in type 2 diabetes. *Journal of clinical periodontology*, *47*(6), pp.737-746.

Presta, M., Dell'Era, P., Mitola, S., Moroni, E., Ronca, R. and Rusnati, M., 2005. Fibroblast growth factor/fibroblast growth factor receptor system in angiogenesis. *Cytokine & growth factor reviews*, *16*(2), pp.159-178.

Pretheeban, T., Lemos, D.R., Paylor, B., Zhang, R.H. and Rossi, F.M., 2012. Role of stem/progenitor cells in reparative disorders. *Fibrogenesis & tissue repair*, *5*(1), pp.1-12.

Pringle, J.H. and Fletcher, M.A.D.I.L.Y.N., 1986. Influence of substratum hydration and adsorbed macromolecules on bacterial attachment to surfaces. *Applied and environmental microbiology*, *51*(6), pp.1321-1325.

Procházková, D., Boušová, I. and Wilhelmová, N., 2011. Antioxidant and prooxidant properties of flavonoids. *Fitoterapia*, *82*(4), pp.513-523.
properties related to bonding. *Journal of dentistry*, *25*(6), pp.441-458.

Qiu, Z., Zhou, B., Jin, L., Yu, H., Liu, L., Liu, Y., Qin, C., Xie, S. and Zhu, F., 2013. In vitro antioxidant and antiproliferative effects of ellagic acid and its colonic metabolite, urolithins, on human bladder cancer T24 cells. *Food and Chemical Toxicology*, *59*, pp.428-437.

Qu, W., Breksa III, A.P., Pan, Z. and Ma, H., 2012. Quantitative determination of major polyphenol constituents in pomegranate products. *Food Chemistry*, *132*(3), pp.1585-1591.

Quintero-Villegas, M.I., Aam, B.B., Rupnow, J., Sørlie, M., Eijsink, V.G. and Hutkins, R.W., 2013. Adherence inhibition of enteropathogenic Escherichia coli by chitooligosaccharides with specific degrees of acetylation and polymerization. *Journal of agricultural and food chemistry*, *61*(11), pp.2748-2754.

Quirynen, M., Vogels, R., Peeters, W., van Steenberghe, D., Naert, I. and Haffajee, A., 2006. Dynamics of initial subgingival colonization of 'pristine'peri-implant pockets. *Clinical oral implants research*, *17*(1), pp.25-37.

Rafieian-Kopaei, M., Baradaran, A. and Rafieian, M., 2013. Plants antioxidants: From laboratory to clinic. *Journal of nephropathology*, *2*(2).

Rafiq, Z., Narasimhan, S., Vennila, R. and Vaidyanathan, R., 2016. Punigratane, a novel pyrrolidine alkaloid from Punica granatum rind with putative efflux inhibition activity. *Natural product research*, *30*(23), pp.2682-2687.

Rahman, K., 2007. Studies on free radicals, antioxidants, and co-factors. *Clinical interventions in aging*, *2*(2), p.219.

Rahmani, A.H., Alsahli, M.A. and Almatroodi, S.A., 2017. Active constituents of pomegranates (Punica granatum) as potential candidates in the management of health through modulation of biological activities. *Pharmacognosy Journal*, *9*(5).

Rainsford, K.D., Kean, W.F. and Ehrlich, G.E., 2008. Review of the pharmaceutical properties and clinical effects of the topical NSAID formulation, diclofenac epolamine. *Current medical research and opinion*, *24*(10), pp.2967-2992.

Rajha, H.N., Abi-Khattar, A.M., El Kantar, S., Boussetta, N., Lebovka, N., Maroun, R.G., Louka, N. and Vorobiev, E., 2019. Comparison of aqueous extraction efficiency and biological activities of polyphenols from pomegranate peels assisted by infrared, ultrasound, pulsed electric fields and high-voltage electrical discharges. *Innovative Food Science & Emerging Technologies*, *58*, p.102212.

Ramage, G., Bachmann, S., Patterson, T.F., Wickes, B.L. and López-Ribot, J.L., 2002. Investigation of multidrug efflux pumps in relation to fluconazole resistance in Candida albicans biofilms. *Journal of Antimicrobial Chemotherapy*, *49*(6), pp.973-980.

Ramboarina, S., Garnett, J.A., Zhou, M., Li, Y., Peng, Z., Taylor, J.D., Lee, W.C., Bodey, A., Murray, J.W., Alguel, Y. and Bergeron, J., 2010. Structural insights into serine-rich fimbriae from Gram-positive bacteria. *Journal of Biological Chemistry*, *285*(42), pp.32446-32457.

Rana, J. C., Pradheep, K., & Verma, V. D., 2007. Naturally occurring wild relatives of temperate fruits in Western Himalayan region of India: an analysis. *Biodiversity and conservation*, *16*(14), 3963-3991.

Rasheed, Z., Akhtar, N., Anbazhagan, A.N., Ramamurthy, S., Shukla, M. and Haqqi, T.M., 2009. Polyphenol-rich pomegranate fruit extract (POMx) suppresses PMACI-induced expression of pro-inflammatory cytokines by inhibiting the activation of MAP Kinases and NF-κB in human KU812 cells. *Journal of inflammation*, *6*(1), pp.1-12.

Rautemaa, R., Lauhio, A., Cullinan, M.P. and Seymour, G.J., 2007. Oral infections and systemic disease—an emerging problem in medicine. *Clinical Microbiology and Infection*, *13*(11), pp.1041-1047.

Ravanti, L., Häkkinen, L., Larjava, H., Saarialho-Kere, U., Foschi, M., Han, J. and Kähäri, V.M., 1999. Transforming growth factor-β induces collagenase-3 expression by human gingival fibroblasts via p38 mitogen-activated protein kinase. *Journal of Biological Chemistry*, *274*(52), pp.37292-37300.

Razavi, A., Gmür, R., Imfeld, T. and Zehnder, M., 2007. Recovery of Enterococcus faecalis from cheese in the oral cavity of healthy subjects. *Oral microbiology and immunology*, *22*(4), pp.248-251.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M. and Rice-Evans, C., 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free radical biology and medicine*, *26*(9-10), pp.1231-1237.

Reid, L.M., Woodcock, T., O'Donnell, C.P., Kelly, J.D. and Downey, G., 2005. Differentiation of apple juice samples on the basis of heat treatment and variety using chemometric analysis of MIR and NIR data. *Food Research International*, *38*(10), pp.1109-1115.

Reis, C., Da Costa, A.V., Guimarães, J.T., Tuna, D., Braga, A.C., Pacheco, J.J., Arosa, F.A., Salazar, F. and Cardoso, E.M., 2014. Clinical improvement following therapy for periodontitis: Association with a decrease in IL-1 and IL-6. *Experimental and therapeutic medicine*, *8*(1), pp.323-327.

Rheinwald, J.G. and Green, H., 1975. Formation of a keratinizing epithelium in culture by a cloned cell line derived from a teratoma. *Cell*, *6*(3), pp.317-330.

Rheinwald, J.G. and Green, H., Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. Cell [Internet]. 1975 Nov [cited 2016 Jul 5]; 6 (3): 331–43.

Rhie, G.E., Hwang, C.S., Brady, M., Kim, S.T., Kim, Y.R., Huh, W.K., Baek, Y.U., Lee, B.H., Lee, J.S. and Kang, S.O., 1999. Manganese-containing superoxide dismutase and its gene from Candida albicans. *Biochimica et Biophysica Acta (BBA)-General Subjects*, *1426*(3), pp.409-419.

Ricci, D., Giamperi, L., Bucchini, A. and Fraternale, D., 2006. Antioxidant activity of Punica granatum fruits. *Fitoterapia*, *77*(4), pp.310-312.

Richards, D. and Rutherford, R.B., 1988. The effects of interleukin 1 on collagenolytic activity and prostaglandin-E secretion by human periodontal-ligament and gingival fibroblast. *Archives of Oral Biology*, *33*(4), pp.237-243.

Rifkin, B.R., Vernillo, A.T. and Golub, L.M., 1993. Blocking periodontal disease progression by inhibiting tissue-destructive enzymes: a potential therapeutic role for tetracyclines and their chemically-modified analogs. *Journal of periodontology*, *64*, pp.819-827.

Rinn, J.L., Wang, J.K., Liu, H., Montgomery, K., Van De Rijn, M. and Chang, H.Y., 2008. A systems biology approach to anatomic diversity of skin. *Journal of Investigative Dermatology*, *128*(4), pp.776-782.

Rmaile, A., Carugo, D., Capretto, L., Aspiras, M., De Jager, M., Ward, M. and Stoodley, P., 2014. Removal of interproximal dental biofilms by high-velocity water microdrops. *Journal of dental research*, *93*(1), pp.68-73.

Roberts, J.L., Maillard, J.Y., Waddington, R.J., Denyer, S.P., Lynch, C.D. and Sloan, A.J., 2013. Development of an ex vivo coculture system to model pulpal infection by Streptococcus anginosus group bacteria. *Journal of endodontics*, *39*(1), pp.49-56.

Robinson, C., Kirkham, J. and Shore, R.C. eds., 2017. *Dental enamel formation to destruction*. CRC press.

Rodrigues, F., Lehmann, M., do Amaral, V.S., Reguly, M.L. and de Andrade, H.H.R., 2007. Genotoxicity of three mouthwash products, Cepacol®, Periogard®, and Plax®, in the Drosophila wing-spot test. *Environmental and molecular mutagenesis*, *48*(8), pp.644-649.

Rogers III, R.S., 1997, December. Recurrent aphthous stomatitis: clinical characteristics and associated systemic disorders. In *Seminars in cutaneous medicine and surgery* (Vol. 16, No. 4, pp. 278-283). No longer published by Elsevier.

Romeo, F.V., Ballistreri, G., Fabroni, S., Pangallo, S., Nicosia, M.G.L.D., Schena, L. and Rapisarda, P., 2015. Chemical characterization of different sumac and pomegranate extracts effective against Botrytis cinerea rots. *Molecules*, *20*(7), pp.11941-11958.

Rongai, D., Pulcini, P., Di Lernia, G., Nota, P., Preka, P. and Milano, F., 2019. Punicalagin content and antifungal activity of different pomegranate (Punica ganatum L.) genotypes. *Horticulturae*, *5*(3), p.52.

Rosier, B.T., De Jager, M., Zaura, E. and Krom, B.P., 2014. Historical and contemporary hypotheses on the development of oral diseases: are we there yet?. *Frontiers in cellular and infection microbiology*, *4*, p.92.

Rout, G.R. and Das, P., 2009. Effect of metal toxicity on plant growth and metabolism: I. Zinc. *Sustainable agriculture*, pp.873-884.

Rovai, E.S., Souto, M.L.S., Ganhito, J.A., Holzhausen, M., Chambrone, L. and Pannuti, C.M., 2016. Efficacy of local antimicrobials in the non-surgical treatment of patients with periodontitis and diabetes: a systematic review. *Journal of periodontology*, *87*(12), pp.1406-1417.

Rudney, J.D., Staikov, R.K. and Johnson, J.D., 2009. Potential biomarkers of human salivary function: a modified proteomic approach. *Archives of oral biology*, *54*(1), pp.91-100.

Rudolf, E., Rudolf, K., Radocha, J., Peychl, J. and Červinka, M., 2003. The role of intracellular zinc in modulation of life and death of Hep-2 cells. *Biometals*, *16*(2), pp.295-309.

Ruhl, S., Sandberg, A.L. and Cisar, J.O., 2004. Salivary receptors for the proline-rich protein-binding and lectin-like adhesins of oral actinomyces and streptococci. *Journal of dental research*, *83*(6), pp.505-510.

Russell, A.D., 2004. Whither triclosan?. *Journal of Antimicrobial Chemotherapy*, *53*(5), pp.693-695.

Russell, M.W., Childers, N.K., Michalek, S.M., Smith, D.J. and Taubman, M.A., 2004. A caries vaccine?. *Caries research*, *38*(3), pp.230-235.

Russo, M., Cacciola, F., Arena, K., Mangraviti, D., de Gara, L., Dugo, P. and Mondello, L., 2020. Characterization of the polyphenolic fraction of pomegranate samples by comprehensive two-dimensional liquid chromatography coupled to mass spectrometry detection. *Natural product research*, *34*(1), pp.39-45.

Rusyanti, Y., Widyaputra, S. and Maskoen, A.M., 2019. Periodontal tissue destruction in aggressive periodontitis: Determination of gene or environmental factors. *The Saudi dental journal*, *31*(2), pp.290-299.

Safiaghdam, H., Oveissi, V., Bahramsoltani, R., Farzaei, M.H. and Rahimi, R., 2018. Medicinal plants for gingivitis: a review of clinical trials. *Iranian journal of basic medical sciences*, *21*(10), p.978.

Sakagami, H., Jiang, Y., Kusama, K., Atsumi, T., Ueha, T., Toguchi, M., Iwakura, I., Satoh, K., Ito, H., Hatano, T. and Yoshida, T., 2000. Cytotoxic activity of hydrolyzable tannins against human oral tumor cell lines—a possible mechanism. *Phytomedicine*, *7*(1), pp.39-47.

Saito, S., Rosol, T.J., Saito, M., Ngan, P.W., Shanfeld, J. and Davidovitch, Z., 1990a. Bone-resorbing activity and prostaglandin E produced by human periodontal ligament cells in vitro. *Journal of Bone and Mineral Research*, *5*(10), pp.1013-1018.

Saito, S., Saito, M., Ngan, P., Lanese, R., Shanfeld, J. and Davidovitch, Z., 1990b. Effects of parathyroid hormone and cytokines on prostaglandin E synthesis and bone resorption by human periodontal ligament fibroblasts. *Archives of oral biology*, *35*(10), pp.845-855.

Sakihama, Y., Cohen, M.F., Grace, S.C. and Yamasaki, H., 2002. Plant phenolic antioxidant and prooxidant activities: phenolics-induced oxidative damage mediated by metals in plants. *Toxicology*, *177*(1), pp.67-80.

Salari, Z., Ameri, A., Forootanfar, H., Adeli-Sardou, M., Jafari, M., Mehrabani, M. and Shakibaie, M., 2017. Microwave-assisted biosynthesis of zinc nanoparticles and their cytotoxic and antioxidant activity. *Journal of Trace Elements in Medicine and Biology*, *39*, pp.116-123.

Salehi, B., Lopez-Jornet, P., Pons-Fuster López, E., Calina, D., Sharifi-Rad, M., Ramírez-Alarcón, K., Forman, K., Fernández, M., Martorell, M., Setzer, W.N. and Martins, N., 2019. Plant-derived bioactives in oral mucosal lesions: A key emphasis to curcumin, lycopene, chamomile, aloe vera, green tea and coffee properties. *Biomolecules*, *9*(3), p.106.

Salgado, J.M., Ferreira, T.R.B., de Oliveira Biazotto, F. and dos Santos Dias, C.T., 2012. Increased antioxidant content in juice enriched with dried extract of pomegranate (Punica granatum) peel. *Plant foods for human nutrition*, *67*(1), pp.39-43.

Saliasi, I., Llodra, J.C., Bravo, M., Tramini, P., Dussart, C., Viennot, S. and Carrouel, F., 2018. Effect of a toothpaste/mouthwash containing Carica papaya leaf extract on interdental gingival bleeding: A randomized controlled trial. *International journal of environmental research and public health*, *15*(12), p.2660.

Salminen, A., Huuskonen, J., Ojala, J., Kauppinen, A., Kaarniranta, K. and Suuronen, T., 2008. Activation of innate immunity system during aging: NF-kB signaling is the molecular culprit of inflamm-aging. *Ageing research reviews*, *7*(2), pp.83-105.

Šamec, D., Durgo, K., Grúz, J., Kremer, D., Kosalec, I., Piljac-Žegarac, J. and Salopek-Sondi, B., 2015. Genetic and phytochemical variability of six Teucrium arduini L. populations and their antioxidant/prooxidant behaviour examined by biochemical, macromolecule-and cell-based approaches. *Food chemistry*, *186*, pp.298-305.

Samsom, J.N., 2004. Regulation of antigen-specific regulatory T-cell induction via nasal and oral mucosa. *Critical Reviews™ in Immunology*, *24*(3).

Sandasi, M., Leonard, C.M. and Viljoen, A.M., 2010. The in vitro antibiofilm activity of selected culinary herbs and medicinal plants against Listeria monocytogenes. *Letters in applied microbiology*, *50*(1), pp.30-35.

Sandulache, V.C., Dohar, J.E. and Hebda, P.A., 2005. Adult-fetal fibroblast interactions: effects on cell migration and implications for cell transplantation.

Santos, A., 2003. Evidence-based control of plaque and gingivitis. *Journal of clinical periodontology*, *30*, pp.13-16.

Sarabhai, S., Sharma, P. and Capalash, N., 2013. Ellagic acid derivatives from Terminalia chebula Retz. downregulate the expression of quorum sensing genes to attenuate Pseudomonas aeruginosa PAO1 virulence. *PLoS one*, *8*(1), p.e53441.

Saraf, S., 2012. Formulating moisturizers using natural raw materials. In *Treatment of Dry Skin Syndrome* (pp. 379-397). Springer, Berlin, Heidelberg.

Saraf, S., Kour Chhabra, S., Deep Kaur, C. and Saraf, S., 2012. Development of photochemoprotective herbs containing cosmetic formulations for improving skin properties. *Journal of cosmetic science*, *63*(2), p.119.

Saraste, A. and Pulkki, K., 2000. Morphologic and biochemical hallmarks of apoptosis. *Cardiovascular research*, *45*(3), pp.528-537.

Sardesai, V.M., 2002. Herbal medicines: Poisons or potions?. *Journal of Laboratory and Clinical Medicine*, *139*(6), pp.343-348.

Sarrà, M., Casas, C., Poch, M. and Gòdia, F., 1999. A simple structured model for continuous production of a hybrid antibiotic by Streptomyces lividans pellets in a fluidized-bed bioreactor. *Applied biochemistry and biotechnology*, *80*(1), pp.39-50.

Sasaki, H., Matsumoto, M., Tanaka, T., Maeda, M., Nakai, M., Hamada, S. and Ooshima, T., 2004. Antibacterial activity of polyphenol components in oolong tea extract against Streptococcus mutans. *Caries Research*, *38*(1), pp.2-8.

Satomi, H., Umemura, K., Ueno, A., Hatano, T., Okuda, T. and Noro, T., 1993. Carbonic anhydrase inhibitors from the pericarps of Punica granatum L. *Biological and Pharmaceutical Bulletin*, *16*(8), pp.787-790.

Satomi, H., Umemura, K., Ueno, A., Hatano, T., Okuda, T. and Noro, T., 1993. Carbonic anhydrase inhibitors from the pericarps of Punica granatum L. *Biological and Pharmaceutical Bulletin*, *16*(8), pp.787-790.

Scannapieco, F.A., Bergey, E.J., Reddy, M.S. and Levine, M.J., 1989. Characterization of salivary alpha-amylase binding to Streptococcus sanguis. *Infection and immunity*, *57*(9), pp.2853-2863.

Schäfer, M. and Werner, S., 2007. Transcriptional control of wound repair. *Annu. Rev. Cell Dev. Biol.*, *23*, pp.69-92.

Schmuch, J., Beckert, S., Brandt, S., Löhr, G., Hermann, F., Schmidt, T.J., Beikler, T. and Hensel, A., 2015. Extract from Rumex acetosa L. for prophylaxis of periodontitis: inhibition of bacterial in vitro adhesion and of gingipains of Porphyromonas gingivalis by epicatechin-3-O-(4β→ 8)-epicatechin-3-O-gallate (procyanidin-B2-Di-gallate). *Plos one*, *10*(3), p.e0120130.

Schroeder, H.E. and Shanley, D., 1969. Formation and inhibition of dental calculus. *Journal of Periodontology-Periodontics*, *40*(11), pp.643-646.

Schroeder, H.E., 1981. References/Author Index/Subject Index. In *Differentiation of Human Oral Stratified Epithelia* (pp. 269-306). Karger Publishers.

Schwartz, J.R., Marsh, R.G. and Draelos, Z.D., 2005. Zinc and skin health: overview of physiology and pharmacology. *Dermatologic surgery*, *31*, pp.837-847.

Sciubba, J.J., Waterhouse, J.P. and Meyer, J., 1978. A fine structural comparison of the healing of incisional wounds of mucosa and skin. *Journal of Oral Pathology & Medicine*, *7*(4), pp.214-227.

Scully, C., Ei-Kabir, M. and Samaranayake, L.P., 1994. Candida and oral candidosis: a review. *Critical Reviews in Oral Biology & Medicine*, *5*(2), pp.125-157.

Scutera, S., Zucca, M. and Savoia, D., 2014. Novel approaches for the design and discovery of quorum-sensing inhibitors. *Expert Opinion on Drug Discovery*, *9*(4), pp.353-366.

Seeram, N., Lee, R., Hardy, M. and Heber, D., 2005. Rapid large scale purification of ellagitannins from pomegranate husk, a by-product of the commercial juice industry. *Separation and purification technology*, *41*(1), pp.49-55.

Seeram, N.P., Adams, L.S., Henning, S.M., Niu, Y., Zhang, Y., Nair, M.G. and Heber, D., 2005. In vitro antiproliferative, apoptotic and antioxidant activities of punicalagin, ellagic acid and a total pomegranate tannin extract are enhanced in combination with other polyphenols as found in pomegranate juice. *The Journal of nutritional biochemistry*, *16*(6), pp.360-367.

Seeram, N.P., Zhang, Y. and Heber, D., 2006. 12 Commercialization of Pomegranates: Fresh Fruit, Beverages, and Botanical Extracts. *Pomegranates*, p.187.

Segal, A. and Wong, D.T., 2008. Salivary diagnostics: enhancing disease detection and making medicine better. *European journal of dental education: official journal of the Association for Dental Education in Europe*, *12*(Suppl 1), p.22.

Selwitz, R.H., Ismail, A.I. and Pitts, N.B., 2007. Dental caries. *The Lancet*, *369*(9555), pp.51-59.

Sentandreu, E., Cerdán-Calero, M. and Sendra, J.M., 2013. Phenolic profile characterization of pomegranate (Punica granatum) juice by high-performance liquid chromatography with diode array detection coupled to an electrospray ion trap mass analyzer. *Journal of Food Composition and Analysis*, *30*(1), pp.32-40.

Serra, M.B., Barroso, W.A., Silva, N.N.D., Silva, S.D.N., Borges, A.C.R., Abreu, I.C. and Borges, M.O.D.R., 2017. From inflammation to current and alternative therapies involved in wound healing. *International Journal of Inflammation*, *2017*.

Serrano, C., Torres, N., Valdivieso, C., Castaño, C., Barrera, M. and Cabrales, A., 2009. Antibiotic resistance of periodontal pathogens obtained from frequent antibiotic users. *Acta odontológica latinoamericana: AOL*, *22*(2), pp.99-104.

Seymour, G.J., Ford, P.J., Cullinan, M.P., Leishman, S. and Yamazaki, K., 2007. Relationship between periodontal infections and systemic disease. *Clinical Microbiology and Infection*, *13*, pp.3-10.

Share Dental Care (2021) *Gum disease.* Available at: https://sharedentalcare.com/gingivitis-vs-periodontitis/ (Accessed: 12 September 2021).

Sharma, N.C., Lyle, D.M., Qaqish, J.G., Galustians, J. and Schuller, R., 2008. Effect of a dental water jet with orthodontic tip on plaque and bleeding in adolescent patients with fixed orthodontic appliances. *American Journal of Orthodontics and Dentofacial Orthopedics*, *133*(4), pp.565-571.

Sharma, S. and Sabnis, S., 2010. Study of anti adhesive properties of fruit juices and plant extracts on urinary tract pathogens. *Asian J Exp Biol Sci Spl*, *3*, pp.100-3.

Sharon, N. and Ofek, I., 2000. Safe as mother's milk: carbohydrates as future anti-adhesion drugs for bacterial diseases. *Glycoconjugate journal*, *17*(7), pp.659-664.

Sharon, N., 1987. Bacterial lectins, cell-cell recognition and infectious disease. *FEBS letters*, *217*(2), pp.145-157.

Shi, D., Mi, G., Wang, M. and Webster, T.J., 2019. In vitro and ex vivo systems at the forefront of infection modeling and drug discovery. *Biomaterials*, *198*, pp.228-249.

Shi, X., Hanley, S.A., Faray-Kele, M.C., Fawell, S.C., Aduse-Opoku, J., Whiley, R.A., Curtis, M.A. and Hall, L.M., 2007. The rag locus of Porphyromonas gingivalis contributes to virulence in a murine model of soft tissue destruction. *Infection and immunity*, *75*(4), pp.2071-2074.

Shigeta, Y., Ogawa, T., Tomoko, I., Clark, G.T. and Enciso, R., 2010. Soft palate length and upper airway relationship in OSA and non-OSA subjects. *Sleep and Breathing*, *14*(4), pp.353-358.

Shimkets, L.J. and Brun, Y.V., 1999. Prokaryotic development: strategies to enhance survival. *Prokaryotic development*, pp.1-7.

Shirode, A.B., Bharali, D.J., Nallanthighal, S., Coon, J.K., Mousa, S.A. and Reliene, R., 2015. Nanoencapsulation of pomegranate bioactive compounds for breast cancer chemoprevention. *International journal of nanomedicine*, *10*, p.475.

Shirode, A.B., Kovvuru, P., Chittur, S.V., Henning, S.M., Heber, D. and Reliene, R., 2014. Antiproliferative effects of pomegranate extract in MCF-7 breast cancer cells are associated with reduced DNA repair gene expression and induction of double strand breaks. *Molecular carcinogenesis*, *53*(6), pp.458-470.

Shukla, M., Gupta, K., Rasheed, Z., Khan, K.A. and Haqqi, T.M., 2008. Consumption of hydrolyzable tannins-rich pomegranate extract suppresses inflammation and joint damage in rheumatoid arthritis. *Nutrition*, *24*(7-8), pp.733-743.

Siang, S. T., 1983. Use of combined traditional Chinese and Western medicine in the management of burns. *Panminerva medica*, *25*(3), 197-202.

Signoretto, C., Bianchi, F., Burlacchini, G., Sivieri, F., Spratt, D. and Canepari, P., 2010. Drinking habits are associated with changes in the dental plaque microbial community. *Journal of clinical microbiology*, *48*(2), pp.347-356.

Signoretto, C., Burlacchini, G., Bianchi, F., Cavalleri, G. and Canepari, P., 2006. Differences in microbiological composition of saliva and dental plaque in subjects with different drinking habits. *The new microbiologica*, *29*(4), pp.293-302.

Signoretto, C., Canepari, P., Stauder, M., Vezzulli, L. and Pruzzo, C., 2012. Functional foods and strategies contrasting bacterial adhesion. *Current opinion in biotechnology*, *23*(2), pp.160-167.

Silver, S., 1996. Bacterial resistances to toxic metal ions-a review. *Gene*, *179*(1), pp.9-19.

Simões, L.C., Simões, M. and Vieira, M.J., 2007. Biofilm interactions between distinct bacterial genera isolated from drinking water. *Applied and environmental microbiology*, *73*(19), pp.6192-6200.

Simon, S., Smith, A.J., Lumley, P.J., Berdal, A., Smith, G., Finney, S. and Cooper, P.R., 2009. Molecular characterization of young and mature odontoblasts. *Bone*, *45*(4), pp.693-703.

Singh, B., Singh, J.P., Kaur, A. and Singh, N., 2019. Antimicrobial potential of pomegranate peel: a review. *International Journal of Food Science & Technology*, *54*(4), pp.959-965.

Singh, G. and Triadafilopoulos, G., 1999. Epidemiology of NSAID induced gastrointestinal complications. *The Journal of rheumatology. Supplement*, *56*, pp.18-24.

Singh, K., Jaggi, A.S. and Singh, N., 2009. Exploring the ameliorative potential of Punica granatum in dextran sulfate sodium induced ulcerative colitis in mice. *Phytotherapy Research*, *23*(11), pp.1565-1574.

Singh, R.P., Chidambara Murthy, K.N. and Jayaprakasha, G.K., 2002. Studies on the antioxidant activity of pomegranate (Punica granatum) peel and seed extracts using in vitro models. *Journal of agricultural and food chemistry*, *50*(1), pp.81-86.

Singh, V.P. and Katiyar, A., 2008. Synthesis, spectral characterization and antimicrobial activity of some transition metal (II) complexes with acetone p-amino acetophenone benzoylhydrazone. *Pesticide Biochemistry and Physiology*, *92*(1), pp.8-14.

Singh, Y. N., 1986. Traditional medicine in Fiji: some herbal folk cures used by Fiji Indians. *Journal of ethnopharmacology*, *15*(1), 57-88.

Siqueira, W.L., Bakkal, M., Xiao, Y., Sutton, J.N. and Mendes, F.M., 2012. Quantitative proteomic analysis of the effect of fluoride on the acquired enamel pellicle. *PloS one*, *7*(8), p.e42204.

Sis, N.M., Semsari, M.C., Eshratkhah, B., Sadaghian, M., Gorbani, A. and Hassanpour, S., 2011. during naturally acquired mixed nematode infections in Moghani sheep. *Annals of Biological Research*, *2*(2), pp.170-174.

Slimestad, R., Fossen, T. and Vågen, I.M., 2007. Onions: a source of unique dietary flavonoids. *Journal of agricultural and food chemistry*, *55*(25), pp.10067-10080.

Sloan, A.J. and Waddington, R.J., 2009. Dental pulp stem cells: what, where, how?. *International Journal of Paediatric Dentistry*, *19*(1), pp.61-70.

Sloan, A.J., Shelton, R.M., Hann, A.C., Moxham, B.J. and Smith, A.J., 1998. An in vitro approach for the study of dentinogenesis by organ culture of the dentine–pulp complex from rat incisor teeth. *Archives of oral biology*, *43*(6), pp.421-430.

Sloan, A.J., Taylor, S.Y., Smith, E.L., Roberts, J.L., Chen, L., Wei, X.Q. and Waddington, R.J., 2013. A novel ex vivo culture model for inflammatory bone destruction. *Journal of dental research*, *92*(8), pp.728-734.

Slots, J. and Gibbons, R.J., 1978. Attachment of Bacteroides melaninogenicus subsp. asaccharolyticus to oral surfaces and its possible role in colonization of the mouth and of periodontal pockets. *Infection and immunity*, *19*(1), pp.254-264.

Smartt, J., 1976. *Evolution of crop plants* (No. 631.58 E9/2 pt.). N. W. Simmonds (Ed.). London: Longman.

Smith, E.L., Locke, M., Waddington, R.J. and Sloan, A.J., 2010. An ex vivo rodent mandible culture model for bone repair. *Tissue Engineering Part C: Methods*, *16*(6), pp.1287-1296.

Smith, R.S., Smith, T.J., Blieden, T.M. and Phipps, R.P., 1997. Fibroblasts as sentinel cells. Synthesis of chemokines and regulation of inflammation. *The American journal of pathology*, *151*(2), p.317.

Smullen, J., Koutsou, G.A., Foster, H.A., Zumbé, A. and Storey, D.M., 2007. The antibacterial activity of plant extracts containing polyphenols against Streptococcus mutans. *Caries research*, *41*(5), pp.342-349.

Socransky, S.S. and Haffajee, A.D., 2005. Periodontal microbial ecology. *Periodontology 2000*, *38*(1), pp.135-187.

Socransky, S.S., 1977. Microbiology of periodontal disease—present status and future considerations. *Journal of periodontology*, *48*(9), pp.497-504.

Socransky, S.S., Haffajee, A.D., Cugini, M.A., Smith, C. and Kent Jr, R.L., 1998. Microbial complexes in subgingival plaque. *Journal of clinical periodontology*, *25*(2), pp.134-144.

Socransky, S.S., Manganiello, A.D., Propas, D., Oram, V. and Van Houte, J., 1977. Bacteriological studies of developing supragingival dental plaque. *Journal of periodontal research*, *12*(2), pp.90-106.

Solanki, G., 2012. A general overview of gingiva. *IJBR*, *3*(02), pp.79-82.

Song, J., Choi, B., Jin, E.J., Yoon, Y. and Choi, K.H., 2012. Curcumin suppresses Streptococcus mutans adherence to human tooth surfaces and extracellular matrix proteins. *European journal of clinical microbiology & infectious diseases*, *31*(7), pp.1347-1352.

Sorrenti, V., Randazzo, C.L., Caggia, C., Ballistreri, G., Romeo, F.V., Fabroni, S., Timpanaro, N., Raffaele, M. and Vanella, L., 2019. Beneficial effects of pomegranate peel extract and probiotics on pre-adipocyte differentiation. *Frontiers in microbiology*, *10*, p.660.

Sparvoli, F., Martin, C., Scienza, A., Gavazzi, G. and Tonelli, C., 1994. Cloning and molecular analysis of structural genes involved in flavonoid and stilbene biosynthesis in grape (Vitis vinifera L.). *Plant molecular biology*, *24*(5), pp.743-755.

Springman, E.B., Nagase, H., Birkedal-Hansen, H. and Van Wart, H.E., 1995. Zinc content and function in human fibroblast collagenase. *Biochemistry*, *34*(48), pp.15713-15720.

Squier, C. and Brogden, K., 2010. *Human oral mucosa: development, structure and function*. John Wiley & Sons.

Squier, C.A. and Kremer, M.J., 2001. Biology of oral mucosa and esophagus. *JNCI Monographs*, *2001*(29), pp.7-15.

Sreenivasan, P. and Gaffar, A., 2002. Antiplaque biocides and bacterial resistance: a review. *Journal of clinical periodontology*, *29*(11), pp.965-974.

Staab, J.F., Bradway, S.D., Fidel, P.L. and Sundstrom, P., 1999. Adhesive and mammalian transglutaminase substrate properties of Candida albicans Hwp1. *Science*, *283*(5407), pp.1535-1538.

Stadler, A.F., Angst, P.D., Arce, R.M., Gomes, S.C., Oppermann, R.V. and Susin, C., 2016. Gingival crevicular fluid levels of cytokines/chemokines in chronic periodontitis: a meta-analysis. *Journal of clinical periodontology*, *43*(9), pp.727-745.

Stanley, P.M., 1983. Factors affecting the irreversible attachment of Pseudomonas aeruginosa to stainless steel. *Canadian journal of microbiology*, *29*(11), pp.1493-1499.

Stavri, M., Piddock, L.J. and Gibbons, S., 2007. Bacterial efflux pump inhibitors from natural sources. *Journal of antimicrobial chemotherapy*, *59*(6), pp.1247-1260.

Stavropoulos, A., Sculean, A., Bosshardt, D.D., Buser, D. and Klinge, B., 2015. Pre-clinical in vivo models for the screening of bone biomaterials for oral/craniofacial indications: focus on small-animal models. *Periodontology 2000*, *68*(1), pp.55-65.

Steeve, K.T., Marc, P., Sandrine, T., Dominique, H. and Yannick, F., 2004. IL-6, RANKL, TNF-alpha/IL-1: interrelations in bone resorption pathophysiology. *Cytokine & growth factor reviews*, *15*(1), pp.49-60.

Steinberg, D., Feldman, M., Ofek, I. and Weiss, E.I., 2004. Effect of a high-molecular-weight component of cranberry on constituents of dental biofilm. *Journal of Antimicrobial Chemotherapy*, *54*(1), pp.86-89.

Steinberg, D., Feldman, M., Ofek, I. and Weiss, E.I., 2005. Cranberry high molecular weight constituents promote Streptococcus sobrinus desorption from artificial biofilm. *International Journal of Antimicrobial Agents*, *25*(3), pp.247-251.

Stenudd, C., Nordlund, A., Ryberg, M., Johansson, I., KallestÅl, C. and Strömberg, N., 2001. The association of bacterial adhesion with dental caries. *Journal of dental research*, *80*(11), pp.2005-2010.

Stephen, K.W., Creanor, S.L., Russell, J.I., Burchell, C.K., Huntington, E. and Downie, C.F., 1988. A 3-year oral health dose-response study of sodium monofluoro-phosphate dentifrices with and without zinc citrate: anti-caries results. *Community dentistry and oral epidemiology*, *16*(6), pp.321-325.

Stephens, P. and Genever, P., 2007. Non-epithelial oral mucosal progenitor cell populations. *Oral diseases*, *13*(1), pp.1-10.

Sterzenbach, T., Helbig, R., Hannig, C. and Hannig, M., 2020. Bioadhesion in the oral cavity and approaches for biofilm management by surface modifications. *Clinical Oral Investigations*, pp.1-24.

Stewart, G.S., Jassim, S.A., Denyer, S.P., Newby, P., Linley, K. and Dhir, V.K., 1998. The specific and sensitive detection of bacterial pathogens within 4 h using bacteriophage amplification. *Journal of applied microbiology*, *84*(5), pp.777-783.

Stewart, P.S. and Costerton, J.W., 2001. Antibiotic resistance of bacteria in biofilms. *The lancet*, *358*(9276), pp.135-138.

Stewart, P.S. and Franklin, M.J., 2008. Physiological heterogeneity in biofilms. *Nature Reviews Microbiology*, *6*(3), pp.199-210.

Stewart, P.S., 2001. Multicellular resistance: biofilms. *Trends in microbiology*, *9*(5), p.204.

Still, D. W., 2006. 13 Pomegranates: A Botanical Perspective. *Pomegranates*, 199.

Stinson, M.W., Safulko, K. and Levine, M.J., 1991. Adherence of Porphyromonas (Bacteroides) gingivalis to Streptococcus sanguis in vitro. *Infection and Immunity*, *59*(1), pp.102-108.

Stoeken, J.E., Paraskevas, S. and Van Der Weijden, G.A., 2007. The long-term effect of a mouthrinse containing essential oils on dental plaque and gingivitis: a systematic review. *Journal of periodontology*, *78*(7), pp.1218-1228.

Stone, G., Wood, P., Dixon, L., Keyhan, M. and Matin, A., 2002. Tetracycline rapidly reaches all the constituent cells of uropathogenic Escherichia coli biofilms. *Antimicrobial agents and chemotherapy*, *46*(8), pp.2458-2461.

Stoodley, P., Lewandowski, Z., Boyle, J.D. and Lappin-Scott, H.M., 1999. The formation of migratory ripples in a mixed species bacterial biofilm growing in turbulent flow. *Environmental microbiology*, *1*(5), pp.447-455.

STUART, L.F., 1997. The history of oral hygiene products: how far have we come in 6000 years?. *Periodontology 2000*, *15*, pp.7-14.

Su, X., Sangster, M.Y. and D'Souza, D.H., 2010. In vitro effects of pomegranate juice and pomegranate polyphenols on foodborne viral surrogates. *Foodborne Pathogens and Disease*, *7*(12), pp.1473-1479.

Sudbeck, B.D., Pilcher, B.K., Welgus, H.G. and Parks, W.C., 1997. Induction and repression of collagenase-1 by keratinocytes is controlled by distinct components of different extracellular matrix compartments. *Journal of Biological Chemistry*, *272*(35), pp.22103-22110.

Sudbery, P.E., 2011. Growth of Candida albicans hyphae. *Nature Reviews Microbiology*, *9*(10), pp.737-748.

Sugawara, S., Uehara, A., Tamai, R. and Takada, H., 2002. Innate immune responses in oral mucosa. *Journal of endotoxin research*, *8*(6), pp.465-468.

Sugiyama, A., Uehara, A., Iki, K., Matsushita, K., Nakamura, R., Ogawa, T., Sugawara, S. and Takada, H., 2002. Activation of human gingival epithelial cells by cell-surface components of black-pigmented bacteria: augmentation of production of interleukin-8, granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor and expression of intercellular adhesion molecule 1. *Journal of medical microbiology*, *51*(1), pp.27-33.

Sum, E.Y., O'Reilly, L.A., Jonas, N., Lindeman, G.J. and Visvader, J.E., 2005. The LIM domain protein Lmo4 is highly expressed in proliferating mouse epithelial tissues. *Journal of Histochemistry & Cytochemistry*, *53*(4), pp.475-486.

Sun, J.N., Solis, N.V., Phan, Q.T., Bajwa, J.S., Kashleva, H., Thompson, A., Liu, Y., Dongari-Bagtzoglou, A., Edgerton, M. and Filler, S.G., 2010. Host cell invasion and virulence mediated by Candida albicans Ssa1. *PLoS Pathog*, *6*(11), p.e1001181.

Sundararajan, A., Ganapathy, R., Huan, L., Dunlap, J.R., Webby, R.J., Kotwal, G.J. and Sangster, M.Y., 2010. Influenza virus variation in susceptibility to inactivation by pomegranate polyphenols is determined by envelope glycoproteins. *Antiviral research*, *88*(1), pp.1-9.

Sundstrom, P., Cutler, J.E. and Staab, J.F., 2002. Reevaluation of the role of HWP1 in systemic candidiasis by use of Candida albicans strains with selectable marker URA3 targeted to the ENO1 locus. *Infection and immunity*, *70*(6), pp.3281-3283.

Sutherland, I.W., 2001. Microbial polysaccharides from Gram-negative bacteria. *International Dairy Journal*, *11*(9), pp.663-674.

Sutherland, I.W., 2001. The biofilm matrix–an immobilized but dynamic microbial environment. *Trends in microbiology*, *9*(5), pp.222-227.

Svensson, M., Platt, F.M. and Svanborg, C., 2006. Glycolipid receptor depletion as an approach to specific antimicrobial therapy. *FEMS microbiology letters*, *258*(1), pp.1-8.

Syed, S.A. and Loesche, W.J., 1978. Bacteriology of human experimental gingivitis: effect of plaque age. *Infection and immunity*, *21*(3), pp.821-829.

Szabo, J., Trombitas, K. and Szabo, I., 1984. The odontoblast process and its branches in human teeth observed by scanning electron microscopy. *Archives of oral biology*, *29*(4), pp.331-333.

Szczuka, E., Urbańska, K., Pietryka, M. and Kaznowski, A., 2013. Biofilm density and detection of biofilm-producing genes in methicillin-resistant Staphylococcus aureus strains. *Folia microbiologica*, *58*(1), pp.47-52.

Szpaderska, A.M., Walsh, C.G., Steinberg, M.J. and DiPietro, L.A., 2005. Distinct patterns of angiogenesis in oral and skin wounds. *Journal of dental research*, *84*(4), pp.309-314.

Szpaderska, A.M., Zuckerman, J.D. and DiPietro, L.A., 2003. Differential injury responses in oral mucosal and cutaneous wounds. *Journal of dental research*, *82*(8), pp.621-626.

Taha, A. A., Patel, M. P., Hill, R. G., & Fleming, P. S. (2017). The effect of bioactive glasses on enamel remineralization: A systematic review. *Journal of dentistry*, *67*, 9-17.

Tagboto, S. and Townson, S., 2001. Antiparasitic properties of medicinal plants and other naturally occurring products.

Takada, H., Mihara, J., Morisaki, I. and Hamada, S., 1991. Induction of interleukin-1 and-6 in human gingival fibroblast cultures stimulated with Bacteroides lipopolysaccharides. *Infection and immunity*, *59*(1), pp.295-301.

Takahashi, N. and Nyvad, B., 2011. The role of bacteria in the caries process: ecological perspectives. *Journal of dental research*, *90*(3), pp.294-303.

Takenaka, S., Ohshima, H., Ohsumi, T. and Okiji, T., 2012. Current and future strategies for the control of mature oral biofilms—Shift from a bacteria-targeting to a matrix-targeting approach. *Journal of Oral Biosciences*, *54*(4), pp.173-179.

Tamura, M., Tokuda, M., Nagaoka, S. and Takada, H., 1992. Lipopolysaccharides of Bacteroides intermedius (Prevotella intermedia) and Bacteroides (Porphyromonas) gingivalis induce interleukin-8 gene expression in human gingival fibroblast cultures. *Infection and immunity*, *60*(11), pp.4932-4937.

Tanaka, T., Nonaka, G.I. and Nishioka, I., 1986. Tannins and Related Compounds. XL.: Revision of the Structures of Punicalin and Punicalagin, and Isolation and Characterization of 2-O-Galloylpunicalin from the Bark of Punica granatum L. *Chemical and Pharmaceutical Bulletin*, *34*(2), pp.650-655.

Tang, J., Li, B., Hong, S., Liu, C., Min, J., Hu, M., Li, Y., Liu, Y. and Hong, L., 2017. Punicalagin suppresses the proliferation and invasion of cervical cancer cells through inhibition of the β-catenin pathway. *Molecular medicine reports*, *16*(2), pp.1439-1444.

Tao, L. and Tanzer, J.M., 2002. Novel sucrose-dependent adhesion co-factors in Streptococcus mutans. *Journal of dental research*, *81*(7), pp.505-510.

Tapiero, H. and Tew, K.D., 2003. Trace elements in human physiology and pathology: zinc and metallothioneins. *Biomedicine & Pharmacotherapy*, *57*(9), pp.399-411.

Tenovuo, J., Grahn, E., Lehtonen, O.P., Hyyppa, T., Karhuvaara, L. and Vilja, P., 1987. Antimicrobial factors in saliva: ontogeny and relation to oral health. *Journal of dental research*, *66*(2), pp.475-479.

Tezal, M., Sullivan, M.A., Hyland, A., Marshall, J.R., Stoler, D., Reid, M.E., Loree, T.R., Rigual, N.R., Merzianu, M., Hauck, L. and Lillis, C., 2009. Chronic periodontitis and the incidence of head and neck squamous cell carcinoma. *Cancer epidemiology and prevention biomarkers*, *18*(9), pp.2406-2412.

The European Committee on Antimicrobial Susceptibility Testing (2013). *EUCAST*. Available at: https://eucast.org/

Theilade, E., 1986. The non-specific theory in microbial etiology of inflammatory periodontal diseases. *Journal of clinical periodontology*, *13*(10), pp.905-911.

Thermo Scientific, *Pierce LDH Cytotoxicity Assay Kit.* Available at: https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0011851_Pierce_LDH_Cytotoxicity_Asy_UG.pdf (Accessed: 11 October 2021).

Tichy, J. and Novak, J., 1998. Extraction, assay, and analysis of antimicrobials from plants with activity against dental pathogens (Streptococcus sp.). *The Journal of Alternative and Complementary Medicine*, *4*(1), pp.39-45.

Tilburt, J.C. and Kaptchuk, T.J., 2008. Herbal medicine research and global health: an ethical analysis. *Bulletin of the World Health Organization*, *86*, pp.594-599.

Tintelnot, K., Haase, G., Seibold, M., Bergmann, F., Staemmler, M., Franz, T. and Naumann, D., 2000. Evaluation of phenotypic markers for selection and identification of Candida dubliniensis. *Journal of Clinical Microbiology*, *38*(4), pp.1599-1608.

Tomás-Barberán, F.A. and Espín, J.C., 2001. Phenolic compounds and related enzymes as determinants of quality in fruits and vegetables. *Journal of the Science of Food and Agriculture*, *81*(9), pp.853-876.

Tonetti, M.S., Jepsen, S., Jin, L. and Otomo-Corgel, J., 2017. Impact of the global burden of periodontal diseases on health, nutrition and wellbeing of mankind: A call for global action. *Journal of clinical periodontology*, *44*(5), pp.456-462.

Travis, J., Pike, R., Imamura, T. and Potempa, J., 1997. Porphyromonas gingivalis proteinases as virulence factors in the development of periodontitis. *Journal of periodontal research*, *32*(1), pp.120-125.

Tribble, G.D., Kerr, J.E. and Wang, B.Y., 2013. Genetic diversity in the oral pathogen Porphyromonas gingivalis: molecular mechanisms and biological consequences. *Future microbiology*, *8*(5), pp.607-620.

Trowbridge, H.O., Emling, R.C. and Fornatora, M., 1997. Inflammation. A review of the process. *Implant dentistry*, *6*(3), p.238.

Truong, V.K., Lapovok, R., Estrin, Y.S., Rundell, S., Wang, J.Y., Fluke, C.J., Crawford, R.J. and Ivanova, E.P., 2010. The influence of nano-scale surface roughness on bacterial adhesion to ultrafine-grained titanium. *Biomaterials*, *31*(13), pp.3674-3683.

Tsao, R., 2010. Chemistry and biochemistry of dietary polyphenols. *Nutrients*, *2*(12), pp.1231-1246.

Turabelidze, A., Guo, S., Chung, A.Y., Chen, L., Dai, Y., Marucha, P.T. and DiPietro, L.A., 2014. Intrinsic differences between oral and skin keratinocytes. *PloS one*, *9*(9), p.e101480.

Turgeneva, L.B., Novikov, V.E. and Tsenov, L.M., 1997. A clinico-pharmacological study of olifen in periodontal inflammation. *Eksperimental'naia i klinicheskaia farmakologiia*, *60*(2), pp.75-77.

Türkyılmaz, M., 2013. Anthocyanin and organic acid profiles of pomegranate (Punica granatum L.) juices from registered varieties in Turkey. *International journal of food science & technology*, *48*(10), pp.2086-2095.

Turnbaugh, P.J., Ley, R.E., Hamady, M., Fraser-Liggett, C.M., Knight, R. and Gordon, J.I., 2007. The human microbiome project. *Nature*, *449*(7164), pp.804-810.

Turrini, F., Malaspina, P., Giordani, P., Catena, S., Zunin, P. and Boggia, R., 2020. Traditional decoction and PUAE aqueous extracts of pomegranate peels as potential low-cost anti-tyrosinase ingredients. *Applied Sciences*, *10*(8), p.2795.

Tyagi, P., Dodwad, V., Kukreja, B.J. and Kukreja, P., 2021. A comparison of the efficacy of scaling and root planning with application of pomegranate chip, pomegranate gel, and scaling and root planing in sufferers with adult periodontitis–A prospective study. *Journal of Indian Society of Periodontology*, *25*(1), p.41.

Tyldesley, W.R. and Field, E.A., 1995. Oral medicine.

Tyszka-Czochara, M., Paśko, P., Reczyński, W., Szlósarczyk, M., Bystrowska, B. and Opoka, W., 2014. Zinc and propolis reduces cytotoxicity and proliferation in skin fibroblast cell culture: total polyphenol content and antioxidant capacity of propolis. *Biological trace element research*, *160*(1), pp.123-131.

Tzulker, R., Glazer, I., Bar-Ilan, I., Holland, D., Aviram, M. and Amir, R., 2007. Antioxidant activity, polyphenol content, and related compounds in different fruit juices and homogenates prepared from 29 different pomegranate accessions. *Journal of Agricultural and Food Chemistry*, *55*(23), pp.9559-9570.

Uivarosi, V., 2013. Metal complexes of quinolone antibiotics and their applications: an update. *Molecules*, *18*(9), pp.11153-11197.

Umar, D., Dilshad, B., Farhan, M., Ali, A. and Baroudi, K., 2016. The effect of pomegranate mouthrinse on Streptococcus mutans count and salivary pH: An in vivo study. *Journal of advanced pharmaceutical technology & research*, *7*(1), p.13.

Usha, C., Ramarao, S., John, B.M. and Babu, M.E., 2017. Anticariogenicity of Stevia rebaudiana extract when used as a mouthwash in high caries risk patients: Randomized controlled clinical trial. *World*, *8*(5), pp.364-369.

Vacchina, V., Lobinski, R., Oven, M. and Zenk, M.H., 2000. Signal identification in size-exclusion HPLC-ICP-MS chromatograms of plant extracts by electrospray tandem mass spectrometry (ES MS/MS). *Journal of Analytical Atomic Spectrometry*, *15*(5), pp.529-534.

Vallee, B.L. and Falchuk, K.H., 1993. The biochemical basis of zinc physiology. *Physiological reviews*, *73*(1), pp.79-118.

Valsaraj, R., Pushpangadan, P., Smitt, U.W., Adsersen, A. and Nyman, U., 1997. Antimicrobial screening of selected medicinal plants from India. *Journal of Ethnopharmacology*, *58*(2), pp.75-83.

Van Elswijk, D.A., Schobel, U.P., Lansky, E.P., Irth, H. and van der Greef, J., 2004. Rapid dereplication of estrogenic compounds in pomegranate (Punica granatum) using on-line biochemical detection coupled to mass spectrometry. *Phytochemistry*, *65*(2), pp.233-241.

Van Houdt, R. and Michiels, C.W., 2005. Role of bacterial cell surface structures in Escherichia coli biofilm formation. *Research in microbiology*, *156*(5-6), pp.626-633.

Van Houte, J., 1994. Role of micro-organisms in caries etiology. *Journal of dental research*, *73*(3), pp.672-681.

van Putten, S., Shafieyan, Y. and Hinz, B., 2016. Mechanical control of cardiac myofibroblasts. *Journal of molecular and cellular cardiology*, *93*, pp.133-142.

van Roon, J.A., van Roy, J.L., Gmelig-Meyling, F.H., Lafeber, F.P. and Bijlsma, J.W., 1996. Prevention and reversal of cartilage degradation in rheumatoid arthritis by interleukin-10 and interleukin-4. *Arthritis & Rheumatism*, *39*(5), pp.829-835.

Vandenbosch, D., Braeckmans, K., Nelis, H.J. and Coenye, T., 2010. Fungicidal activity of miconazole against Candida spp. biofilms. *Journal of Antimicrobial Chemotherapy*, *65*(4), pp.694-700.

Vanderouderaa, F. and Cummins, D., 1989. Delivery systems for agents in supra-gingival and sub-gingival plaque control. *Journal of Dental Research*, *68*, pp.1617-1624.

Vasconcelos, L.C.D.S., Sampaio, F.C., Sampaio, M.C.C., Pereira, M.D.S.V., Higino, J.S. and Peixoto, M.H.P., 2006. Minimum inhibitory concentration of adherence of Punica granatum Linn (pomegranate) gel against S. mutans, S. mitis and C. albicans. *Brazilian dental journal*, *17*, pp.223-227.

Vasudevan, R., 2014. Biofilms: microbial cities of scientific significance. *J Microbiol Exp*, *1*(3), p.00014.

Verma, N., Mohanty, A. and Lal, A., 2010. Pomegranate genetic resources and germplasm conservation: a review. *Fruit, Vegetable and Cereal Science and Biotechnology*, *4*(2), pp.120-125.

Verpoorte, R., 1998. Exploration of nature's chemodiversity: the role of secondary metabolites as leads in drug development. *Drug Discovery Today*, *3*(5), pp.232-238.

Verstrepen, K.J. and Klis, F.M., 2006. Flocculation, adhesion and biofilm formation in yeasts. *Molecular microbiology*, *60*(1), pp.5-15.

Viuda-Martos, M., Fernández-López, J. and Pérez-Álvarez, J.A., 2010. Pomegranate and its many functional components as related to human health: a review. *Comprehensive reviews in food science and food safety*, *9*(6), pp.635-654.

VIUDA-MARTOS, M.A.N.U.E.L., PÉREZ-ÁLVAREZ, J.A., Sendra, E. and FERNÁNDEZ-LÓPEZ, J.U.A.N.A., 2013. In vitro antioxidant properties of pomegranate (Punica granatum) peel powder extract obtained as coproduct in the juice extraction process. *Journal of Food Processing and Preservation*, *37*(5), pp.772-776.

von Bubnoff, D., Matz, H., Frahnert, C., Rao, M.L., Hanau, D., de la Salle, H. and Bieber, T., 2002. FcεRI induces the tryptophan degradation pathway involved in regulating T cell responses. *The Journal of Immunology*, *169*(4), pp.1810-1816.

Voravuthikunchai, S.P., Limsuwan, S. and Mitchell, H., 2006. Effects of Punica granatum pericarps and Quercus infectoria nutgalls on cell surface hydrophobicity and cell survival of Helicobacter pylori. *Journal of health science*, *52*(2), pp.154-159.

Vriens, A.P., Waaijman, T., Van Den Hoogenband, H.M., De Boer, E.M., Scheper, R.J. and Gibbs, S., 2008. Comparison of autologous full-thickness gingiva and skin substitutes for wound healing. *Cell Transplantation*, *17*(10-11), pp.1199-1209.

Wächtler, B., Wilson, D., Haedicke, K., Dalle, F. and Hube, B., 2011. From attachment to damage: defined genes of Candida albicans mediate adhesion, invasion and damage during interaction with oral epithelial cells. *PloS one*, *6*(2), p.e17046.

Waddington, R.J., Moseley, R. and Embery, G., 2000. Periodontal Disease Mechanisms: Reactive oxygen species: a potential role in the pathogenesis of periodontal diseases. *Oral diseases*, *6*(3), pp.138-151.

Wafa, B.A., Makni, M., Ammar, S., Khannous, L., Hassana, A.B., Bouaziz, M., Es-Safi, N.E. and Gdoura, R., 2017. Antimicrobial effect of the Tunisian Nana variety Punica granatum L. extracts against Salmonella enterica (serovars Kentucky and Enteritidis) isolated from chicken meat and phenolic composition of its peel extract. *International journal of food microbiology*, *241*, pp.123-131.

Wahab, S., Fiki, N.M.E.I., Mostafa, S. and Hassan, A., 1998. Characterization of certain steroid hormones in Punica granatum L. seeds.

Wajant, H., Pfizenmaier, K. and Scheurich, P., 2003. Tumor necrosis factor signaling. *Cell Death & Differentiation*, *10*(1), pp.45-65.

Walmsley, A.D., Walsh, T.F., Lumley, P., Burke, F.T., Shortall, A.C., Hayes-Hall, R. and Pretty, I., 2007. *Restorative dentistry*. elsevier health sciences.

Walmsley, M., Katsikis, P.D., Abney, E., Parry, S., Williams, R.O., Maini, R.N. and Feldmann, M., 1996. Interleukin-10 inhibition of the progression of established collagen-induced arthritis. *Arthritis & Rheumatism: Official Journal of the American College of Rheumatology*, *39*(3), pp.495-503.

Walsh, L.J., 2003. Mast cells and oral inflammation. *Critical reviews in oral biology & medicine*, *14*(3), pp.188-198.

Walsh, L.J., L'Estrange, P.R. and Seymour, G.J., 1996. High magnification in situ viewing of wound healing in oral mucosa. *Australian dental journal*, *41*(2), pp.75-79.

Walsh, T.N., Noonan, N., Hollywood, D., Kelly, A., Keeling, N. and Hennessy, T.P., 1996. A comparison of multimodal therapy and surgery for esophageal adenocarcinoma. *New England Journal of Medicine*, *335*(7), pp.462-467.

Walters III, M.C., Roe, F., Bugnicourt, A., Franklin, M.J. and Stewart, P.S., 2003. Contributions of antibiotic penetration, oxygen limitation, and low metabolic activity to tolerance of Pseudomonas aeruginosa biofilms to ciprofloxacin and tobramycin. *Antimicrobial agents and chemotherapy*, *47*(1), pp.317-323.

Wang, T.H., Zhang, J., Qiu, X.H., Bai, J.Q., Gao, Y.H. and Xu, W., 2016. Application of ultra-high-performance liquid chromatography coupled with LTQ-orbitrap mass spectrometry for the qualitative and quantitative analysis of Polygonum multiflorum Thumb. and its processed products. *Molecules*, *21*(1), p.40.

Wang, Y. and Ma, S., 2014. Small molecules modulating AHL-based quorum sensing to attenuate bacteria virulence and biofilms as promising antimicrobial drugs. *Current medicinal chemistry*, *21*(3), pp.296-311.

Ward, O.P., 2010. Microbial biosurfactants and biodegradation. *Biosurfactants*, pp.65-74.

Wasil, M., Halliwell, B. and Moorhouse, C.P., 1988. Scavenging of hypochlorous acid by tetracycline, rifampicin and some other antibiotics: a possible antioxidant action of rifampicin and tetracycline?. *Biochemical pharmacology*, *37*(4), pp.775-778.

Watabe, K., Nishi, M., Miyake, H. and Hirata, K., 1998. Lifestyle and gastric cancer: a case-control study. *Oncology reports*, *5*(5), pp.1191-1195.

Waters, C.M. and Bassler, B.L., 2005. Quorum sensing: cell-to-cell communication in bacteria. *Annu. Rev. Cell Dev. Biol.*, *21*, pp.319-346.

Watnick, P. and Kolter, R., 2000. Biofilm, city of microbes. *Journal of bacteriology*, *182*(10), pp.2675-2679.

Wayne, P.A., 2005. Clinical and laboratory standards institute; 2007. *Performance standards for antimicrobial susceptibility testing. CLSI document M100-S17*.

Wei, Y.Y., Yan, D., Japar, A., Qu, S.S., Haji, A.A. and Parhat, K., 2013. Effects of pomegranate flowers polyphenols on liver PON expression of diabetes combining non-alcoholic fat liver diseases rats. *Yao xue xue bao= Acta pharmaceutica Sinica*, *48*(1), pp.71-76.

Weiss, E.I., London, J., Kolenbrander, P.E., Hand, A.R. and Siraganian, R., 1988. Localization and enumeration of fimbria-associated adhesins of Bacteroides loescheii. *Journal of bacteriology*, *170*(3), pp.1123-1128.

Weissman, L., Garty, J. and Hochman, A., 2005. Rehydration of the lichen Ramalina lacera results in production of reactive oxygen species and nitric oxide and a decrease in antioxidants. *Applied and Environmental Microbiology*, *71*(4), pp.2121-2129.

Wen, Z.T., Yates, D., Ahn, S.J. and Burne, R.A., 2010. Biofilm formation and virulence expression by Streptococcus mutans are altered when grown in dual-species model. *BMC microbiology*, *10*(1), pp.1-9.

Wexler, H.M., Molitoris, E., Murray, P.R., Washington, J., Zabransky, R.J., Edelstein, P.H. and Finegold, S.M., 1996. Comparison of spiral gradient endpoint and agar dilution methods for susceptibility testing of anaerobic bacteria: a multilaboratory collaborative evaluation. *Journal of clinical microbiology*, *34*(1), pp.170-174.

Whaley, S.G., Berkow, E.L., Rybak, J.M., Nishimoto, A.T., Barker, K.S. and Rogers, P.D., 2017. Azole antifungal resistance in Candida albicans and emerging non-albicans Candida species. *Frontiers in microbiology*, *7*, p.2173.

Whitby, D.J. and Ferguson, M.W., 1991. The extracellular matrix of lip wounds in fetal, neonatal and adult mice. *Development*, *112*(2), pp.651-668.

White, T.C., Holleman, S., Dy, F., Mirels, L.F. and Stevens, D.A., 2002. Resistance mechanisms in clinical isolates of Candida albicans. *Antimicrobial agents and chemotherapy*, *46*(6), pp.1704-1713.

Whiteman, M. and Halliwell, B., 1997. Prevention of peroxynitrite-dependent tyrosine nitration and inactivation of α1-antiproteinase by antibiotics. *Free radical research*, *26*(1), pp.49-56.

Widyarman, A.S., Suhalim, O.P., Nandary, D. and Theodorea, C.F., 2018. Pomegranate juice inhibits periodontal pathogens biofilm in vitro. *Scientific Dental Journal*, *2*(3), pp.101-108.

Wikoff, W.R., Anfora, A.T., Liu, J., Schultz, P.G., Lesley, S.A., Peters, E.C. and Siuzdak, G., 2009. Metabolomics analysis reveals large effects of gut microflora on mammalian blood metabolites. *Proceedings of the national academy of sciences*, *106*(10), pp.3698-3703.

Wimpenny, J.W. and Colasanti, R., 1997. A unifying hypothesis for the structure of microbial biofilms based on cellular automaton models. *FEMS microbiology ecology*, *22*(1), pp.1-16.

Wolfaardt, G.M., Lawrence, J.R., Robarts, R.D., Caldwell, S.J. and Caldwell, D.E., 1994. Multicellular organization in a degradative biofilm community. *Applied and Environmental Microbiology*, *60*(2), pp.434-446.

Wolf, C.M. and Eastman, A., 1999. The Temporal Relationship between Protein Phosphatase, Mitochondrial CytochromecRelease, and Caspase Activation in Apoptosis. *Experimental cell research*, *247*(2), pp.505-513.

Wolff, M.S. and Larson, C., 2009. The cariogenic dental biofilm: good, bad or just something to control?. *Brazilian oral research*, *23*, pp.31-38.

Wölfle, U., Seelinger, G., Bauer, G., Meinke, M.C., Lademann, J. and Schempp, C.M., 2014. Reactive molecule species and antioxidative mechanisms in normal skin and skin aging. *Skin pharmacology and physiology*, *27*(6), pp.316-332.

Wong, J.W., Gallant-Behm, C., Wiebe, C., Mak, K., Hart, D.A., Larjava, H. and Häkkinen, L., 2009. Wound healing in oral mucosa results in reduced scar formation as compared with skin: evidence from the red Duroc pig model and humans. *Wound repair and regeneration*, *17*(5), pp.717-729.
Wood, T.K., Knabel, S.J. and Kwan, B.W., 2013. Bacterial persister cell formation and dormancy. *Applied and environmental microbiology*, *79*(23), pp.7116-7121.

Wren, R.C., 1956. Potter's new cyclopaedia of botanical drugs and preparations.

Wyrepkowski, C.C., Gomes da Costa, D.L.M., Sinhorin, A.P., Vilegas, W., De Grandis, R.A., Resende, F.A., Varanda, E.A. and Dos Santos, L.C., 2014. Characterization and quantification of the compounds of the ethanolic extract from Caesalpinia ferrea stem bark and evaluation of their mutagenic activity. *Molecules*, *19*(10), pp.16039-16057.

Xavier, J.B., Picioreanu, C., Rani, S.A., Van Loosdrecht, M. and Stewart, P.S., 2005. Biofilm control strategies based on enzymic disruption of the extracellular polymeric substance matrix-a modeling study. *Microbiology*, *151*(12), pp.3817-3832.

Xiao, C.J., Yu, X.J., Xie, J.L., Liu, S. and Li, S., 2018. Protective effect and related mechanisms of curcumin in rat experimental periodontitis. *Head & face medicine*, *14*(1), pp.1-8.

Xiao, J., Huang, X., Alkhers, N., Alzamil, H., Alzoubi, S., Wu, T.T., Castillo, D.A., Campbell, F., Davis, J., Herzog, K. and Billings, R., 2018. Candida albicans and early childhood caries: a systematic review and meta-analysis. *Caries research*, *52*(1-2), pp.102-112.

Xie, Y., Chen, J., He, J., Miao, X., Xu, M., Wu, X., Xu, B., Yu, L. and Zhang, W., 2014. Antimicrobial resistance and prevalence of resistance genes of obligate anaerobes isolated from periodontal abscesses. *Journal of periodontology*, *85*(2), pp.327-334.

Ximénez-Fyvie, L.A., Haffajee, A.D. and Socransky, S.S., 2000. Comparison of the microbiota of supra- and subgingival plaque in health and periodontitis. *Journal of clinical periodontology*, *27*(9), pp.648-657.

Xu, L.C. and Siedlecki, C.A., 2012. Submicron-textured biomaterial surface reduces staphylococcal bacterial adhesion and biofilm formation. *Acta biomaterialia*, *8*(1), pp.72-81.

Xu, X., Yin, P., Wan, C., Chong, X., Liu, M., Cheng, P., Chen, J., Liu, F. and Xu, J., 2014. Punicalagin inhibits inflammation in LPS-induced RAW264. 7 macrophages via the suppression of TLR4-mediated MAPKs and NF-κB activation. *Inflammation*, *37*(3), pp.956-965.

Yadav, M.K., Kwon, S.K., Cho, C.G., Park, S.W., Chae, S.W. and Song, J.J., 2012. Gene expression profile of early in vitro biofilms of Streptococcus pneumoniae. *Microbiology and immunology*, *56*(9), pp.621-629.

Yamasaki, H. and Grace, S.C., 1998. EPR detection of phytophenoxyl radicals stabilized by zinc ions: evidence for the redox coupling of plant phenolics with ascorbate in the H_2O_2-peroxidase system. *FEBS letters*, *422*(3), pp.377-380.

Yan, L., Zhou, X., Shi, L., Shalimu, D., Ma, C. and Liu, Y., 2017. Phenolic profiles and antioxidant activities of six Chinese pomegranate (Punica granatum L.) cultivars. *International journal of food properties*, *20*(sup1), pp.S94-S107.

Yang, L., Liu, Y., Wu, H., Høiby, N., Molin, S. and Song, Z.J., 2011. Current understanding of multi-species biofilms. *International journal of oral science*, *3*(2), pp.74-81.

Yang, X., Xie, L., Li, Y. and Wei, C., 2009. More than 9,000,000 unique genes in human gut bacterial community: estimating gene numbers inside a human body. *PloS one*, *4*(6), p.e6074.

Yang, Y., Mao, M., Lei, L., Li, M., Yin, J., Ma, X., Tao, X., Yang, Y. and Hu, T., 2019. Regulation of water-soluble glucan synthesis by the Streptococcus mutans dexA gene effects biofilm aggregation and cariogenic pathogenicity. *Molecular oral microbiology*, *34*(2), pp.51-63.

Yao, E.S., Lament, R.J., Leu, S.P. and Weinberg, A., 1996. Interbacterial binding among strains of pathogenic and commensal oral bacterial species. *Oral microbiology and immunology*, *11*(1), pp.35-41.

Yasoubi, P., Barzegar, M., SAHARI, M.A. and Azizi, M.H., 2007. Total phenolic contents and antioxidant activity of pomegranate (Punica granatum L.) peel extracts.

Yoneda, M., Hirofuji, T., Anan, H., Matsumoto, A., Hamachi, T., Nakayama, K. and Maeda, K., 2001. Mixed infection of Porphyromonas gingivalis and Bacteroides forsythus in a murine abscess model: involvement of gingipains in a synergistic effect. *Journal of periodontal research*, *36*(4), pp.237-243.

Yones, D.A., Badary, D.M., Sayed, H., Bayoumi, S.A., Khalifa, A.A. and El-Moghazy, A.M., 2016. Comparative evaluation of anthelmintic activity of edible and ornamental pomegranate ethanolic extracts against Schistosoma mansoni. *BioMed research international*, *2016*.

Yuan, Z. and Fang, Y., 2018. Flavonol s and F lavone s C hanges in P omegranate (Punica granatum L.) Fruit P eel during F ruit D evelopment.

Yucel-Lindberg, T. and Båge, T., 2013. Inflammatory mediators in the pathogenesis of periodontitis. *Expert reviews in molecular medicine*, *15*.

Zahin, M., Aqil, F. and Ahmad, I., 2010. Broad spectrum antimutagenic activity of antioxidant active fraction of Punica granatum L. peel extracts. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, *703*(2), pp.99-107.

Zaid, M.A., Afaq, F., Syed, D.N., Dreher, M. and Mukhtar, H., 2007. Inhibition of UVB-mediated oxidative stress and markers of photoaging in immortalized HaCaT keratinocytes by pomegranate polyphenol extract POMx. *Photochemistry and Photobiology*, *83*(4), pp.882-888.

Zakikhany, K., Naglik, J.R., Schmidt-Westhausen, A., Holland, G., Schaller, M. and Hube, B., 2007. In vivo transcript profiling of Candida albicans identifies a gene essential for interepithelial dissemination. *Cellular microbiology*, *9*(12), pp.2938-2954.

Zaouay, F., Mena, P., Garcia-Viguera, C. and Mars, M., 2012. Antioxidant activity and physico-chemical properties of Tunisian grown pomegranate (Punica granatum L.) cultivars. *Industrial Crops and Products*, *40*, pp.81-89.

Zarkan, A., Macklyne, H.R., Truman, A.W., Hesketh, A.R. and Hong, H.J., 2016. The frontline antibiotic vancomycin induces a zinc starvation response in bacteria by binding to Zn (II). *Scientific reports*, *6*(1), pp.1-9.

Zegarelli, D.J., 1993. Fungal infections of the oral cavity. *Otolaryngologic Clinics of North America*, *26*(6), pp.1069-1089.

Zeilhofer, H.U. and Brune, K., 2006. Analgesic strategies beyond the inhibition of cyclooxygenases. *Trends in pharmacological sciences*, *27*(9), pp.467-474.

Zelles, T., Purushotham, K.R., Macauley, S.P., Oxford, G.E. and Humphreys-Beher, M.G., 1995. Concise review: saliva and growth factors: the fountain of youth resides in us all. *Journal of dental research*, *74*(12), pp.1826-1832.

Zenobia, C., Luo, X.L., Hashim, A., Abe, T., Jin, L., Chang, Y., Jin, Z.C., Sun, J.X., Hajishengallis, G., Curtis, M.A. and Darveau, R.P., 2013. Commensal bacteria-dependent select expression of CXCL 2 contributes to periodontal tissue homeostasis. *Cellular microbiology*, *15*(8), pp.1419-1426.

Zhang, H. and Tsao, R., 2016. Dietary polyphenols, oxidative stress and antioxidant and anti-inflammatory effects. *Current Opinion in Food Science*, *8*, pp.33-42.

Zhang, J., Guan, S., Sun, J., Liu, T., Chen, P., Feng, R., Chen, X., Wu, W., Yang, M. and Guo, D.A., 2015. Characterization and profiling of phenolic amides from Cortex Lycii by ultra-high performance liquid chromatography coupled with LTQ-Orbitrap mass spectrometry. *Analytical and bioanalytical chemistry*, *407*(2), pp.581-595.

Zhang, J., Zhan, B., Yao, X., Gao, Y. and Shong, J., 1995. Antiviral activity of tannin from the pericarp of Punica granatum L. against genital Herpes virus in vitro. *Zhongguo Zhong yao za zhi= Zhongguo zhongyao zazhi= China journal of Chinese materia medica*, *20*(9), pp.556-8.

Zhang, Q., Nijampatnam, B., Hua, Z., Nguyen, T., Zou, J., Cai, X., Michalek, S.M., Velu, S.E. and Wu, H., 2017. Structure-based discovery of small molecule inhibitors of cariogenic virulence. *Scientific reports*, *7*(1), pp.1-10.

Zhang, X. and World Health Organization, 2002. Traditional medicine strategy 2002 2005.

Zhang, X., Kang, X., Jin, L., Bai, J., Liu, W. and Wang, Z., 2018. Stimulation of wound healing using bioinspired hydrogels with basic fibroblast growth factor (bFGF). *International journal of nanomedicine*, *13*, p.3897.

Zhang, X., Kohli, M., Zhou, Q., Graves, D.T. and Amar, S., 2004. Short-and long-term effects of IL-1 and TNF antagonists on periodontal wound healing. *The journal of immunology*, *173*(5), pp.3514-3523.

Zhao, F., Pang, W., Zhang, Z., Zhao, J., Wang, X., Liu, Y., Wang, X., Feng, Z., Zhang, Y., Sun, W. and Liu, J., 2016. Pomegranate extract and exercise provide additive benefits on improvement of immune function by inhibiting inflammation and oxidative stress in high-fat-diet-induced obesity in rats. *The Journal of nutritional biochemistry*, *32*, pp.20-28.

Zhao, L., Chu, P.K., Zhang, Y. and Wu, Z., 2009. Antibacterial coatings on titanium implants. *Journal of Biomedical Materials Research Part B: Applied Biomaterials*, *91*(1), pp.470-480.

Zhao, L.X., Li, D.D., Hu, D.D., Hu, G.H., Yan, L., Wang, Y. and Jiang, Y.Y., 2013. Effect of tetrandrine against Candida albicans biofilms. *PLoS One*, *8*(11), p.e79671.

Zhao, X., Yuan, Z., Fang, Y., Yin, Y. and Feng, L., 2013. Characterization and evaluation of major anthocyanins in pomegranate (Punica granatum L.) peel of different cultivars and their development phases. *European Food Research and Technology*, *236*(1), pp.109-117.

Zhao, Y., Tan, Y., Dai, J., Li, B., Guo, L., Cui, J., Wang, G., Shi, X., Zhang, X., Mellen, N. and Li, W., 2011. Exacerbation of diabetes-induced testicular apoptosis by zinc deficiency is most likely associated with oxidative stress, p38 MAPK activation, and p53 activation in mice. *Toxicology letters*, *200*(1-2), pp.100-106.

Zheng, L.F., Dai, F., Zhou, B., Yang, L. and Liu, Z.L., 2008. Prooxidant activity of hydroxycinnamic acids on DNA damage in the presence of Cu (II) ions: mechanism and structure–activity relationship. *Food and Chemical Toxicology*, *46*(1), pp.149-156.

Zhou, J., Qi, L. and Li, P., 2008. Quality control of Chinese herbal medicines with chromatographic fingerprints. *Se pu= Chinese journal of chromatography*, *26*(2), pp.153-159.

Zhou, J.L., Qi, L.W. and Li, P., 2009. Herbal medicine analysis by liquid chromatography/time-of-flight mass spectrometry. *Journal of chromatography A*, *1216*(44), pp.7582-7594.

Zhu, C.H., Ying, D.J., Mi, J.H., Zhang, W., Dong, S.W., Sun, J.S. and Zhang, J.P., 2004. The zinc finger protein A20 protects endothelial cells from burns serum injury. *Burns*, *30*(2), pp.127-133.

Zhu, J., Krom, B.P., Sanglard, D., Intapa, C., Dawson, C.C., Peters, B.M., Shirtliff, M.E. and Jabra-Rizk, M.A., 2011. Farnesol-induced apoptosis in Candida albicans is mediated by Cdr1-p extrusion and depletion of intracellular glutathione. *PloS one*, *6*(12), p.e28830.

Zhu, W. and Filler, S.G., 2010. Interactions of Candida albicans with epithelial cells. *Cellular microbiology*, *12*(3), pp.273-282.

Zhukovskii, P. M., 1971. Cultivated plants and their wild relatives. *Cultivated plants and their wild relatives.*

Zijnge, V., van Leeuwen, M.B.M., Degener, J.E., Abbas, F., Thurnheer, T., Gmür, R. and Harmsen, H.J., 2010. Oral biofilm architecture on natural teeth. *PloS one*, *5*(2), p.e9321.

Zobell, C.E., 1937. The influence of solid surface upon the physiological activities of bacteria in sea water. *J. Bacteriol*, *33*, p.86.

Zordan, R. and Cormack, B., 2011. Adhesins in opportunistic fungal pathogens. *Candida and candidiasis*, pp.243-P2.

Zschüttig, A., Zimmermann, K., Blom, J., Goesmann, A., Pöhlmann, C. and Gunzer, F., 2012. Identification and characterization of microcin S, a new antibacterial peptide produced by probiotic Escherichia coli G3/10. *PloS one*, *7*(3), p.e33351.

Appendix

Appendix 1: Experimental procedure for IL-1β ELISA assay.

PEPROTECH

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Rat IL-1-β
ELISA Development Kit
900-K91 Lot# 0212091
Expiration one year from date of receipt

Description: Rat IL-1-β ELISA development kit contains the key components required for the quantitative measurement of natural and/or recombinant rIL-1-β in a sandwich ELISA format within the range of 63–4000pg/ml. Using the ELISA protocol described below, the recommended microplates, reagents and solutions, the components supplied in this kit are sufficient to assay rIL-1- β in approximately 1000 ELISA plate wells.

RECONSTITUTION & STORAGE

Capture Antibody: 200µg of antigen-affinity purified rabbit anti-rIl-1 β + 2.5mg D-mannitol. Centrifuge vial prior to opening. Reconstitute in 1.0ml sterile water for a concentration of 200µg/ml.

Detection Antibody: 50µg of biotinylated antigen-affinity purified rabbit anti-rIL-1- β + 2.5mg D-mannitol. Centrifuge vial prior to opening. Reconstitute in 0.50ml sterile water for a concentration of 100µg/ml.

Rat IL-1- β Standard: 1µg of recombinant rIL-1- β + 2.2mg BSA + 11.0mg D-mannitol. Centrifuge vial prior to opening. Reconstitute in 1ml sterile water for a concentration of 1µg/ml.

Note: The reconstituted components are stable for 2 weeks when stored at 2-8°C. Components that have been reconstituted and aliquoted can be stored at -20°C for up to 6 months.

Avidin-HRP Conjugate: 60µl vial. Upon receipt, avidin-HRP conjugate should be aliquoted into ten 6µl vials and stored at ≤-20ºC. Aliquots stored frozen at ≤-20ºC are stable for up to 2 years form date of receipt. Avoid more than one freeze-thaw cycle. **Avidin should be used in conjunction with ABTS only.**

RECOMMENDED MATERIALS (or purchase PeproTech's ELISA Buffer Kit: Cat. # 900-K00)

ELISA microplates (Nunc MaxiSorp Prod. # 439454, or Corning Prod # 3590);
Tween-20 (Sigma Cat. # P-7949);
BSA (Sigma Cat # A-7030);
ABTS Liquid Substrate Solution (Sigma Cat. # A3219);
Dulbecco's PBS [10x] (Gibco BRL Cat. # 14200-075).

RECOMMENDED SOLUTIONS

All solutions should be at ambient temperature prior to use.
PBS: dilute 10xPBS to 1xPBS, pH 7.20 in sterile water.
Wash Buffer: 0.05% Tween-20 in PBS
Block Buffer: 1% BSA in PBS *
Diluent: 0.05% Tween-20, 0.1% BSA in PBS *
**** Sterile filter and store at 4ºC for up to 1 week.***

PLATE PREPARATION

1. Dilute capture antibody with PBS to a concentration of 2.0µg/ml. Immediately, add 100µl to each ELISA plate well. Seal the plate and incubate overnight at room temperature.
2. Aspirate the wells to remove liquid and wash the plate 4 times using 300µl of wash buffer per well. After the last wash invert plate to remove residual buffer and blot on paper towel.
3. Add 300µl block buffer to each well. Incubate for at least 1 hour at room temperature.
4. Aspirate and wash plate 4 times.

ELISA PROTOCOL

Standard/Sample: Dilute standard from 4ng/ml to zero in diluent. Immediately add 100µl of standard or sample to each well in triplicate. Incubate at room temperature for at least 2 hours.

Detection: Aspirate and wash plate 4 times. Dilute detection antibody in diluent to a concentration of 0.50µg/ml. Add 100µl per well. Incubate at room temperature for 2 hours.

Avidin Peroxidase: Aspirate and wash plate 4 times. Dilute one 5.5µl aliquot of Avidin Peroxidase 1:2000 in diluent for total volume of 11ml. Add 100µl per well. Incubate 30 minutes at room temperature.

ABTS Liquid Substrate:

(ABTS Substrate should be at ambient temperature prior to use)
Aspirate and wash plate 4 times. Add 100µl of substrate solution to each well. Incubate at room temperature for color development. Monitor color development with an ELISA plate reader at 405 nm with wavelength correction set at 650 nm.

NOTE: Reliable standard curves are obtained when either O.D. readings do not exceed 0.2 units for the zero standard concentrations, or 1.4 units for the highest standard concentration. The plate should be monitored at 5-minute intervals for approximately 30 minutes.

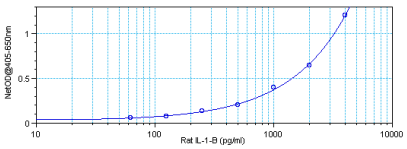
CROSS REACTIVITY

When tested at 50ng/ml the following antigens exhibited less than 5% cross reactivity:

Murine IL-1β

When tested at 50ng/ml the following antigens did not exhibit significant cross reactivity:

Human IL-1α, IL-2, sIL-2R, IL-4, sIL-4R
Murine IL-1α, IL-2, IL-4, IL-10
Rat GM-CSF, IL-1α, IL-2, IL-4, IL-10, SCF, TNFα



Appendix 2: Experimental procedure for TNF-α ELISA assay.

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Data Sheet

Rat TNF-α Mini ABTS ELISA Development Kit

Catalog# 900-M73
Lot# 0521073-M

Range of Detection: 47–3000 pg/ml

DESCRIPTION

ELISA Development Kit contains the key components required for the quantitative measurement of natural and/or recombinant Rat TNF-α in a sandwich ELISA format within the range of 47–3000 pg/ml. Using the ELISA protocol described below, the recommended microplates, reagents and solutions, the components supplied in this kit are sufficient to assay approximately 200 ELISA plate wells.

RECONSTITUTION & STORAGE

- **Capture Antibody*:** 21µg of Rabbit Anti-Rat TNF-α + 0.5mg D-mannitol. Centrifuge vial prior to opening. Reconstitute in 210µl sterile water for a concentration of 100µg/ml.
- **Detection Antibody*:** 21µg of Biotinylated Rabbit Anti-Rat TNF-α + 0.5mg D-mannitol. Centrifuge vial prior to opening. Reconstitute in 210µl PBS containing 0.1% BSA for a concentration of 100µg/ml.
- **Standard*:** 1µg of Recombinant Rat TNF-α + 2.2mg BSA + 11.0mg D-mannitol. Centrifuge vial prior to opening. Reconstitute in 1ml sterile water for a concentration of 1µg/ml.

***Note:* Lyophilized components can be stored at -20°C for up to a year from receipt. Reconstituted components are stable for 2 weeks when stored at 2-8°C. Aliquots of reconstituted components can be stored at -20°C for up to 6 months.**

- **Avidin-HRP Conjugate**:** 18µl vial. Expiration date on vial applies to unaliquoted material stored at 2-8°C. Centrifuge vial prior to opening. Upon receipt, avidin-HRP conjugate should be aliquoted into two 9µl vials and stored at ≤-20˚C. Aliquots stored frozen at ≤-20˚C are stable for up to 2 years from date of receipt. Avoid more than one freeze-thaw cycle.

***Note: Avidin should be used in conjunction with ABTS only.**

RECOMMENDED MATERIALS

Available in PeproTech's
ABTS ELISA Buffer Kit (Cat# 900-K00)

ELISA microplates: 96-well, flat-bottom
(Nunc MaxiSorp Prod. # 439454 or Corning Prod. # 3590)
Tween-20 (Sigma Cat. # P-7949)
BSA (Sigma Cat. # A-7030)
ABTS Liquid Substrate Solution (Sigma Cat. # A3219)
Dulbecco's PBS [10x] (Gibco BRL Cat. # 14200-075)

RECOMMENDED SOLUTIONS

All solutions should be at ambient temperature prior to use.

PBS: dilute 10xPBS to 1xPBS, pH 7.20 in sterile water.
Wash Buffer: 0.05% Tween-20 in PBS
Block Buffer: 1% BSA in PBS *
Diluent: 0.05% Tween-20, 0.1% BSA in PBS *
Sterile filter and store at 4˚C for up to 1 week.

PLATE PREPARATION

1. Dilute capture antibody with PBS to a concentration of 1.0µg/ml. Immediately, add 100µl to each ELISA plate well. Seal the plate and incubate overnight at room temperature.
2. Aspirate the wells to remove liquid and wash the plate 4 times using 300µl of wash buffer per well. After the last wash invert plate to remove residual buffer and blot on paper towel.
3. Add 300µl block buffer to each well. Incubate for at least 1 hour at room temperature.
4. Aspirate and wash plate 4 times.

ELISA PROTOCOL

Standard/Sample:
Dilute standard from 3000pg/ml to zero in diluent. Immediately add 100µl of standard or sample to each well in triplicate. Incubate at room temperature for at least 2 hours.

Detection:
Aspirate and wash plate 4 times. Dilute detection antibody in diluent to a concentration of 1.0µg/ml. Add 100µl per well. Incubate at room temperature for 2 hours.

Avidin-HRP Conjugate:
Aspirate and wash plate 4 times. Dilute 5.5µl of Avidin HRP-Conjugate 1:2000 in diluent for total volume of 11ml. Add 100µl per well. Incubate 30 minutes at room temperature.

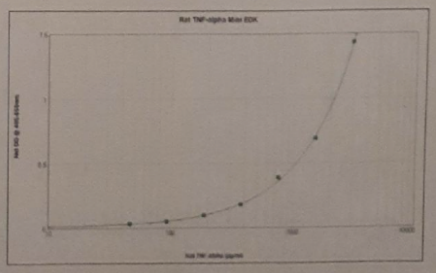
ABTS Liquid Substrate:
(ABTS Substrate should be at ambient temperature prior to use)
Aspirate and wash plate 4 times. Add 100µl of substrate solution to each well. Incubate at room temperature for color development. Monitor color development with an ELISA plate reader at 405nm with wavelength correction set at 650nm.

NOTE: Reliable standard curves are obtained when either O.D. readings do not exceed 0.2 units for the zero standard concentrations, or 1.4 units for the highest standard concentration. The plate should be monitored at 5-minute intervals for approximately 25 minutes. *O.D. readings may vary.*

CROSS REACTIVITY

When tested at 50ng/ml the following antigens exhibited complete O.D. saturation:
Murine: TNF-α

When tested at 50ng/ml the following antigens did not exhibit significant cross reactivity:
Human: TNF-α, TNF-β, sTNF Receptor Type I, sTNF Receptor Type II
Rat: CNTF, IFN-γ, IL-1α, IL-1β, IL-13, SCF



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Appendix 3: Experimental procedure for IL-6 ELISA assay.

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Data Sheet

Rat IL-6 Mini ABTS ELISA Development Kit

Catalog# 900-M86
Lot# 0415086-M

Range of Detection: 31–2000 pg/ml

DESCRIPTION

ELISA Development Kit contains the key components required for the quantitative measurement of natural and/or recombinant Rat IL-6 in a sandwich ELISA format within the range of 31–2000 pg/ml. Using the ELISA protocol described below, the recommended microplates, reagents and solutions, the components supplied in this kit are sufficient to assay approximately 200 ELISA plate wells.

RECONSTITUTION & STORAGE

- **Capture Antibody*:** 25µg of Goat Anti-Rat IL-6 + 0.5mg D-mannitol. Centrifuge vial prior to opening. Reconstitute in 250µl sterile water for a concentration of 100µg/ml.
- **Detection Antibody*:** 6µg of Biotinylated Goat Anti-Rat IL-6 + 0.5mg D-mannitol. Centrifuge vial prior to opening. Reconstitute in 60µl sterile water for a concentration of 100µg/ml.
- **Standard*:** 1µg of Recombinant Rat IL-6 + 2.2mg BSA + 11.0mg D-mannitol. Centrifuge vial prior to opening. Reconstitute in 1ml sterile water for a concentration of 1µg/ml.

***Note:* Lyophilized components can be stored at -20°C for up to a year from receipt. Reconstituted components are stable for 2 weeks when stored at 2-8°C. Aliquots of reconstituted components can be stored at -20°C for up to 6 months.**

- **Avidin-HRP Conjugate**:** 18µl vial. Expiration date on vial applies to unaliquoted material stored at 2-8°C. Centrifuge vial prior to opening. Upon receipt, avidin-HRP conjugate should be aliquoted into two 9µl vials and stored at ≤-20˚C. Aliquots stored frozen at ≤-20˚C are stable for up to 2 years from date of receipt. Avoid more than one freeze-thaw cycle.

***Note: Avidin should be used in conjunction with ABTS only.**

RECOMMENDED MATERIALS

Available in PeproTech's
ABTS ELISA Buffer Kit (Cat# 900-K00)

ELISA microplates: 96-well, flat-bottom
(Nunc MaxiSorp Prod. # 439454 or Corning Prod. # 3590)
Tween-20 (Sigma Cat. # P-7949)
BSA (Sigma Cat. # A-7030)
ABTS Liquid Substrate Solution (Sigma Cat. # A3219)
Dulbecco's PBS [10x] (Gibco BRL Cat. # 14200-075)

RECOMMENDED SOLUTIONS

All solutions should be at ambient temperature prior to use.

PBS: dilute 10xPBS to 1xPBS, pH 7.20 in sterile water.
Wash Buffer: 0.05% Tween-20 in PBS
Block Buffer: 1% BSA in PBS *
Diluent: 0.05% Tween-20, 0.1% BSA in PBS *
Sterile filter and store at 4˚C for up to 1 week.

PLATE PREPARATION

1. Dilute capture antibody with PBS to a concentration of 1.0µg/ml. Immediately, add 100µl to each ELISA plate well. Seal the plate and incubate overnight at room temperature.
2. Aspirate the wells to remove liquid and wash the plate 4 times using 300µl of wash buffer per well. After the last wash invert plate to remove residual buffer and blot on paper towel.
3. Add 300µl block buffer to each well. Incubate for at least 1 hour at room temperature.
4. Aspirate and wash plate 4 times.

ELISA PROTOCOL

Standard/Sample:
Dilute standard from 2000pg/ml to zero in diluent. Immediately add 100µl of standard or sample to each well in triplicate. Incubate at room temperature for at least 2 hours.

Detection:
Aspirate and wash plate 4 times. Dilute detection antibody in diluent to a concentration of 0.25µg/ml. Add 100µl per well. Incubate at room temperature for 2 hours.

Avidin-HRP Conjugate:
Aspirate and wash plate 4 times. Dilute 5.5µl of Avidin HRP-Conjugate 1:2000 in diluent for total volume of 11ml. Add 100µl per well. Incubate 30 minutes at room temperature.

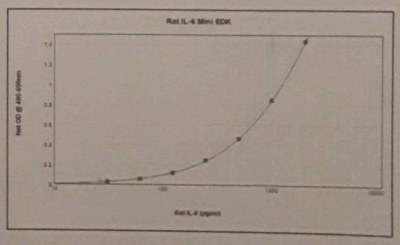
ABTS Liquid Substrate:
(ABTS Substrate should be at ambient temperature prior to use)
Aspirate and wash plate 4 times. Add 100µl of substrate solution to each well. Incubate at room temperature for color development. Monitor color development with an ELISA plate reader at 405nm with wavelength correction set at 650nm.

NOTE: Reliable standard curves are obtained when either O.D. readings do not exceed 0.2 units for the zero standard concentrations, or 1.4 units for the highest standard concentration. The plate should be monitored at 5-minute intervals for approximately 35 minutes. *O.D. readings may vary.*

CROSS REACTIVITY

When tested at 50ng/ml the following antigens exhibited complete O.D. saturation:
Murine: IL-6

When tested at 50ng/ml the following antigens did not exhibit significant cross reactivity:
Human: IL-3, IL-6, sIL-6 Receptor α, IL-11, Cardiotrophin-1, NNT-1/BCSF-3, Oncostatin M
Murine: IL-3, IL-11, Cardiotrophin-1
Rat: IL-3β, CNTF, Oncostatin M



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Appendix 4: Experimental procedure for PGE-2 ELISA assay.

SAMPLE PREPARATION

Cell culture supernate samples require a 3-fold dilution. A suggested 3-fold dilution is 150 µL sample + 300 µL of Calibrator Diluent RD5-39.

All serum, plasma, and urine samples require a 10-fold dilution. A suggested 10-fold dilution is 40 µL sample + 360 µL Calibrator Diluent RD5-39.

Note: *To avoid dilution of samples containing low levels of PGE_2, PGE_2 may be extracted from the sample matrix as outlined in the section below.*

SAMPLE EXTRACTION

1. Acidify the sample with 50 µL of 2 N HCl per 1.0 mL of sample. Allow to sit at 2 - 8° C for 15 minutes. Centrifuge the sample at 16,000 x g for 1 minute to remove any precipitate.

2. Prepare the C_{18} reverse phase column by washing with 10 mL of ethanol followed by 10 mL of deionized water.

3. Apply the sample under a slight positive pressure to obtain a flow rate of about 0.5 mL/minute. Wash the column with 10 mL of cold deionized water, followed by 10 mL of 15% ethanol, and 10 mL of hexane. Elute the sample from the column by the addition of 10 mL of ethyl acetate.

4. If analysis is to be delayed, store the sample as the eluted ethyl acetate solution at ≤ -70° C until the assay is to be run. To prepare the eluate for assay, evaporate the sample under a stream of nitrogen. Reconstitute with at least 250 µL of Calibrator Diluent RD5-39. Vortex well. Extracted samples may be concentrated by reconstituting in less than the original volume.

$$\text{Concentration factor} = \frac{\text{initial volume}}{\text{final volume}}$$

Note: *It is recommended that a control spike be run with each sample to determine the extraction efficiency. The PGE_2 Standard can be used as the spiking material.*

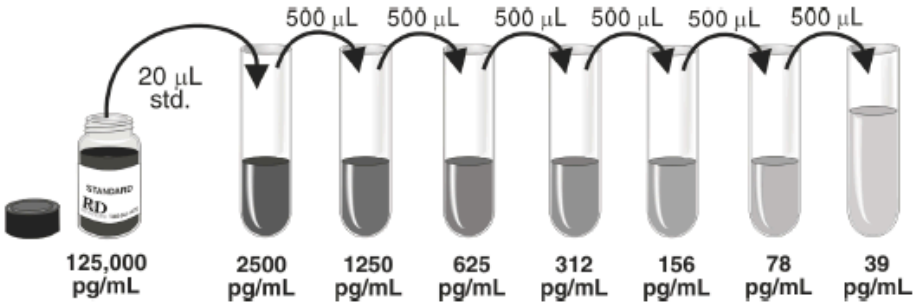
REAGENT PREPARATION

Bring all reagents to room temperature before use.

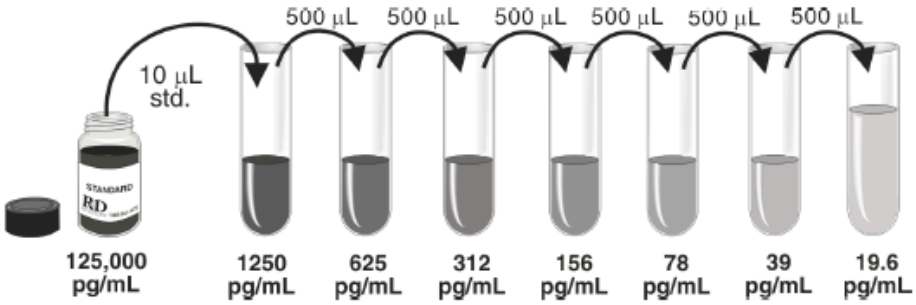
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 µL of the resultant mixture is required per well.

PGE_2 Standard, Regular Sensitivity Option - Pipette 980 µL of Calibrator Diluent RD5-39 into the 2500 pg/mL tube. Pipette 500 µL of Calibrator Diluent RD5-39 into the remaining tubes. Use the 125,000 pg/mL standard stock to produce a dilution series (below). Mix each tube thoroughly and change pipette tips between each transfer. The 2500 pg/mL standard serves as the high standard and Calibrator Diluent RD5-39 serves as the zero standard (B_0) (0 pg/mL). **Use diluted standards within 60 minutes of preparation.**



PGE_2 Standard, High Sensitivity Option - Pipette 990 µL of Calibrator Diluent RD5-39 into the 1250 pg/mL tube. Pipette 500 µL of Calibrator Diluent RD5-39 into the remaining tubes. Use the 125,000 pg/mL standard stock to produce a dilution series (below). Mix each tube thoroughly and change pipette tips between each transfer. The 1250 pg/mL standard serves as the high standard and Calibrator Diluent RD5-39 serves as the zero standard (B_0) (0 pg/mL). **Use diluted standards within 60 minutes of preparation.**



ASSAY PROCEDURE

REGULAR SENSITIVITY ASSAY OPTION

Bring all reagents and samples to room temperature before use. It is recommended that all samples, controls, and standards be assayed in duplicate.

1. Prepare all reagents, working standards and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, reseal.
3. Add 150 µL of Calibrator Diluent RD5-39 to the NSB wells.
4. Add 100 µL of Calibrator Diluent RD5-39 to the zero standard (B_0) wells.
5. Add 100 µL of Standard or sample* to the remaining wells.
6. Add 50 µL of the Primary Antibody Solution to each well (***excluding the NSB wells***). All wells except the NSB wells will now be blue in color.
7. Add 50 µL of PGE_2 Conjugate to each well. All wells except the NSB wells will now be violet in color. Cover with the adhesive strip provided.
8. Incubate for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm.
9. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
10. Add 200 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature **on the benchtop. Protect from light.**
11. Add 50 µL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
12. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 or 570 nm. If wavelength correction is not available, subtract readings at 540 or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

*Samples require dilution or extraction. See Sample Preparation section.

ASSAY PROCEDURE

HIGH SENSITIVITY ASSAY OPTION

Bring all reagents and samples to room temperature before use. It is recommended that all samples, controls, and standards be assayed in duplicate.

1. Prepare all reagents, working standards and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, reseal.
3. Add 200 µL of Calibrator Diluent RD5-39 to the NSB wells.
4. Add 150 µL of Calibrator Diluent RD5-39 to the zero standard (B_0) wells.
5. Add 150 µL of Standard or sample* to the remaining wells.
6. Add 50 µL of the Primary Antibody Solution to each well (***excluding the NSB wells***). All wells except the NSB wells will now be blue in color.
7. Add 50 µL of PGE_2 Conjugate to each well. All wells except the NSB wells will now be violet in color. Cover with the adhesive strip provided.
8. Incubate for 16 - 20 hours at 2 - 8° C.
9. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
10. Add 200 µL of Substrate Solution to each well. Incubate for 20 minutes at room temperature **on the benchtop. Protect from light.**
11. Add 50 µL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
12. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 or 570 nm. If wavelength correction is not available, subtract readings at 540 or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

*Samples require dilution or extraction. See Sample Preparation section.