Potential antimicrobial, anti-glycation and antioxidant properties of bioactive compounds of Indian spices and herbs extracted using ultrasound-assisted extraction process

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Potential antimicrobial, anti-glycation and antioxidant properties of bioactive compounds of Indian spices and herbs extracted using ultrasound-assisted extraction process

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Abstract

Food safety is a fundamental health concern for consumers and an ongoing challenge for food producers particularly in light of recent trends towards 'clean label' and minimally processed foods. Natural antimicrobial such as spice and herbs contain a variety of secondary metabolites such as phenolic acid, flavonoids, and terpenes compounds, which may be present in various parts of the plants possessing antimicrobial properties. These compounds have application in the preservation of foodstuffs, through inhibition of lipid oxidation, and inhibition of foodborne pathogens. The aim of the study was to investigate potential antimicrobial, anti-glycation and antioxidant properties of selected spices and herbs extracts obtained using ultrasound-assisted extraction in relation to their bioactive phenolic component.

Thirty-five India herbs and spice extracts prepared using ultrasound-assisted extraction process were screened for their antimicrobial properties against *Bacillus cereus*, *Listeria monocytogenes, Kocuria rhizohilia, E.coli, Salmonella typhi,* and *Pseudomonas aeruginosa.* Out of the 35 spices and herbs extracts, six spices that showed strong antimicrobial effectiveness, including ajwain, bay leaves, nutmeg, cumin, clove and Indian gooseberry were selected for further study. Antimicrobial properties were evaluated using Zone of Inhibition (ZoI), Minimum inhibition concentration (MIC) and Minimum bacterial concentration (MBC). Scanned electron microscopy (SEM) was used to evaluate the effect of spice extracts on the morphology and changes in cellular structure of the selected microorganisms. Gas-Chromatography Mass-Spectrometry (GCMS) and High-performance liquid chromatography (HPLC) was used to establish the bioactive phytochemical components responsible for antimicrobial properties of different ajwain varieties (which showed the highest antimicrobial effectiveness). The spice and herbs ethanolic extracts were also evaluated for their anti-glycation and antioxidant properties *in vitro*.

The results highlight strong antimicrobial, anti-glycation and antioxidant properties of ajwain due to good source of phenolic compounds. The quantification of bioactive phytochemical component in spice (ajwain) extract showed potential could be relevant to quality maker for producers wishing to use natural plant-based extracts as alternative to synthetic chemical preservatives in food safety and preservation.

Declaration

I hereby declare that the thesis is based on my original work except for citations which have been duly acknowledged. I also declare that it has not been accepted for any degree before and is not currently being submitted in candidature for any degree other than that of PhD of the Manchester Metropolitan University.

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Abbreviations list

ZoI	Zone of inhibition
MIC	Minimum Inhibition concentration
MBC	Minimum bacterial concentration
SEM	Scanned electron microscopy
GCMS	Gas chromatography mass spectrometry
HPLC	High performance liquid chromatography
DAD	Diode array detection
EO	Essential oil
UAE	Ultrasound-assisted extraction
BHT	Butylated hydroxytoluene
AGEs	Advanced glycation end-products
SDS PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel
TPC	Total phenolic content
GAE	Gallic acid equivalent
FRAP	Ferric reducing antioxidant power
DPPH	2,2-diphenyl-2-picrylhydrazyl hydrate
PTFE	Polytetrafluroethylene

Chapter 1

1.1 Introduction

In the last decades, there has been an increase in the consumption of minimally processed foods due to a change in consumer perceptions, leading to an increase in the number of outbreaks associated with foodborne illness (Alegre *et al*., 2013). Foodborne illness has become a pivotal important health concern for consumers and a main challenge for food producers due to the emergence of foodborne disease outbreaks caused by pathogenic microorganisms (Tajkarimi *et al*., 2010; Zhang *et al*., 2016). Numerous foodborne ailments are caused by ingestion of food contaminated with microbial pathogens (Pisoschi *et al*., 2018). Foodborne disease rates are expanding and involve a wide range of ailment including fever, headache, nausea, vomiting, abdominal pain, and diarrhoea (Webb *et al*., 2015). Each year around the world, unsafe food causes an expected 600 million instances of foodborne diseases, about 1 out of 10 individuals in the world become sick subsequent to eating contaminated food and 420,000 die consistently. 30% of foodborne deaths happen among children under 5 years of age. WHO evaluated that subsequent in the loss of 33 million healthy lives are lost globally every year (WHO 2019).There have been reports of foodborne outbreaks due to *Listeria*, *Escherichia coli*, *Staphylococcus aureus*, *Salmonella* and *Campylobacter* in contaminated food causing a serious health risk to humans (Zhang *et al*., 2016; Webb, 2015).

Every year a large number of individuals become infected by the most generally recognized foodborne pathogenic microorganisms such as *Salmonella, Campylobacter, and Escherichia coli*. Symptoms includes fever, headache, nausea, vomiting, abdominal pain and diarrhoea or sometimes serious and lethal results. For instances eggs, poultry and other animal origin products involved in outbreaks of salmonellosis. Raw milk, raw or undercooked poultry and drinking water are associated with foodborne cases with *Campylobacter*. Unpasteurized milk, undercooked meat and fresh fruits and vegetables caused *Escherichia coli* illness (WHO 2019). *E.coli* live in human intestines, most *E.coli* are harmless and some are pathogenic causes food illness, that are transmitted through contaminated water or food (CDC 2019). *E.coli* strains can produces toxicity and causes delayed wound-healing in diabetic patients by altering the mechanical barrier of the skin and vascular abnormalities (Zhang *et al*., 2016; Ahmed *et al*., 2005). It has been the culprit in many foodborne infections outbreaks linked to various foods

and produces a toxin that damages the insulin-producing cells, causing an insulin deficiency in diabetic patients (Smith, 2012).

Food supply chain includes production, handling, processing, distribution, and preparation. The complexity of food supply chain is a challenge for food manufacturers, as contamination can occur at any point along the chain leading to foodborne illnesses (Webb *et al*., 2015). Mostly, foods are heat processed for few seconds to minutes using temperatures differing from 60 to 100°C to destroy living microorganisms (Pisoschi *et al*., 2018; Nerin *et al*., 2016). However, heat treatment that food undergo can produce unwanted responses, leading to undesirable organoleptic and nutritional impacts. Ensuring food safety and in the meantime taking care of such demands for nutrition retention and quality characteristics, has lead to increased demand in interest in alternative method of preservation techniques for extending the shelf life of food product (Tiwari *et al*., 2009). Throughout the food supply chain, microbes are present everywhere and can contaminate food in various ways, such as through irrigation water at the farm, field workers, other insects, and faecal contamination by wild animals like rats, birds, as well as postharvest sources, for example workers handling, vehicles transport, and equipment for processing; water for washing; and cross-contamination from other foods. These microbes cause two main issues to the food supply chain i.e. the foodborne illness can cause hazard to human and the food loss due to food spoilage that is associated with economic losses (Davidson *et al*., 2015).

To inhibit the growth of foodborne pathogenic microorganisms in food products, synthetic chemical preservatives and antimicrobial agents are used by food manufactures (Witkowska *et al.,* 2013; Weerakkody *et al*., 2010). There are number of compounds approved as direct use as antimicrobial substance in food examples of such compounds are p-hydroxybenzoic acid, acetic acid, acetate salts, benzoic acid and benzoate salts, lactic acid and lactate salts, lysozyme, natamycin, nisin, nitrites and nitrates, phosphates, propionic acid and propionate salts, sorbic acid and sorbate salts, and sulfite derivatives (Davidson and Zivanovic, 2003). The death rate of the microorganisms in the presence preservative depend upon a number of factors such as preservative concentration, temperature, environmental conditions, water activity and pH. For instance - nitrites preserve the red colour of meat by reacting with the myoglobin in muscle tissue. The antimicrobial efficacy of nitrites is optimised at low pH.

However, nitrites are suspected to form carcinogenic nitrosamines (Gyawali and Ibrahim, 2014). The utilization of sulphite as preservative in food can cause allergic responses in sulphite sensitive persons. The use of synthetic phenolic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) has been reduced, due to health concerns (Pisoschi *et al*., 2018).

On the other hand, chemical synthetic preservatives cannot reduce foodborne pathogenic microorganisms such as *Listeria monocytogenes* or delay microbial spoilage totally (Liu *et al.,* 2017; Tajkarimi *et al*., 2010). Today consumers have more awareness and concern that synthetic preservatives have some toxic substances that cause adverse health effects (Witkowska *et al*., 2013). In response to increasing consumer opposition to synthetic preservatives in food, the food industry is effectively searching for alternative ways to protect food, while addressing the issue of microbial contamination. Hence, there is high demand for foods that are minimally processed and free from synthetic preservatives and contain natural antimicrobial compounds that provide food safety and preservation (Witkowska *et al*., 2013; Weerakkody *et al*., 2010). Furthermore, increase in microorganisms that are resistant to antibiotics and are tolerant to various food processing and preservation methods are a serious concern and there is demand for alternative antimicrobial agents (Gyawali and Ibrahim, 2014). During last decades, most studies have focussed mainly on the extraction of plant material and isolation of bioactive compounds as a potential natural antimicrobial agents that could inhibit the growth of foodborne pathogenic organisms and extend food shelf life (Tajkarimi *et al*., 2010; Weerakkody *et al*., 2010). Natural antimicrobial compounds present in foods can extend the shelf life of unprocessed food or processed foods by inhibiting the growth of microbes. Natural preservatives from different sources like bacteria, fungi, plant, animals, have the ability to ensure food safety because of their antimicrobial activity against foodborne pathogens (Pisoschi *et al*., 2018).

In the present study, around 35 spices and herbs (most commonly used in India) were assessed with regard to their antimicrobial activities against *Bacillus cereus, Listeria monocytogenes, Kocuria rhizophilia, Salmonella typhymurium, E.coli* and *Pseudomonas aeruginosa*. Screening of spices and herbs were studied for the selection of most efficient spices and herbs. The criteria for selection of spices and herbs were their popular organic use

(recent growing interest in application of organic material) (Moghaddam et al., 2015), availability to people, and ensuring that organic production and collection of samples from organic and primary procurement. Ultrasound-assisted extraction method selected for extraction of spices and herbs, due to high extraction efficiency, reduced extraction time with high reproducibility, which has not been used earlier for extraction of selected spices and herbs and investigated against selected microorganisms. (Tiwari, 2015; Asbahani et al., 2015).

1.1.1 Aims and objectives -

Aims - The present study aims to determine the antimicrobial, anti-glycation and antioxidant properties of spice and herb extracts obtained by ultrasound-assisted extraction.

Objectives –

- To investigate antimicrobial properties and screening of a number of spices and herbs and optimization of a method of extraction of potential bioactive compounds.
- Investigation of antimicrobial activity of spice and herb extracts using minimum inhibition concentration, minimum bacterial concentration, and scanning electron microscopy (SEM).
- Identification of bioactive ingredients of spice and herb extracts using GCMS and HPLC.
- Investigation of anti-glycation activity of extracts obtained from spices and herbs.
- Investigation of antioxidant properties of extracts obtained from spices and herbs.

1.2 Literature review

1.2.1 Chemical preservative

Chemical synthetic preservatives are used as antimicrobial in foods, food packaging or food processing to inhibit or inactivate pathogenic and spoilage microorganisms (Davidson and Zivanovic, 2003). According to Davidson et al (2013), an ideal antimicrobial should be effective at low concentration, be economical, cause no sensory change, inhibit pathogenic and spoilage microbes and be nontoxic. The use of chemical preservative or antimicrobials in food ensure microbioloical safety and already available for use in the food industry. There is the list of traditional regulatory approved compounds such as organic acids that function well only at a low pH. However, many synthetic antimicrobials pose possible toxicological problems, and major problems with food safety (Davidson and Zivanvic, 2003). Food companies seek natural plant-based antimicrobials which likely to be use as antimicrobial with ‘’green ‘’ or ‘’clean’’ labels. There seems to be gap in the knowledge regarding use of natural antimicrobial as food preservatives which can be used by the food industry. For instance, mustard, garlic has been used for thousands of years and improve immune system (Benkenlia, 2004; Rahman, 2007). This is because, there is strict requirement to gain approval for novel direct food additives, it requires to be tested *in vitro* and *in vivo*, can take years and a large amount of money to obtain approval (Davidson and Zivanovic, 2003).

All additives are thoroughly assessed for safety before they are allowed to be used. Approved additives are given a number, and some are also given an ‘E’ if they been accepted for safe use within the European Union (British Nutrition Foundation, 2018). Natural additives are substances found naturally in a foodstuff and are extracted from one food to be used in another. For example, beetroot juice with its bright purple colour can be used to colour other foods such as sweets (British Nutrition Foundation, 2018). Although some chemical compounds are found in nature and isolated from plants. These compounds chemically synthesised to gain regulatory approval to be use in food and can no longer called ‘’green label’’ for example, sorbic acid, acetic acid, benzoic acid. For instance, benzoic acid is a substance that is found in nature but is also made synthetically and used as a preservative (Davidson and Zivanovic, 2003).

1.2.2 Natural Antimicrobials

Natural antimicrobials are used in food to control food spoilage and prevent the growth of pathogenic microorganisms (Tajkarimi *et al*., 2010; Davidson and Zivanovic, 2003). Plant-based preservative has proved to be able to delay lipid oxidation, inhibit colour loss, prolonged storage life and ensure food safety. Various natural extracts obtained from plant materials such as herbs, spices, fruits and vegetables has been shown effective food preservation. Firstly, spices and herbs were added to food to change the flavour or to improve taste. There is an increased demand from food industry for more natural compounds that have greater antimicrobial properties and improved protective effects. An effective natural antimicrobial extracted from its natural source has potential benefits. Natural antimicrobials inhibiting a pathogenic organism in food, - must be effective during all stages of food processing, storage and distribution. To analyse antimicrobial activity, a standardised method would be necessary and need to be liable for any claims regarding effectiveness (Davidson and Zivanovic, 2003; Tajkarimi *et al*., 2010; Weerakkody *et al*., 2010). Numerous researchers have reported previously on spices and herbs that contain natural antimicrobial properties and can reduce microbial growth and extend the shelf life of unprocessed (raw) and processed foods (Ahmad *et al*., 2007; Tiwari *et al*., 2009; Tajkarimi *et al*., 2010). Therefore, natural antimicrobials are gaining attention for a number of issues such as consumers demanding natural alternatives to the current synthetic products and the need for new products emerging from microbial resistance to current antimicrobials (Tajkarimi *et al* 2010).

1.2.3 Spices and herbs

Spices and herbs that are being utilized for therapeutic purposes to treat a wide range of diseases (Park, 2011), tend to be strongly flavoured and used in small quantities as either a preservative or as a flavouring in food (Wei *et al*., 2016). Spices and herbs contain secondary metabolites – bioactive components such as phenolic compounds present in various parts of the plants (flowers, buds, seeds, leaves, twigs, bark, wood, fruits and roots) imparting antimicrobial and, antioxidant properties (Zheng and Wang, 2001; Burt, 2004; Witkowska *et al.*, 2013). Some of these substances are likewise known to add to the self-defence of plants against infectious microorganisms (Tiwari *et al*., 2009, Pisoschi *et al*., 2018). The summary of

the characteristics of 35 spices and herbs, common name and part used are enumerated in Table 1.1.

Table 1.1 - The summary of the characteristics of the spices and herbs used in this study, taken from the literature

Spices and Herbs	**Biological name and Family name**	**Physical characteristics and general description**	**Image of spice and herb and part used**
Ajwain	*Trachyspermum ammi* Apicaceae (Umbelliferae)	• Small, oval-shaped seeds (i.e fruits), • bitter and pungent taste with a flavour, • aroma (smell) almost exactly like thyme	 Seeds (i.e Fruit)
Asafoetida	*Asafoetida* Apiaceae	• Dried latex exuded from the rhizome of herb, • mainly cultivated in India. • It has a pungent smell. • It contains about 10-17% volatile oil, which is rich in various organo-sulfide compounds.	 Rhizome http://theepicentre.com/spice/asafetida/
Bay leaves	*Laurus nobilis* Lauraceae	• Light to medium green colour leaves • Contain about 1.3% essential oils.	 Leaves http://olives-organics.co.uk/product/bay-leaves-25g
Black pepper	*Piper nigrum* Piperaceae	• Cultivated in India • World's most traded spice. • 'King of spices'. • Pepper contains piperine	

			Fruit
Black salt	-	• type of rock salt • it is salty and pungent smelling • condiment used in Asia.	 https://www.exportersindia.com/jai-bharat-trading/black-salt-4289636.htm
Brown cardamom	*Amomum* Zingiberaceae	• Brown cardamom pods are used as a spice • Used in Indian cuisines.	 Seed pods
Brown mustard	*Brassica juncea* Brassica	• Small round shape seed • Mainly use in regional food. • Different types –brown, black and white/yellow mustard seeds.	 Seeds https://www.worldspice.com/spices/mustard-seed-yellow
Cinnamon	*Cinnamomum verum* Lauraceae	• Part used is inner bark of the tree • used as aromatic condiment flavouring additive • Used in wide variety of cuisines and traditional foods.	 Bark
Cloves	*Syzygim aromaticum* Myrtaceae	• Aromatic flower buds • cultivated in India. • Cloves are used in the cuisines of Asia, gives aromatic and flavour qualities.	 Flower buds

Coriander	*Coriandrum sativum* Apiaceae	• Annual herb cultivated in India. • Edible plant parts such as leaves and seeds. • Traditionally used in cooking.	 Seeds https://www.worldspice.com/spices/coriander-seed-indian
Cumin	*Cuminum cyminum* Apiaceae	• Dried seeds of a flowering plant • cultivated in India. • distinctive flavour and aroma. • used as ground or whole seeds.	 Seeds
Curry leaves	*Murraya koenigii* Rutaceae	• tropical to sub-tropical tree (Shrub) • native to India. • leaves used in Indian subcontinent.	 Leaves https://www.redrickshaw.com/products/fresh-indian-curry-leaves-15g
Dry ginger powder	*Zingiber officinale* Zingiberaceae	• flowering plant whose rhizome or ginger root used as a spice. • Ginger powder is used in food preparations and medicinal purposes.	 Roots https://en.clipdealer.com/photo/media/A:61577996
Fennel	*Foeniculum vulgare* Umbelliferae	• Fennel is a flowering plant, highly aromatic and flavourful herb • used in many culinary traditions of the world.	 Seeds

			https://timesofindia.indiatimes.com/life-style/food-news/not-just-a-mouth-freshener-fennel-seeds-are-a-health-food-
Fenugreek (seeds)	*Trigonella foenumaraecum* Fabaceae	• Spice responsible for distinctive sharp smell. • India is a major producer.	 Seeds https://www.nutraingredients.com/Article/2016/12/09/
Ginger (paste)	*Zingiber officinale* Zingiberaceae	• flowering plant whose rhizome or ginger root used as a spice. • Ginger powder is used in food preparations and medicinal purposes.	 Roots https://www.thespruceeats.com/ginger-recipes-and-cooking-tips-1807768
Green cardamom	*Elettaria* Zingiberaceae	• It is native to southern India. • green seed pods are used in Indian and other Asian cuisines, either whole or ground.	 Seed pods
Holy basil	*Ocimum enuiflorum* Lamiaceae	• Holy basil or Tulsi, is an aromatic plant native to India. • Tulsi is cultivated for religious and traditional medicines purposes and for its essential oil.	 Leaves https://www.bingenheimersaatgut.de/en/organic-seeds/herbs/basil/holy-basil-k93

Indian gooseberry	*Phyllanthus emblica* Phyllanthaceae	• Indian gooseberry or amla is an edible fruit. • Used in traditional Indian and various Ayurvedic medicine • All plant parts are useful • India is the largest producers and suppliers of Indian gooseberries. • fruits contain high amounts of vitamin C.	 Fruit https://www.etsy.com/listing/711439985/indian-gooseberry-tree-seedphyllanthus
Inkut (Harad)	*Terminalia chebula* Combretaceae	• Harad is a fruit, native to India. • main ingredient in the Ayurvedic medicine (Triphala), used for kidney and liver dysfunctions.	 Fruit https://mangalorespice.com/products/ink nut-haritaki
Long pepper	*Piper longum* Piperaceae	• flowering vine • used as a spice and seasoning. • The plant is a native of India.	 Fruit https://www.123rf.com/photo_96748199_dried-long-pepper-piper-longum-isolated-on-white.html
Mace	*Myristica fragrans* Myristica	• It is reddish seed covering (aril) of the nutmeg seed. • flavour is similar to nutmeg.	 Aril (seed covering) https://5.imimg.com/data5/WE/OF/NK/SELLER-61630555/mace-spice-javitri--500x500.jpg

Mango powder	*Mangifera indica* Anacardiaceae	• Fruity spice powder made from dried unripe green mangoes • used as a seasoning. • produced in India, and is used to flavour foods.	 Unripe fruit https://timesofindia.indiatimes.com/life-style/health-fitness/diet/aamchur-powder-raw-mango-powder
Mint leaves	*Mentha spicata* Lamiaceae	• Mints are perennial herbs. • used as medicinal herb • Menthol derived from mint is an ingredient of many cosmetics and perfumes.	 Leaves https://www.teavivre.com/catalog/product/view/id/756/s/mint-leaf-herbal-tea
Mustard (Black)	*Brassica nigra* Brassica	• They are flavourful, commonly used spice in Indian cuisine.	 Seeds https://www.southspices.co.uk/sample-page/mustard-500g/
Nigella seeds	*Nigella sativa* Ranunculaceae	• Nigella sativa or black cumin or kalonji is an annual flowering plant. • used as a spice in Indian cuisines. • They have a pungent, bitter taste and smell.	 Seeds https://spicesontheweb.co.uk/nigella-sativa-seeds/
Nutmeg	*Myristica fragrans* Myristica	• Grinding of nutmeg seeds use as spice	 Seed

		• Spice have distinctive pungent fragrance and slightly sweet taste • used to flavour baked goods.	https://www.etsy.com/listing/641857647/whole-nutmeg-seeds-organic-all-natural
Pomegranate seeds (dried)	*Punica granatum* Lythraceae	• The pomegranate is a fruit bearing shrub • native to northern India. • Pomegranate seeds are used as a spice known as anar-dana in Indian cuisine.	 Seeds https://www.vivapura.com/Dried-Pomegranate-Seeds-p/vu-044.htm
Poppy seeds	*Papaver somniferum* Papaveraceae	• Poppy seed is an oilseed obtained from the poppy. • Poppy seeds or khus khus are widely used in many regional Indian cuisines.	 Seeds https://www.indiamart.com/proddetail/white-poppy-seeds-khaskhas-21057553362.html
Red chili powder	-	• Chilli pepper pods are used fresh or dried or ground to powder • In India, it is used to flavour most curries and dry dishes.	 Fruit and seeds https://etradepakistan.com/product/buy-organic-red-chili-powder/
Saffron	*Crocus sativus* Iridaceae	• spice derived from the flower (stigma). • harvested by hand, the most expensive spice. • Saffron is widely used in Indian cuisines,	 Stigmas with style

		• it also contributes a luminous yellow-orange colouring in foods.	https://foodal.com/knowledge/herbs-spices/saffron/
Sesame seeds	*Sesamum indicum* Pedaliceae	• Edible seeds that grow in pods. • India is one of the largest producer • Contain highest oil contents, rich nutty flavour. • Used in worldwide cuisines	 Seeds https://foodtolive.com/shop/sesame-seeds/
Star anise	*Illicium verum* Magnoliaceae	• star-shaped pericarps of the fruit. • used as a spice in preparation of biryani and masala chai all over the Indian subcontinent.	 Fruit https://www.thespicehouse.com/products/star-anise-pieces
Tamarind	*Tamaridus indica* Fabaceae	• pod-like fruit that contains an edible pulp called tamarind or Imli, • used in cuisines around the world. • India is the largest producer.	 Fruit https://www.yipisale.com/item/1982/Whole-Dry-Spice-Tamarind-Imli
Turmeric	*Curcuma longa* Zingiberaceae	• Turmeric is a mustard-like, earthy aroma and pungent, slightly bitter flavour to food.	

		• The rhizomes are dried and grounded into deep orange-yellow powder • commonly used as a colouring and flavouring agent. • It is widely used as a spice in India.	 Roots https://thefactoryfresh.com/apna-bazar-turmeric-powder-7-oz-nj.html

(Adapted from Sachan *et* al., 2018). Some Images developed by author.

1.2.4 Common spices and herbs used in food

Ajwain (*Trachyspermum ammi*) is Umbelliferous plant from Apiaceae family, other names of the plant - bishop's weed, ajowan originated in India. Ajwain leaves and the fruit (seeds like) of the plant widely used in Indian cuisines. Ajwain is small, oval-shaped, seed-like fruits, smell like thyme, aromatic and somewhat bitter and pungent in taste. Even a small number of fruits tend to dominate the flavour of a dish. Rajasthan, Gujarat and Andhra Pradesh states in India are largest producer of ajwain. Ajwain is a popular spice and used in traditional Ayurvedic medicine primarily to treat stomach disorders (Chatterjee *et al*., 2012; Sanchan *et al*., 2018).

Bay leaves (*Laurus nobilis,* L.) belongs to Lauraceae family, are dried leaves native to Asia. The spice is used in traditional medicine and possess antioxidant, antimicrobial, anti-inflammatory, and analgesic properties (Polovka *et al.,* 2010).

Clove belongs to Myrtaceae family, aromatic spice. Several studies reported that clove essential oil contain high antimicrobial properties against foodborne pathogenic microorganisms due to a high concentration of its bioactive chemical compounds (Abdali *et al*., 2015). Eugenol (4-allyl-2-methoxyphenol) is the main bioactive compound of clove essential oil composition, which has strong insecticidal, antioxidant, and antifungal activity (Wei *et al*., 2016). The U.S. Food and Drug Administration (FDA) classified eugenol as a food additive substance that is generally regarded as safe (GRAS) (Sanchan *et al.,* 2018).

Cumin (*Cuminum cyminum* L.) is an annual herb from Apiaceae family, widely cultivated in India. Cumin seeds are used as a spice in Indian cuisines and provide taste, flavour to food. Cumin seeds are used in traditional medicines as a stimulant, astringent, diuretic, antimicrobial and antioxidant (Chen *et al.,* 2014).

Emblica officinalis, commonly known as **Indian gooseberry** or Amla belongs to Phyllanthaceae family, is one of the most important medicinal plants in Indian Ayurveda. Amla is highly nutritious and is one of the richest sources of vitamin-C (contains 600mg of vitamin C per 100g). Amla fruit is widely used in the Indian system of medicine in diabetic (Walia *et al*, 2014), stomach problems and for common cold (Sanchan *et al.,* 2018); as alone or in combination with other plants.

Nutmeg (*Myristica fragrans)* belongs to Myristicaceae family. It produces two spices – mace and nutmeg. Nutmeg is the seed kernel inside the fruit and mace is the red covering (aril) on the kernel. The main chemical components of nutmeg include camphene, eugenol, elemicin, isoelemicin, isoeugenol, and methoxyeugenol (Sanchan *et al*., 2018).

1.2.5 Antimicrobial properties of spices and herbs

Some commonly used spices and herb extracts such as cinnamon, cloves, oregano, thyme and rosemary possess strong antimicrobial properties and provide inhibition of foodborne microbes such as *Listeria monocytogenes, Salmonella typhi, E.coli and Bacillus cereus* in food and hence longer shelf life of food (Tajkarimi *et al*., 2010; Weerakkody *et al*., 2010). Over the past few years, research has been carried out to extract and study the bioactive compounds in spices and herbs and their antimicrobial properties. Table 1.2 summarises the research into essential oils of spices and herbs over the past few years. Essential oil such as thymol, carvacrol has been indicated to possess higher antimicrobial activity against Gram-positive bacteria than Gram-negative bacteria (Abdali *et al*., 2015; Burt, 2004). The Gram-negative bacteria such as *E.coli, Salmonella* were the most resistant to essential oils and their compounds because of the presence of lipopolysaccharides (LPS), lipids and proteins in the outer cell membrane of bacteria cells. The Gram-positive bacteria like *Bacillus, listeria* do not

have LPS layer, this decreases the affinity of an antimicrobial and bacterial interface (Abdali *et al*., 2015; Burt, 2004).

Table 1.2: - Research into essential oils of plant antimicrobials (spices and herbs) over 20 years

Spices and herbs	**Applications**	**Effective against**	**Reference**
Oregano, Thyme	Essential oils (EOs) use in food preservation	Enterobacteria, lactic acid bacteria, *B. cereus*, *Pseudomonas* spp	Gutierrez *et al* (2008)
Cinnamon, cloves, cumin	EOs showed strong antimicrobial effect, Food flavouring and preservation	*Staphylococcus aureus, Klebsiella pneumonia aeruginosa, E. coli*	Agaoglu *et al* (2007); Wei *et al* (2016)
Olive leaves	Antimicrobial activity	*Campylobacter jejuni, Helicobacter pylori, Staphylococcus aureus*	Sudjana *et al* (2009)
Oregano, thyme Coriander	Effective antimicrobial components in EOs	*Bacillus cereus, Pseudomonas aeruginosa, E.coli, Listeria monocytogenes*	Almajano *et al* (2008); Gutierrez *et al* (2008)
Bay leaves, coriander, cumin, cinnamon, thyme	Significant inhibition properties for pathogenic and spoilage microorganisms	*Bacillus subtilis, E.coli, L. monocytogenes, Salmonella typhimurium, Staphylococcus aureus*	Burt (2004); Bajpal *et al* (2008); Gutierrez *et al* (2008)
Cumin Thyme, cinnamon, clove	Antimicrobial effect Effective EO components	*Bacillus cereus, L. monocytogenes, Pseudomonas fluorescens, Salmonella enteritidis, Staphylococcus aureus*	Ceylan and Fung (2004) Davidson and Naidu (2000)

Zaika *et al* (1988), reported antimicrobial nature of common spices and herbs. Essential oil of cinnamon, clove, and mustard reported to having strong antimicrobial nature in inhibition of pathogenic microorganisms. Allispice, bay leaves, caraway, coriander, cumin, oregano, rosemary, sage, thyme reported to having medium antimicrobial nature and Black pepper,

red pepper, ginger reported having weak antimicrobial nature on microorganisms (Zaika, 1988; Burt, 2004; Holley and Patel, 2005).

It has been reported that antimicrobial efficiency of plant material depends upon their chemical (phenolic) structure and concentration (Tiwari *et al*., 2009). Most antimicrobial and phenolic compounds found in plant material have been reported to form a part of their mechanism against stress conditions such as high temperature, water supply and drought (Rauha *et al*., 2000). Plants material containing high antimicrobial properties that can inhibit the microbial growth might be due to bioactive chemical compounds (Ibrahim *et at*., 2006). Antimicrobial effects of extracted essential oil from plant materials such as spices and herbs have indicated equivalent impacts to synthetic additives but their applications in the food industry have been limited due to their strong odour, flavour, aroma and relatively high cost (Tajkarimi *et al*., 2010). According to EU registered food flavourings list, food additives such as carvacrol, carvone, cinnamaldehyde, citral, eugenol, p-cymene, limonene, menthol and thymol are some of the essential oils that have been registered and recognized as safe (GRAS – Generally recognized as SAFE) to use as flavourings in food (Tajkarimi *et al*., 2010; Burt, 2004).

1.2.6 Bioactive compounds of essential oils from spices and herbs

Several studies have shown that the bioactive components present in spices and herbs, are responsible for antimicrobial and antioxidant activity (Burt, 2004; Tajkarimi *et al*., 2010; Witkowska *et al*., 2013). Spices and herbs contain secondary metabolites – bioactive components such as phenolics (Flavonoids and non-flavonoids), phenolic acids, phenolic terpenes and tannins present in different parts of the plants imparting antimicrobial and, antioxidant properties (Zheng and Wang, 2001; Burt, 2004; Witkowska *et al*., 2013). The extracts of spices and herbs are comprised mainly of these bioactive compounds (Burt, 2004). The antimicrobial impacts of spices and herbs have been mostly credited to the complex mixture of phenolic compounds. Phenolic refers to a large group of chemical compounds having one or more aromatic ring with at least one hydroxyl (-OH) group attached. The bioactive compounds including phenols, saponin, flavonoids, thiosulfinates, glucosinolates, alcohols, aldehydes, ketones, ethers and hydrocarbons, especially in spices, such as cinnamon, clove, garlic, mustard and onion show inhibition of Gram-positive and Gram-

negative pathogens (Tajkarimi *et al*., 2010). The list of bioactive compounds in spices and herbs that inhibit antimicrobial activity are listed in Table 1.3. These bioactive compounds are responsible for antimicrobial properties including cell wall degradation, disruption of the cytoplasmic membrane, leakage of cell components, alteration of fatty acid and phospholipid constitutes, change in the synthesis of DNA and RNA and destruction of protein translocation (Lambert *et al*., 2001; Shan *et al*., 2007; Witkowska *et al*., 2013).

Table 1.3 - Bioactive (Phenolic) components in spices and herbs with antimicrobial properties

Category	**Class**	**Sub-class**	**Example of spices and herbs**
Polyphenols	Flavonoids	Flavanols (eg. Catechin)	Cinnamon
		Flavanones	Fennel
		Flavones	Onion, Oregano
		Flavonols (eg. Quercetin)	Coriander, Cumin, Black pepper, Onion
	Non- Flavonoids	Phenolic acids	Cloves
Terpenes	Limonene		Fenugreek, Mustard
Vanilloids	Curcumin		Turmeric, Ginger
Organosulphur compound	Disulfides, Thiosulfinates		Garlic, Onion

(Adapted from Bi *et al*., 2017)

Cinnamon spice is widely consumed and has been granted GRAS (Generally Recognized As Safe) by the United States Food and Drug Administration (FDA). Various investigations have indicated that cinnamon powder is rich in bioactive compounds such as cinnamaldehyde, eugenol, phenylpropanoids and their derivatives, which possesses antimicrobial properties (Tajkarimi *et al*., 2010; Asbahani *et al*., 2015; Bi *et al*., 2017). Zaika (1988), reported cinnamon has a strong antimicrobial activity against pathogens. Yuste and Fung (2002) reported that ground cinnamon exhibited a 4.0 - 6.0 log CFU/ml reduction in the growth of *Listeria* in apple juice after 1 h of incubation at 5 and 20 ^{o}C. Another study showed that addition of 0.3% w/v

cinnamon powder to pasteurized apple juice reduced the growth of *E. coli* by 1.6 and 2.0 log CFU/ml when stored at 8 and 25 ^{o}C, respectively (Ceylan and Fung, 2004).

1.2.7 Essential oils and their mechanism of action

Essential oils are complex mixtures of volatile chemical compounds from a large number of plant raw material. An estimated 3000 essential oils are known, of which about 300 are commercially used as flavours and fragrances (Burt, 2004; Asbahani *et al*., 2015). These compounds are hydrophobic in nature and their density is lower than water, they are mostly lipophilic, soluble in organic solvents and, immiscible with water (Asbahani *et al*., 2015). Essential oils (EOs) contain secondary metabolites present in different parts of plants such as, flowers (jasmine, rose and lavender), buds (clove), leaves (Thyme, Bay leaves), fruits (star anise), twigs, bark (cinnamon), seeds (coriander, cardamom), wood (sandal), roots (ginger) (Burt, 2004; Asbahani *et al.*, 2015). These EOs are utilised as natural active agents and have been used to extend the shelf life of food, reducing the load of pathogenic microorganisms, and increasing overall quality of food products (Burt, 2004; Tajkarimi *et al*., 2010). Essential oil composition of plant depends on internal and external factors influencing the plants (Moghaddam *et al*., 2015). In addition, seasonal conditions, developmental stages – age of plant at the time of collection of plant material, harvest methods, plant material processing such as methods of extraction and the analysis conditions influence the yield of bioactive components (Moghaddam *et al*, 2015; Hussain *et al*, 2008). Black pepper contains piperine, β-caryophyllene, ginger contains gingerol, citral, zingiberene, turmeric contains curcumin, cinnamon contains eugenol, cinnamaldehyde, clove contains eugenol, eugenyl acetate, nutmeg contains myristicin, elemicin, coriander contains linalool, cumin contains cuminaldehyde, b-pinene, thyme contains thymol as major chemical components in essential oils of some spices and herbs (Calo *et al,* 2015; Parthasarathy *et al*, 2008; Peter, 2001).

1.2.8 Mode of action of essential oil

Essential oils have been shown to cause morphological structural and functional loss to the bacterial cell membrane (Goni *et al*., 2009). These essential oil affect bacterial cells by numerous antimicrobial mechanisms, such as attacking the phospholipid bilayer of the cell membrane, enzyme systems disruption, genetic material of bacteria compromising, and forming fatty acid hydroperoxidase caused by oxygenation of unsaturated fatty acids (Burt *et*

al., 2007; Tajkarimi *et al.*, 2010). The efficiency of antimicrobial EOs depends on factors including, essential oils extraction method from plant material, inoculum volume used, microorganism growth phase, use of culture medium, intrinsic or extrinsic properties of the food, antioxidants, preservatives, incubation time/temperature and packaging methods as reported by Burt (2004); Brandi *et al*., (2006); and Tajkarimi *et al*., (2010). Generally, Gram-negative microorganisms are less sensitive to antimicrobial EOs because of the presence of a lipopolysaccharide layer on the outer membrane of these organisms, which restricts diffusion of hydrophobic compounds (Burt, 2004; Tajkarimi *et al*., 2010). Plant-based phenolic compounds interfere with the microbial cell membrane and alter the structure and function (Goni *et al*., 2009). It has been shown that phenolic compounds such as thymol, eugenol and carvacrol disrupt the cell membrane, increase membrane permeability, inhibit ATP activity and release cell components (Davidson, 1997; Davidson and Naidu, 2000). In addition, it was reported that the antimicrobial properties of phenolic compounds depend on concentration: low concentration inhibit microbial enzymes activity and high concentration induce denaturation of proteins (Pisoschi *et al*., 2018).

1.2.9 Methods of extraction of bioactive compounds from spices and herbs

Extraction is the crucial first step for the effective separation and purification of bioactive compounds of plant materials. The conventional extraction technologies to extract essential oils from different parts of aromatic plants are solvent extraction and, hydro or steam distillation, etc. (Damyeh *et al*, 2016). In past 2-3 decades, the conventional methods were used in the extraction of essential oil from spices and herbs. However, conventional methods have some disadvantages, including the long extraction time, degradation of chemical compounds in essential oil, low extraction efficacy and high-energy consumption (Tiwari, 2015; Damyeh *et al*, 2016). Various novel technologies have been known for extraction of essential oil, non-conventional methods such as supercritical-fluid extraction providing improved solubility and mass transfer, microwave-assisted hydro-distillation, and ultrasound-assisted extraction that decrease the extraction time (Tiwari, 2015; Damyeh, 2016; Pisoschi *et al*., 2018). Moreover, such innovative technologies have many advantages such as reduced extraction time, better extract quality and essential oil yield obtained (Pisoschi *et* al., 2018). Therefore, there is a need to investigate alternative techniques to prevent the above-

mentioned difficulties of conventional techniques for the extraction of essential oils (Chemat *et al*., 2011; Ma *et al*., 2015).

1.2.9.1 Use of organic solvent in extraction process

The extraction of specific bioactive compound from spices and herbs require the control of parameters such as type of solvent (Tajkarimi *et al*., 2010). The type of solvent used to extract essential oil from plant material such as spices and herbs have a major effect on their antimicrobial activity. The different aromatic and saturated organic chemical compounds extracted using various solvents such as ethanol or methanol (Cowan, 1999; Witkowska *et al*., 2013). Mostly, extraction of spices and herbs with different organic solvents resulted in extracts with higher antimicrobial activity than extraction with water. Studies show that aqueous extracts of all spices and herbs display little or no antimicrobial activity against tested microorganisms (Witkowska *et al*., 2013). The use of ethanol as a solvent is advantageous to use for extraction as it is removed from extracts by evaporation under reduced pressure. This method avoids alterations and chemical products. On the other hand, extracts obtained by other organic solvent such as hexane, contain residues that contaminate the food to which they are included. This compromises the safety of products extracted by this method. Consequently, it is difficult to utilize them for food applications. However, these difficulties could be avoided by using a combination technology of organic solvent with low boiling point and steam distillation process (Asbahani *et al*., 2015).

1.2.9.2 Non-conventional methods of extraction of essential oil

Supercritical fluid extraction: The principle is based on the compression/depression of fluid. By highly compressing and heating, CO_2 reaches the supercritical state and passes through the raw plant material and plant extracts. This step is followed by next depression step: in which the extract is routed to one or more separators, where the CO_2 is gradually decompressed to separate the extract obtained from the fluid. The latter could be turned into a released gas and then could be recycled. The use of this technique for EOs extraction has increased in the last two decades. The only one disadvantage to its development is the high cost of the equipment, its installations and maintenance operations (Asbahani *et al*., 2015).

Microwave-assisted hydrodistillation has the advantage of reduced extraction time, and a better mass transfer due to the microwave power. It is an environmentally friendly technology. Microwaves are electromagnetic based waves with frequency between 300 MHz and 30 GHz and wavelength between 1 cm and 1m. This is rapid extraction method as compared to hydro-distillation. It facilitates energy saving, cleanliness, fast and efficient extraction and reduces waste and avoids water and consumption of solvent (Asbahani *et al*., 2015).

1.2.9.3 Use of ultrasound-assisted extraction

Investigation of novel technologies in the most recent decades has prompted the development of new innovative and more efficient extraction processes. Ultrasound-assisted extraction (UAE) is an emerging potential technology that has been used in the field of plant material extraction when used in combination with other methods such as solvent extraction (Asbahani *et al*., 2015). The plant material is immersed in solvent and exposed to the action of ultrasonic waves which have a frequency of 20 kHz - 1MHz (Asbahani *et al*., 2015). This induces mechanical vibration of the membranes of plant extract including a rapid release of essential oil droplets. The extraction process involves two types: diffusion through the cell walls and washing out the cellular content when cell walls are broken. The essential oil stored in plant in the form of glands filled with essential oil droplets. These plant glands are very thin that can be easily destroyed by ultrasound sonication (Asbahani *et al*., 2015). Some commonly used ultrasonic systems are shown in Figure 1.1 (Chemat *et al*., 2017).

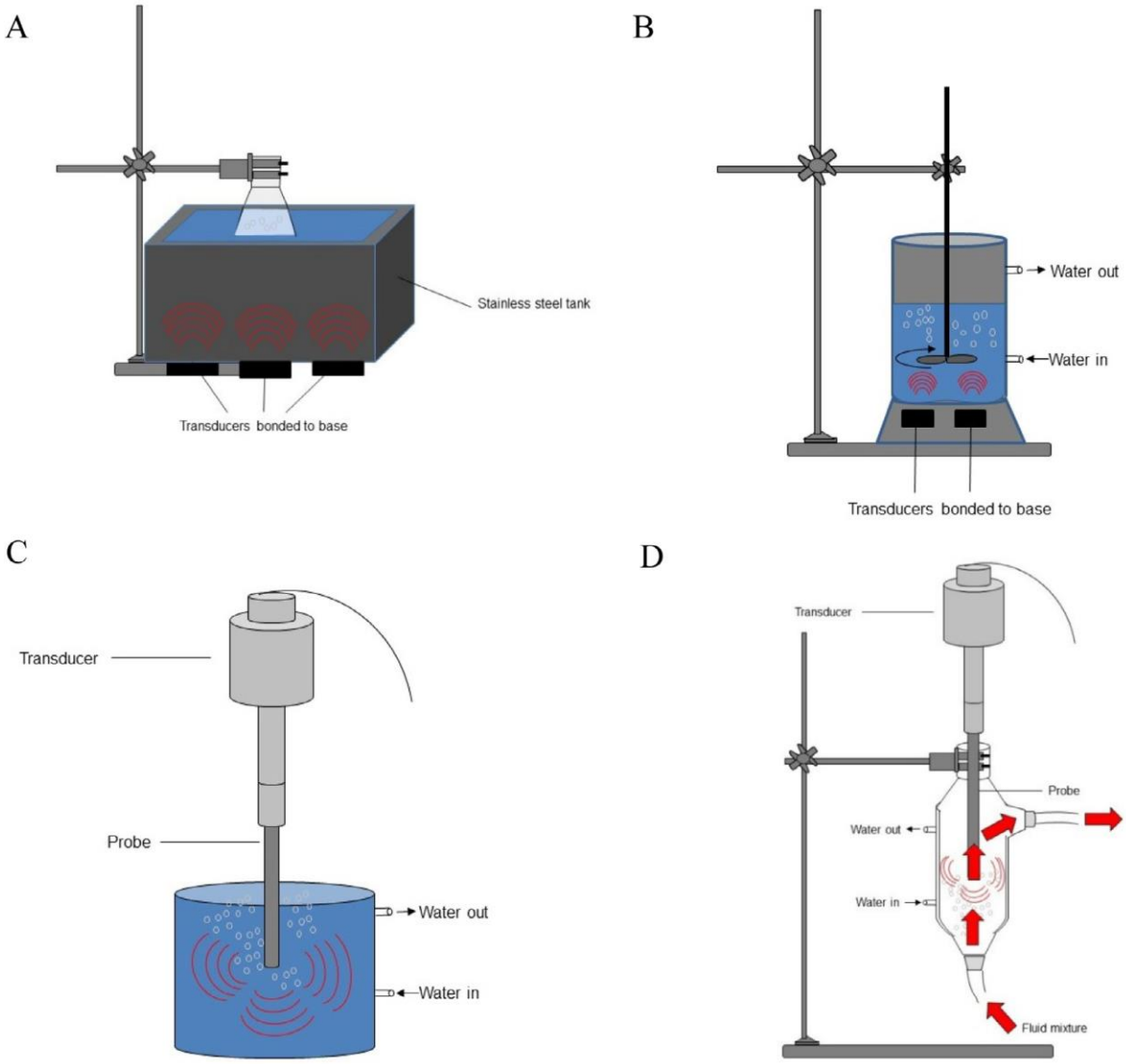


Figure 1.1 - Commonly used ultrasonic systems (A: Ultrasound bath, B: Ultrasound reactor with stirring, C: Ultrasound probe, D: Continuous sonication with ultrasound probe) (Chemat *et al*., 2017)

Ultrasound technology has been utilized in different food applications and it is used to enhance process proficiency (Chemat *et al.,* 2017; Ohja *et al.,* 2016). Application of ultrasound alone, or in combination with other methods, can be utilized to inactivate enzymes and pathogenic microorganisms (Ohja *et al.,* 2016). Mechanism of bubble cavitation phenomenon is shown in Figure 1.2 (Kadam *et al*., 2015) which is applied in food industry (Chemat *et al.,* 2017). The benefits of ultrasound are attributed to acoustic cavitation: i.e. when a mixture (solid material in liquid medium) is subjected to ultrasound, micro-bubbles created in the liquid phase that grow and oscillate due to pressure change and then collapse. These implosions disrupt the surface of the solid matrix, which enhances mass transfer of the

molecules and accelerate diffusion of solid material in liquid phase (Chemat *et al,* 2017; Ohja *et al.,* 2016). Although ultrasound inactivates growth of microorganisms which is due to several physical impacts, including cell membranes disruption, heat localisation, intracellular cavitation and sonolysis of water leading to the production of free radicals (Asbahani *et al.,* 2015; Chemat *et al*., 2017). The capability of ultrasound technique is strongly effected on microorganisms by internal and external parameters such as microbial ecology (such as type of microorganism used, type of medium and composition), ultrasound parameters (such as ultrasound power and frequency), sonication time, pH and temperature (Asbahani *et al*., 2015). In recent years, the use of ultrasound innovation has extended beyond basic inactivation of microorganisms (Chemat *et al.,* 2017).

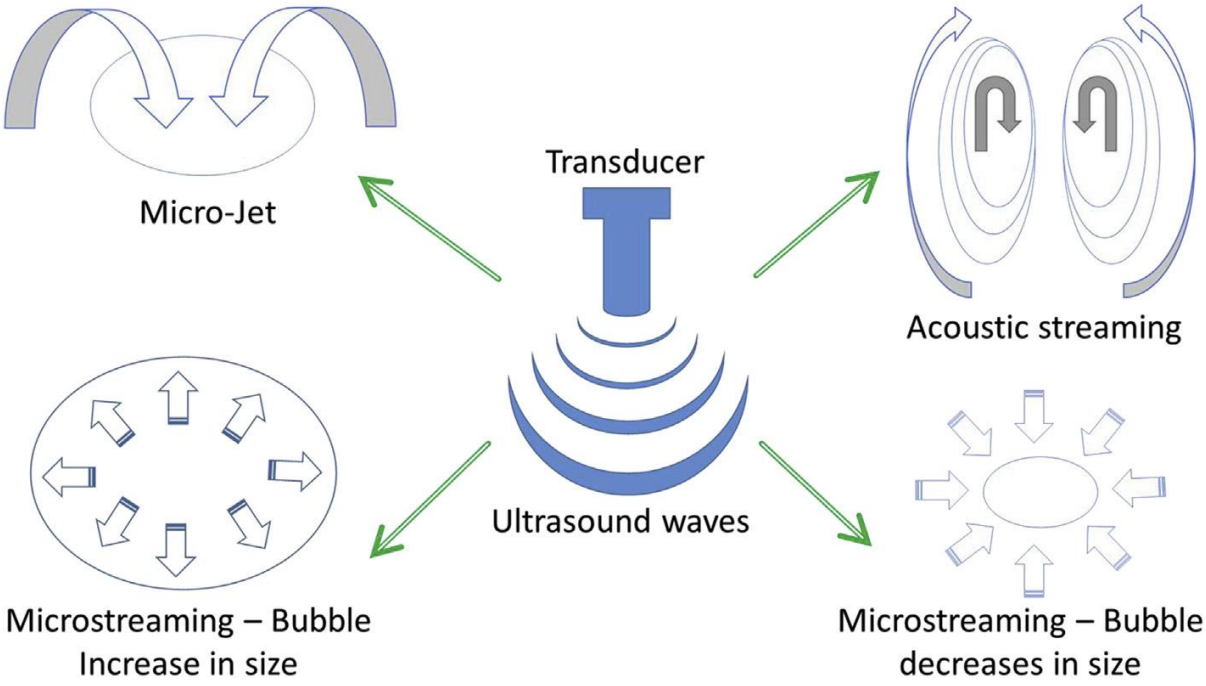


Figure 1.2 - Mechanism of bubble cavitation phenomenon (Kadam *et al*., 2015)

As compared to conventional methods, application of ultrasound for the extraction improves extraction efficiency, reducing the consumption of solvent, reduces extraction time with high reproducibility and improvement of EOs quality (Asbahani *et al*., 2015; Tiwari, 2015). The degree of milling of plant material plays a significant role in the yield obtained. Clearly, decreasing the size of plant material will increase the number of cells exposed to ultrasonically induced cavitations (Asbahani *et al*., 2015). Ultrasound likewise offers a mechanical impact

permitting more penetration of solvent into the matrix of the sample, the contact surface area increases between the solid and liquid phase, and subsequently, the solute rapidly diffuses from the solid phase to the solvent (Hossain *et al*., 2014). The equipment is relatively simple and economical as compared to other techniques such as microwave-assisted extraction (Asbahani *et al*., 2015). To optimise extraction of target compounds using ultrasound various parameters can be controlled including extraction time and temperature, amplitude of ultrasound and solvent extraction. Investigations including the control of all these parameters to optimise extraction can be time-consuming and expensive (Hossain *et al*., 2014).

In a study by Morsy (2015), ultrasound-assisted extraction was performed for cardamom essential oil extraction and was found to be a potential source of essential oil with an aroma. In another study by Ma *et al*. (2015), the extraction of *K. galangal* essential oil was evaluated and optimized with ultrasound extraction, results indicated successful extraction of essential oil, and the ultrasound method is advantageous because of timesaving, environment-friendliness and high efficiency compared to other extraction methods. Damyeh *et al*. (2016), considered comparative extraction methods using hydro-distillation and ultrasound pre-treatment in *Prangos ferulacea Lindl.* and *Satureja macrosiphonia Bornm*- leaves and found that ultrasound pre-treatment accelerates the essential oil extraction process and produces an essential oil with superior characteristics. However, there are few studies on the antimicrobial activities of spices and herb extracts by ultrasound-assisted extraction.

1.2.10 Anti-glycation properties of spices and herbs

The human diet is responsible for life-style related diseases such as diabetes. Glucose is a main energy source for the human body and plays a key role in maintaining health. The metabolism of glucose molecules in most cells forms adenosine triphosphate (ATP) molecules that are fuel for almost every cellular process. A constant level of glucose is required for cells to function (Deng, 2012). The level of glucose above normal that is due to inadequate secretion or insufficiency of insulin is defined as hyperglycaemia. Research on clinical and animal models has indicated that excess glucose levels above the baseline cause diabetic microvascular and macrovascular complications (Verzelloni *et al*, 2011). In humans, insulin

and glucagon are important hormones in controlling glucose levels between 3.0 and 7.8 mmol/L. However, initial hyperglycaemic clinical features appear at concentrations of between 15-20 mmol/L (Cox *et al*, 2005). Hyperglycaemia leads to increased levels of intracellular sugars such as glucose, fructose, fructose-3-phosphate (F-3-P), glucose-6-phosphate (G-6-P) and the accumulation of highly reactive dicarbonyl molecules, for example, methylglyoxal and glyoxal.

1.2.10.1 Diabetes mellitus

Diabetes mellitus is another enormous general health problem around the world. As per Diabetes.org figures, the number of people living with diabetes in the UK has reached 4.7 million. The cost of drugs and treatment for diabetes is very high (Diabetes UK, 2019). Therefore, there is a need to investigate natural plant-based products with antidiabetic properties and investigate to treat diabetic complications.

Diabetes mellitus (DM) is a metabolic disorder characterised by increased blood glucose concentrations (called hyperglycaemia) resulting from a lack or partial deficiency of insulin, or insulin resistance. Diabetic patients are susceptible to the risk of long-term complications such as nephropathy, retinopathy, atherosclerosis and delayed wound-healing (Ahmed, 2005). In diabetes, delayed wound-healing is a major problem. Diabetes can affect various organs and systems such as the gastrointestinal tract causing improper function and making the affected individual more susceptible to infection. Hyperglycaemia plays a critical role in the pathogenesis of long-term complications and its toxicity is mediated by increased protein glycation. Protein glycation is a simple chemical reaction initiated by a nucleophilic addition reaction between a free amino group from a protein and carbonyl groups of sugars to form freely reversible Schiff bases that subsequently form more stable and relatively irreversible ketoamine or Amadori products. The Amadori product goes through a number of reactions involving dicarbonyl intermediates, such as 3-deoxyglucosones and methylglyoxal, and eventually gives rise to poorly characterised structures called advanced glycation end products (AGEs) (Ahmed, 2005) shown in Figure 1.3. The AGEs form as a result of non-enzymatic reactions between the sugars and their derivatives (Mashilipa *et al*, 2011). Glycation causes damage to proteins, lipids and nucleic acids and leads to oxidative stress in chronic conditions and accumulations of AGEs in the body causes long-term diabetic mellitus

complications (Elosta *et al*, 2012). AGEs are complex, heterogeneous molecules that cause protein cross-linking, exhibit browning and generate fluorescence. Formation of AGEs generates auto-oxidation reactions and yield free oxygen radicals (Ahmed, 2005) which can be controlled with antioxidants. This process is known as the Maillard reaction, shown in Figure 1.4. AGEs are critical factors for the diabetes and its complications. Most studies have focussed on developing natural plant-based products that could effectively inhibit AGE formation and have antioxidant properties.

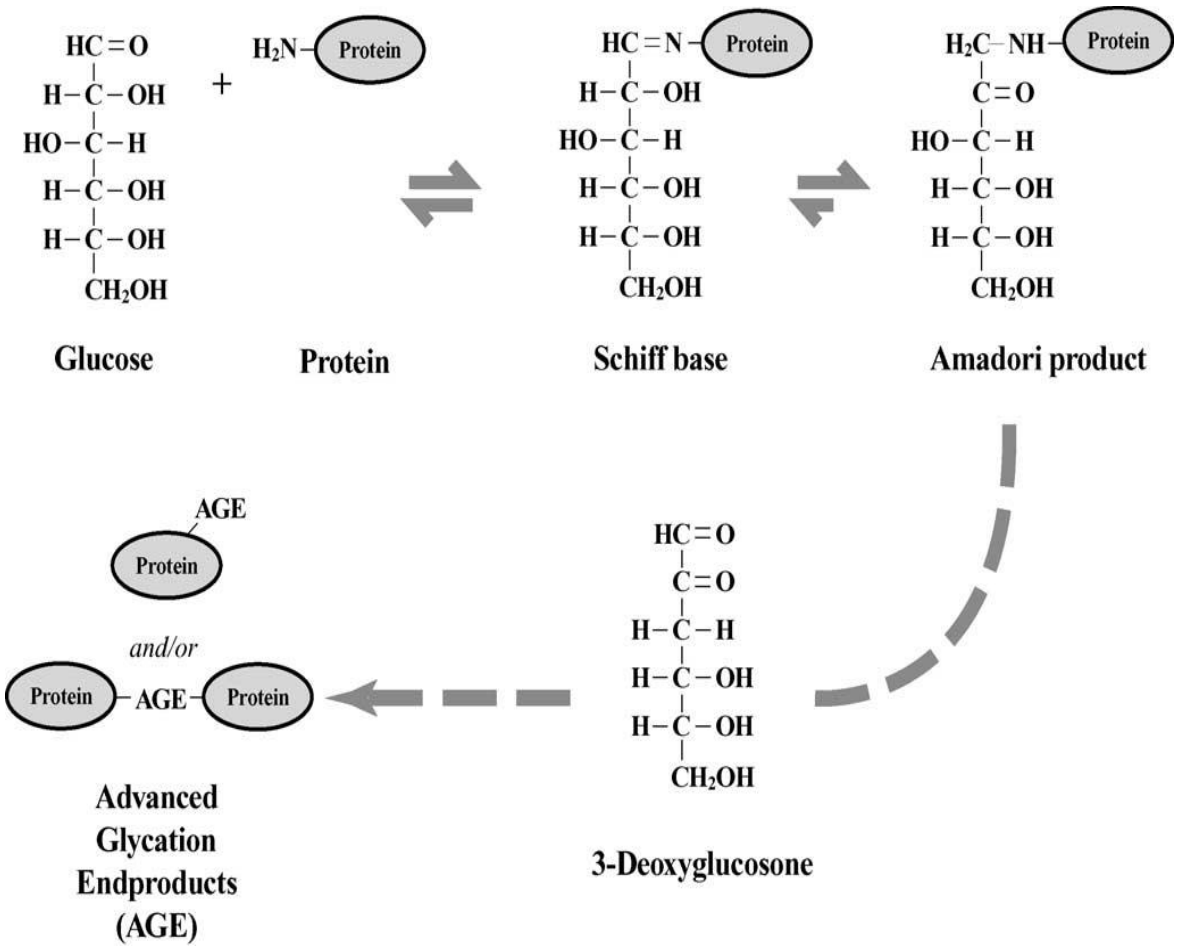


Figure 1.3: Glycation of a protein by glucose and the subsequent formation of AGEs. The initial reaction between glucose and protein amino groups forms a reversible Schiff base that rearranges to a ketoamine or Amadori product. With time, these Amadori products form AGEs via dicarbonyl intermediates such as 3-DG (Ahmed, 2005)

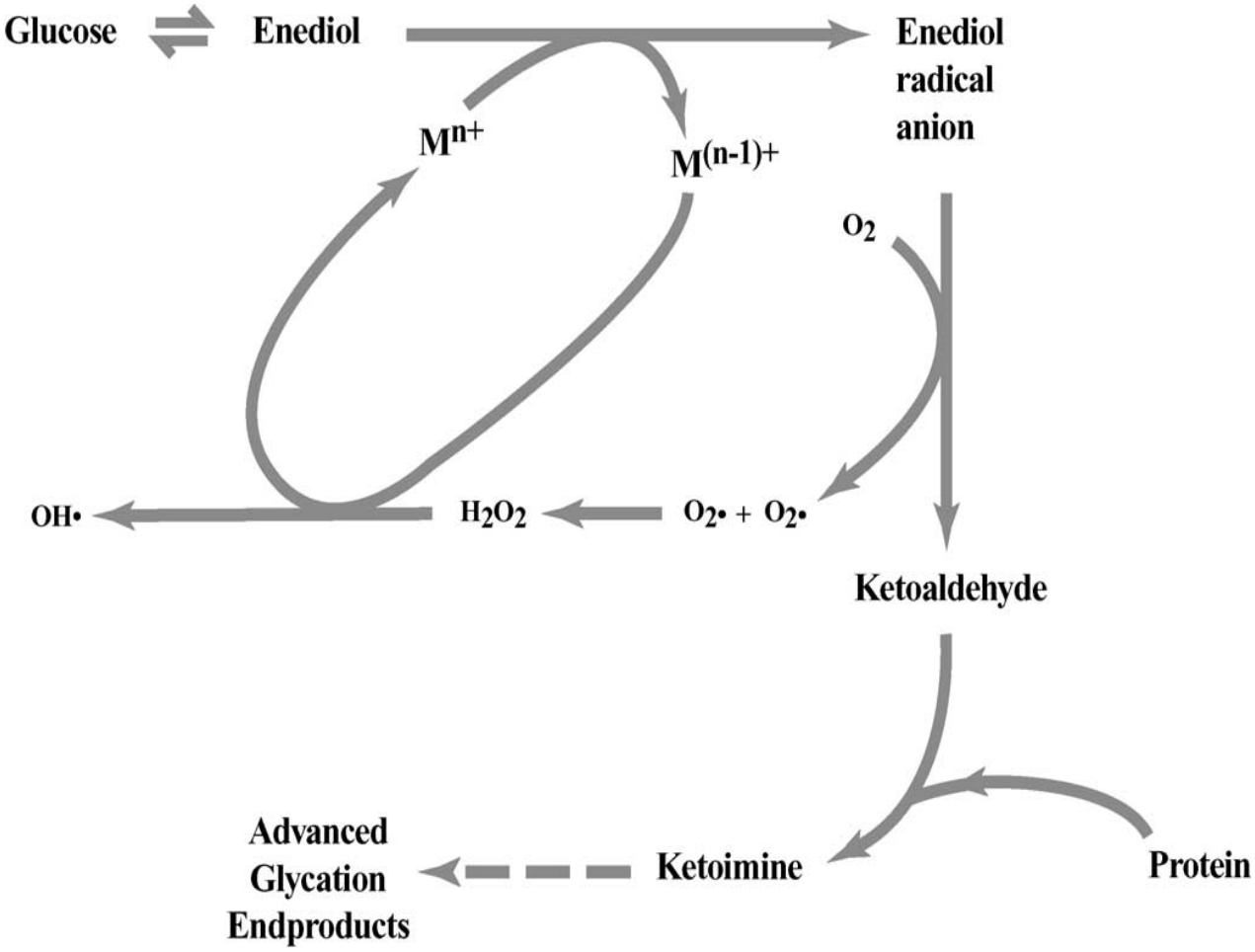


Figure 1.4: Metal-catalysed autoxidation of glucose molecules to protein-reactive dicarbonyls is paralled by the production of superoxide (O_2). Superoxide free radicals can dismutate to hydrogen peroxide (H_2O_2), then are changed to reactive hydroxyl radicals in the presence of transition metals (Ahmed, 2005)

1.2.10.2 Medicinal plants and diabetic mellitus

Spices and herbs such as garlic, cinnamon, thyme, clove are some of the traditionally used medicinal plants that used to treat diabetes in developing countries, where conventional diabetes treatment is expensive and unaffordable (Bi *et al*., 2017). Due to its therapeutic significance, spices and herbs have been a subject of research interest in the field of the plant-based remedy to treat diabetic complications. Many studies have been focused on developing natural plant-based products having less side effects on prevention of AGE formation. Screening and investigation of novel compounds, which offer, combined antioxidant and anti-glycation properties will be beneficial in the treatment of diabetes mellitus. Polyphenolic

contents of spices and herbs can be useful for treatment of hyperglycemia (Jaiswal *et al*., 2009). Phenolic compounds present in plant extracts exhibit antioxidant and anti-glycation properties, reported by Ramkisson *et al*. (2013). The antioxidant ability of polyphenol substances can inhibit AGEs. Many researchers have recommended that phenolic compounds may play a significant role in reducing the risk of diabetes. Therefore, antioxidative agents may inhibit the process of AGE formation by preventing further oxidation of Amadori product and metal-catalyzed glucose oxidation (Ramkisson *et al*., 2013). Several natural plant materials contain chemical compounds possessing antioxidant properties and have been shown to prevent AGEs formation *in vitro* and *in vivo*. Plant-based foods can improve glucose metabolism as well as enhance the overall health of diabetic patients (Saxena *et al*., 2010).

Various different studies have reported antioxidant activity and antidiabetic properties of spice and herbs that may provide benefits for diabetic patients (Bi *et al*., 2017; Adisakwattana *et al*., 2010; Ahmed *et al*., 2005). However, there is not much work done to show that spices and herb extract can inhibit protein glycation induced by different types of reducing monosaccharides. The effect of different spices and herbs extract on AGEs formation deserves consideration, because plant-based preparations could potentially be used as cost-effective, non-toxic supplements with anti-glycation activity to prevent or delay the onset of diabetic complications. Spices and herbs are the main source of phenolic components. The polyphenols in about 80 different spices have been proven to exhibit anti-glycation activity, which contribute to the prevention and management of diabetes. Therefore, the seasoning of foods with spices has been suggested not only to increase antimicrobial properties of a meal but also to have the antioxidant and antiglycation effect (Bi *et al.*, 2017). Therefore, further research in the investigation of antiglycation, antioxidant properties of spices and herbs bioactive extracts is required.

1.2.11 Antioxidant properties of spices and herbs

Antioxidant are bioactive components that can inhibit or delay the oxidation of molecules. Antioxidant are either natural or synthetic. Some synthetic antioxidant such as BHT, BHA are commonly used. Many researchers have concerns about safety because of health concerns cause by synthetic antioxidant use. Therefore, development of natural antioxidant from

plant-based source have been in demand. Some spices and herbs such as cinnamon, cardamom, tamarind, lemon grass have been shown to contain antioxidant properties (Altemimi *et al*, 2017).

Spices and herbs found to exhibit antimicrobial and antioxidant properties. Spices and herbs have been used for flavouring, colour and aroma for more than 2000 years. Spice and herbs have been used for food and beverage preservation due to bioactive phytochemicals profile (Embuscado, 2015). Plant extracts have strong hydrogen (H+) donating activity thus making them extremely effective antioxidants which is often due to phenolic acids (gallic, protocatechuic, caffeic, and rosmarinic acids), phenolic diterpenes (carnosol, carnosic acid, rosmanol, and rosmadial), flavonoids (quercetin, catechin, naringenin, and kaempferol), and volatile oils (eugenol, carvacrol, thymol, and menthol) (Brewer, 2011). The spices and herbs have been used as antioxidants as whole or ground spice and herbs extract because they possess excellent antioxidant properties. Spices and herbs have shown high efficiency as antioxidants and are classified as all natural, an attractive quality for consumers. Spices and herbs may be used as a means to control lipid oxidation in foods (Embuscado, 2015).

Chapter 2

Antimicrobial screening of Indian spices and herbs

2.1 Introduction

Human diet has changed consumer perceptions and there is increasing high demand of safe food due to increased number of foodborne outbreaks caused by pathogenic microbes (Tajkarimi *et al*., 2010). Many bacterial strains have developed resistance to Ampicillin, penicillin and streptomycin. This seriously hinders the management of diseases in food (Liu *et* al., 2017; Witkowska *et al* 2013; Weerakkody *et al*., 2010). There is urgent need for alternative agents for the management of pathogenic spoilage microorganisms in food. Food processing techniques and use of chemical preservatives such as benzoic acid and sorbic acids in food industry are not capable to kill foodborne pathogens such as *Listeria monocytogenes* completely causes adverse health effects, and nutritional and quality loss of food (Liu *et al.,* 2017; Gutierrez *et al*., 2009). Hence, there is high demand of natural antimicrobial for two major reasons: to provide natural preservation and to inhibit the growth of foodborne pathogenic microbes (Tajkarimi *et al.*, 2010). In last decades, most researches have focussed mainly on the finding potential natural antimicrobial agents that could inhibit the growth of pathogenic microorganisms and provide natural preservation for longer periods (Tajkarimi *et al*., 2010; Weerakkody *et al*., 2010).

India is the land of spices and is the largest producer, exporter and consumer of spices in the world and has great future in the coming years emphasis on quality and food safety. India has a wide variety of spice crops that have significant contribution in food industry and Indian spices are popular for their excellent aroma, flavour and spiciness. Out of 109 spices recognized by the International Organization for Standardization (ISO) in the world, more than 60 spice crops are grown in India. India contributes about 20-25% of the world trade in spices (Sharma, 2017). In ancient India, natural spices and herbs were consumed either in food, or used as medicine. Spices such as clove (used for toothache, fever, and pain), cinnamon (used for nervous problems, stomach/intestine infections), mustard, garlic (used for antiseptic, diuretic), ginger (used for digestive, cold), mint etc. have been reported to possess medicinal properties (Sanchan *et al*., 2018). Large number of spices and herbs has

been used to cure different types of diseases and possess antimicrobial activity, antioxidant and anti-inflammation actions (Weerakkody *et al.,* 2010; Embuscado, 2015).

For initial comparative screening of spices and herbs, five processed and packed spices were selected and compared to five unprocessed, unpacked Indian spices and herbs. The extracts of unprocessed spices and herbs namely, coriander, thyme, cumin, cinnamon, bay leaves, fenugreek and mustard were assessed and compared with processed spices and herbs. In last decades, research on natural plant material has been targeted as research interest. The selection of these spices and herbs were made on organic production, widely used in Indian cuisines, popular in common population, cultivated in India and most spices are native to India. Mostly, processed spices are treated with gamma irradiation that helps to extend the shelf life of herbs and to reduce the food-related health hazards caused by pathogenic microorganisms and impurities were removed and packed in ambient temperature (Oraon et al., 2017). Whereas unprocessed spices were collected directly from farms/fields after harvesting, not processed (some may be sundried), contain dry straws stalks of plant, dirt and traces of other spices. The purpose of this research is to find out which spices and herbs would be good for consumption based on microbial profile either processed and packed verse unprocessed and unpacked. These processed and unprocessed Indian origin spices and herbs were compared against initial microbial profile analysis such as total viable count, *Enterobacteriaceae* and *Listeria* count. The purpose of initial microbial profile analysis is to check whether there is any microbial contamination in the sample or not? Are these raw organic spices and herbs are safe to use or not? Is any initial treatment is required to purify these spices and herbs?

The spices and herbs were extracted using different methods and different solvents. The extracts were optimized for different solvent used (water or ethanol), different ratios of spice powder as solute to solvent ratio (1:10 to 1:50), different extraction methods (separating funnel, stirring and ultrasound-assisted extraction). Ultrasound-assisted extraction method selected for extraction of spices and herbs, due to high extraction efficiency, reduced extraction time with high reproducibility, which has not been used earlier for extraction of selected spices and herbs and investigated against selected microorganisms (Tiwari, 2015;

Ashabani et al., 2015)**.** In the present study, the extracts of unprocessed spices and herbs were assessed and compared with processed spices and herbs with regard to their antimicrobial activities against foodborne pathogens - *Bacillus cereus, Listeria monocytogenes, Kocuria rhizophilia, Salmonella typhymurium, E.coli and Pseudomonas aeruginosa.* All the spices and herbs extracts were analyzed for antimicrobial properties using agar disc diffusion assay.

For final screening, thirty-five unprocessed Indian origin spices and herbs were studied for the selection of most efficient spices and herbs based on antimicrobial properties. All 35 spices and herbs were unprocessed collected directly from farms/fields after harvesting, not processed (some may be sundried), contain dry straws stalks of plant, dirt and traces of other spices. The criteria for selection of 35 spices and herbs were their popular use, mostly cultivation and native to India, widely use in Indian cuisines, availability to people, and collection of samples from primary procurement- unprocessed and unpacked spices and herbs used for this study. Each spice extract compared with other spice extract, type of microorganism (Gram-positive or Gram-negative) and with positive (antibiotic) and negative controls (water or ethanol alone).

There are 3 aspects of this study: 1) Comparing selected processed herbs and spices to unprocessed India herbs and spices 2) Optimising the extraction method 3) Screening of 35 unprocessed India herbs and spices based on antimicrobial properties. The main objective of this study was to evaluate the potential of plant (spice and herb) extracts and bioactive components on foodborne microorganisms. Beside antimicrobial properties of spices and herbs, there is gap in knowledge of setting quality standards for spices trading. There is urgent need to create awareness among primary producers and traders about the potential bioactive components present in these spices extract which impart antimicrobial and antioxidant properties. How it can be set for selecting quality biomarkers is a challenge. This research was carried out to find out link between potential bioactive component in spices and herbs extracts, imparting antimicrobial properties.

2.2 Aims and objectives -

Aims- Screening of extracts of 35 Indian spices and herbs for their antimicrobial properties.

Objectives-

- To produce crude extracts from processed and unprocessed Indian spices and herbs using water and ethanol and different extraction methods (separating funnel, stirring and ultrasound enhanced extraction).
- To optimize ratio of solid (spices or herbs) to water and ethanol used for extraction.
- To determine and compare the antimicrobial activities of the extracts from 5 processed and 5 unprocessed Indian origin spices and herb against pathogenic microbes, including *Listeria monocytogenes, Escherichia coli, Bacillus cereus, Salmonella enterica, Pseudomonas aeruginosa* and *Kocuria rhizophila* using agar disc diffusion method.
- To determine and screen the antimicrobial activities of the extracts from a pool of 35 unprocessed Indian origin spices and herb against pathogenic microbes, including *Listeria monocytogenes, Escherichia coli, Bacillus cereus, Salmonella enterica, Pseudomonas aeruginosa* and *Kocuria rhizophila* using agar disc diffusion method.

2.3 Methodology

2.3.1 Sample preparation and treatment of spices and herbs

Spices and herbs that have been processed and packaged were obtained from an organic shop (Holland and Barrett in Manchester) and Indian origin spices and herbs were procured from primary producers in India in the unprocessed form from wholegrain market Faridkot, India. Bay leaves, cinnamon, thyme, cumin, coriander seeds, fenugreek and mustard were considered for initial preliminary study and screening. For final screening of Indian spices and herbs, 35 samples were selected and purchased in unprocessed form from wholegrain market Faridkot, India. All the spices and herbs samples were milled using ZM100 mill (RETSCH, Germany) with a 1 mm seive at 14000 rpm speed and few spices were milled in IKA A11 basic miller (to obtain small quantity for analysis) and then were sieved through 500µm to obtain

a maximum particle size below 500µm and stored in airtight containers at room temperature until used. It has been demonstrated that, effect of different particle size could affect efficiency of extraction (Putra *et. al*, 2018). However, the particle size distribution below 500µm was not determined in the current study. This may be a limitation in the methodology and could affect some of the differences seen in the results.

2.3.2 Microbial profile of spices and herbs

Microbiological analysis of the spices and herbs samples (processed and unprocessed) were carried out to determine their microbial profile before extraction. Total plate count (TPC), total coliform, and *Listeria* spp, were evaluated. TPC was enumerated on Plate count agar (PCA-Oxoid CM0325) incubated at 30^{o}C for 48hr to evaluate growth of organisms. Coliforms was enumerated on Violet red bile glucose agar (VRBGA – Oxoid CM0485) and *Listeria* on brilliance listeria agar (BLA-Oxoid CM1080) incubated at 37^{o}C for 24hr to evaluate growth of organisms (Young *et al.,* 2014). This initial screening of raw spices and herbs was done to determine any contamination in raw sample and to monitor the hygienic condition of the spices and herbs.

2.3.3 Extraction optimization of spices and herbs

Different extraction method such as magnetic stirring, ultrasound-assited and separating funnel extraction were used to prepare crude extracts of spices and herbs. The spices and herbs were extracted with water or ethanol (method described by Witkowska *et al.,* 2013 with some modification). Spices and herbs such as coriander, cumin, thyme, fenugreek and mustard were studied using three methods and extracts were analyse for antimicrobial properties. The effect of different ratio combinations 1:10, 1:20, 1:30, 1:40 and 1:50 of solute (spice/herb powder) to solvent (water or ethanol) were examined on coriander, thyme, cumin, fenugreek and mustard against selected different microorganisms. For preparing 1:10 ratio, water or ethanol extracts were prepared by adding 1g of spice or herb to 9ml of distilled water or ethanol respectively. Similarly, 1:20 ratio prepared using 1g of spice powder to 19ml solvent. For 1:30 ratio, 1g of spice in 29ml solvent used. For 1:40 ratio, 1g spice in 39ml solvent and for 1:50 ratio, 1g of spice in 49ml solvent used. Different ethanol concentration 50%, 70% and absolute ethanol (98% industrial grade) was studied for the extraction of coriander.

Stirring extraction was done using the power magnetic stirrer IKA WERKE RT 15 (IKA, United Kingdom). Spice powder was added to conical flask followed by solvent (distilled water or ethanol) in required proportion (depend upon tested ratio of solute to solvent) and mixed well. Magnetic stirrer bar of size 30mm x 8mm was added to the flask. The power magnetic stirrer was adjusted at speed between 500 to 600 rpm at room temperature. The mixture was left on magnetic stirrer for 24 hours.

Ultrasound-assisted extraction method (UAE) was done using ultrasound apparatus UP400S Ultrasonic processor (Hielscher, Teltow, Germany). Spice powder was added in solvent (distilled water or ethanol) in required proportion (depend upon tested ratio of solute to solvent) and transferred to the double jacketed sonication beaker. The ultrasound probe was dipped in the mixture and a gap of at least 10mm was left between the base of the beaker and the probe in order to avoid the ultrasound shattering the beaker. The mixture of spice powder and solvent was sonicated at frequency of 24 kHz, cycle 0, 400W power at 20% or 100% amplitude for 5 minutes. Ultrasound temperature was controlled by circulation of cold water in outer jacket of sonication beaker.

In the third method, separating funnel extraction was carried out by adding 1g of spice powder to a separating funnel and 9ml of solvent (distilled water or ethanol) was then added from the top, ensure that the stopcock at the bottom was closed. After that, stopper at the top of separating funnel was closed tight and then funnel was vigorously shaken for multiple times. The funnel was left set on a clamp for one hour. This allows mixture to settle and solvent to diffuse into solute. After one hour, the bottom stopcock was opened, and top stopper was loosened to release the vacuum and to allow draining of extract by gravitation. The extraction was left overnight, and extract was collected in conical flask.

After extraction, spice and herb extracts were centrifuged at 9000 RPM for 10min using Rotanta 460R Centrifuge (Hettich lab technology, Tuttlingen, Germany). The supernatants were vacuum filtered using Whatman filter No 4. The filtrates were evaporated at 70^{o}C for water extracts and 40^{o}C for ethanol extracts (to less than 50% concentration of total weight of the sample) in a rotary evaporator (BUCHI 461 water bath). The concentrated extracts obtained were stored at 4^{o}C in airtight container for future used.

2.3.4 Test microorganisms and growth conditions

The antimicrobial activity of all spices and herbs extracts were determined against selected foodborne pathogenic microorganisms. The selection of these microorganisms was based on commonly found in improper food handling. These infectious pathogens and causes food poisoning that is characterised by abdominal cramps, diarrhoea, fever, and vomiting. Bacterial cultures of Gram-positive microorganisms such as - *Bacillus cereus* (ATCC 11778), *Listeria monocytogenes* (ATCC 7644), *Kocuria rhizophilia* (ATCC 9341), and Gram-negative microorganisms such as - *Escherichia coli* (ATCC 25922), *Salmonella enterica sv typhimurium* (ATCC 14028) and *Pseudomonas aeruginosa* (ATCC 27853), - used in the study were obtained from Remel Europe, Ltd. (Dartford, Kent, United Kingdom). The antimicrobial activity of ethanol extracts of selected Indian spices and herbs against *E.coli* Multi resistant strain (Leeds MR C822687) which is the most antibiotic resistant Gram-negative bacteria, was also studied. All microorganisms were maintained at -80^{o}C in cryovials and sub-cultured on Nutrient agar (NA) followed by incubation at 37^{o}C for all strains except at 25^{o}C for *Pseudomonas aeruginosa*. Working cultures were prepared from subcultures and grown on Nutrient agar under optimal conditions for each microorganism. For antimicrobial determination, a single colony of each strain was grown in 10ml Mueller Hilton broth (MHB) for 18±2 hr at 37^{o}C.

2.3.5 Determination of antimicrobial activity of spices and herb using agar disc diffusion assay

Extracts of spices and herbs were tested for antimicrobial activity using an agar disc diffusion method. In this method, the Muller Hilton (MH) agar was used as inoculated agar, different cultures grown for 18hr and turbidity was adjusted to 0.5 McFarland standard (Figure 2.1 mentioned in Balouiri *et al*., 2016), were serially diluted in 9ml of 0.1% peptone to obtain 10^8 cfu/ml (concentration used for all tested microorganisms except for *Bacillus cereus*, which is 10^6 cfu/ml used) and 100 µl (or 0.1ml) inoculum was spread on the surface of MH agar plates and allowed to dry for 5min. Twenty µl of spice or herb extracts was pipetted onto a dry sterile paper disc on the agar surface and incubated at 37^{o}C for 24-48hr. Disc diameter was 6mm, which was excluded from all readings. Microbial inhibition was determined by measuring the diameter of the clear zone of inhibition of growth around each disc and recorded as zone of inhibition (ZoI) and measure in mm (Weerakkody *et al.,* 2010).

Antibiotic such as – Ampicillin (10mcg), Erythromycin (15mcg), Chlorophenicol (30mcg), Streptomycin (10mcg) and Nalidixic acid (30mcg) were studied against each tested microorganism. For positive controls - Ampicillin and Erythromycin were used to compare the susceptible antimicrobial agent. For negative controls - water and ethanol were used. Ethanol alone is effective in inhibition of respective microorganisms. The ethanol readings are included in interpretation of antimicrobial results.

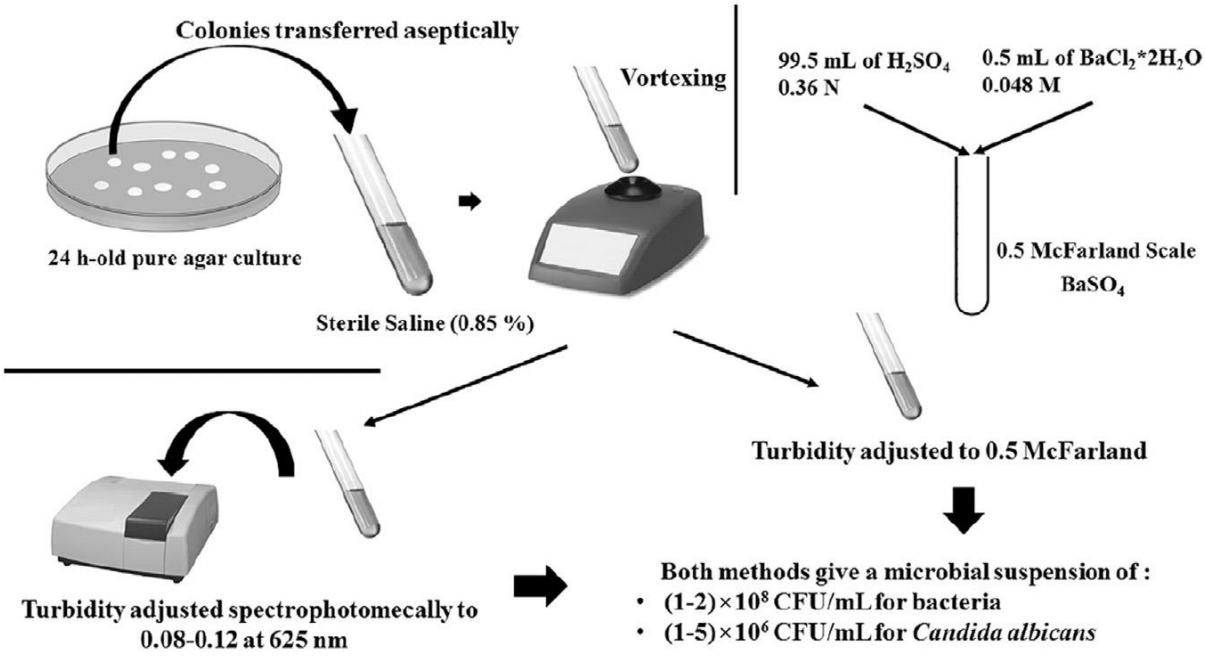


Figure 2.1: 0.5 McFarland microbial inoculum preparation by the direct colony suspension as recommended by CLSI guidelines (Balouiri *et al*., 2016)

2.3.6 Antimicrobial susceptibility test interpretive category

A classification of susceptible test is based on an *in vitro* response of a microorganism to an antimicrobial agent (spices and herbs extracts) at levels corresponding to prescribed agent (antibiotic discs). Susceptible is a category that implies that microorganism are inhibited by the achievable concentrations of antimicrobial agent. Intermediate is a category that includes microorganisms with antimicrobial agent that response rates may be lower than susceptible. Resistant is a category that implies that microorganisms are not inhibited by the achievable concentrations of antimicrobial agent. The microorganism's susceptibility to the antimicrobial is classified according to the criteria shown in the table 2.1. The figure 2.2 shows the

presentation of antibiotic disc on Mueller Hilton agar plates and result interpretation after incubation period.

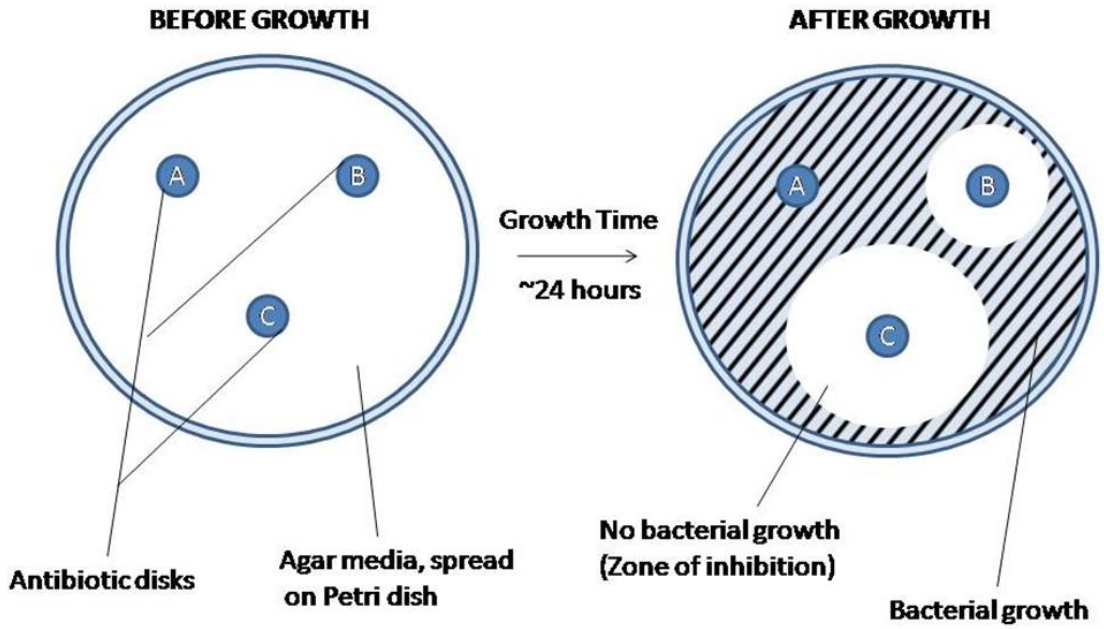


Figure 2.2 - After growth interpretation – A Zone means resistant category. B zone means intermediate and C zone means susceptible category. (Source of Image - EUCAST, January 2021)

Table 2.1 – The table showing the interpretive criteria of the zones of inhibition

	Zone of Inhibition (mm)
Susceptible	≥ 20
Intermediate	15-19
Resistant	≤ 14

(Reference - Adapted from CLSI protocol, 2012)

Ethanol alone is effective in inhibition of microorganism. So, for the calculations of zone of inhibition of microorganisms by antimicrobial agent (spice and herbs extract), ethanol zone of inhibition against respective microorganism was calculated and included in the readings. However, * is given to the readings showing ZoI above ethanol alone reading (negative control). Moreover, if the ZoI is higher than tested antibiotic readings (positive control) in that case ** is used. For instance in this study, if ethanol alone reading were excluded and

antimicrobial agent (spice and herb extract) is effective to inhibit respective microorganism >5mm ZoI then it was considered as Susceptible (* is given) if it was 5mm then Intermediate and resistant for 0mm ZoI.

2.3.7 Screening of processed and unprocessed spices and herbs based on antimicrobial properties

Processed spices and herbs were compared with Indian origin unprocessed spices and herbs based on antimicrobial properties. Spices and herbs such as coriander, cumin, thyme, bay leaves and cinnamon were studied. From extraction optimization, stirring (24hr) and 20% ultrasound-assisted for 5min extraction method were used, 1:10 ratio of spice to solvent were used in this study. For result discussion, spices and herbs were compared within type of solvent used (either water or ethanol), different extraction method (stirring and ultrasound assisted extraction method) and within selected Gram-positive and Gram-negative microorganisms. All the spices and herbs extracts were analyzed for antimicrobial properties using agar disc diffusion assay.

2.3.8 Screening of 35 spices and herbs based on antimicrobial properties

All the spices and herbs extracts were analyzed for antimicrobial properties using agar disc diffusion assay. All spice extract were compared for effective extraction method (stirring or ultrasound-assisted extraction) used and within effect on selected microorganism. Spices and herbs extract, giving highest antimicrobial inhibition of pathogenic microorganisms were selected for further antimicrobial analysis and further studies (Chapter 3).

2.3.9 Experimental design and Statistical analysis

This study was designed to obtain optimize extraction method using water and ethanol as extraction solvents. Extracts were compare based on the antimicrobial efficacy of processed and unprocessed Indian spices and herbs against six foodborne pathogenic organisms. All the experiments were performed in duplicates. The antimicrobial assays were performed with two replicates (i.e for each sample n=4). The final screening of 35 spices and herbs were compared using ethanol extracts prepared using stirring and ultrasound-assisted extraction methods and compared against six foodborne pathogenic microorganisms. The data was analysed using Microsoft office Excel and results were expressed as mean ± standard deviation.

2.4 Results

2.4.1 Initial microbial profile of processed and unprocessed Indian spices and herbs

Microbial profile of all tested processed and unprocessed Indian spices and herbs were evaluated. Total viable count (TVC), presence of *enterobacteriaceae* and *Listeria* was examined in all raw and ground samples (Table 2.2). It was observed that the average TVC of unprocessed Indian spices and herbs had less microbial contamination (< 3 log cfu/g) as compared to processed spices and herbs (> 3 log cfu/g). It was noticed that all unprocessed Indian raw spices and herbs were free from *Enterobacteriaceae* and *Listeria* whereas in processed samples presence of *Enterobacteriaceae* was detected. However, no *Listeria* was observed in processed samples (Table 2.2).

Table 2.2- Initial total viable count, *Enterobacteriaceae* and *Listeria* counts of processed and unprocessed spices and herbs in log cfu/g

Spices and herbs	Processed			Unprocessed		
	Total viable count	*Enterobacteriaceae*	*Listeria*	Total viable count	*Enterobacteriaceae*	*Listeria*
Coriander	ND	< log 2	ND	ND	ND	ND
Cumin	<3 log	< log 2	ND	ND	ND	ND
Thyme	ND	< log 2	ND	ND	ND	ND
Bay leaves	>3 log	> log 3	ND	< 3 log	ND	ND
Cinnamon	>3 log	> log 3	ND	< 3 log	ND	ND

ND- Not detected in the sample. Results are expressed in log cfu/g.

2.4.2 Antimicrobial properties of spices and herbs extracts

Results of antibiotics including Erythromycin (15 µg), Ampicillin (10 µg) and some other antibiotics were tested against all organisms which was shown in Table 2.3.

Table 2.3- Antimicrobial properties (Zone of inhibition) of controls against tested organisms

Control	*Bacillus*	*Listeria*	*Kocuria*	*E.coli*	*Salmonella*	*Pseudomonas*
Water	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Ethanol	9 ± 0	6 ± 0	6 ± 0	9 ± 0	9 ± 0	4 ± 0
Erythromycin	24 ± 0	18 ± 0	34 ± 0	6 ± 0	6 ± 0	14 ± 0
Ampicillin	4 ± 0	18 ± 0	34 ± 0	9 ± 0	14 ± 0	0 ± 0
Streptomycin	18 ± 0	9 ± 0	14 ± 0	14 ± 0	6 ± 0	6 ± 0
Chlorophenicol	14 ± 0	14 ± 0	29 ± 0	18 ± 0	14 ± 0	0 ± 0
Nalidixic acid	9 ± 0	0 ± 0	0 ± 0	14 ± 0	14 ± 0	0 ± 0

All the results shown in table were in mm and diameter of disc (6mm) was excluded. (0) means no zone of inhibition observed in the sample. Values represent mean of triplicates n=3 ± SD.

2.4.3 Optimization of extraction process

1. **Optimization of spice and herbs based on different ratios**

Selected spice and herbs (coriander, cumin, thyme, fenugreek and mustard) water extracts prepared using stirring method at different ratios of solute to solvent 1:10, 1:20, 1:30 ratios (except fenugreek – tested ratios were 1:10 to 1:50) were studied against antimicrobial activity. Out of the ratios tested for coriander, 1:10 was found to be most efficient in inhibiting growth of Gram-negative microorganisms such as *E.coli, Salmonella* and *Pseudomonas*. Similar results obtained for cumin and thyme spice water extracts prepared using stirring method (result tables presented in Appendix A). Different ratios of water extracts of fenugreek were prepared using stirring and ultrasound method were studied against antimicrobial activity. It was found that 1:10 ratio, water extract of fenugreek is not good sample as water gets absorbed by fenugreek seed powder and no/little extract obtained showed no ZoI. Ethanol extracts of fenugreek prepared using stirring method shows some good results on inhibition of microorganisms (result tables provided in Appendix A). However, 1:10 ratio was found to be most efficient as compare to other ratios in which higher amount of ethanol was used. Different ratios for mustard ethanol extracts were studied. It was found that 1:10 ratio was most effective in inhibition of the selected microorganisms. Therefore, from ZoI results of tested spices extracts, 1:10 ratio (spice to solvent) was finally selected for all further analysis.

2. Optimization of spice and herb extracts using different ethanol concentration

Coriander ethanol extracts were prepared using stirring method and studied for different ethanol concentration. It was found that absolute ethanol concentration is highly effective in inhibition of microorganisms as compared to 50% and 70% ethanol concentration (result provided in table 3 Appendix A).

3. Optimization of spice and herb extraction using different methods

For the selection of best extraction method, fenugreek ethanol extracts (1:10 ratio used). Fenugreek ethanol extracts were prepared using 1:10 ratio and studied for the selection of best extraction method. Separating funnel, stirring and ultrasound method were studied. Water extracts of fenugreek is not suitable for separating funnel method, it was found that water gets absorbed by fenugreek powder and it blocked the separating funnel nozzel and stopped the extraction method. Same condition was observed with water extracts of cumin, thyme and bay leaves (result table in Appendix A). Limitation of the work might be only 1:10 ratio of water samples were considered for this study. For ethanol extracts, few crude extract was collected from separating funnel and analysed. However, showed no effect on inhibition as compared to control ethanol and other extraction methods. The use of more ethanol in extraction shows some extraction (result table in Appendix A). However, use of higher amount of ethanol was not selected for this study. Hence, this method was not considered for further experiments (Table 4 in Appendix A). Coriander water and ethanol extract, 1:10 ratio was used and studied for different extraction method – Stirring, ultrasound and separating funnel method. It was found that ultrasound-assisted extraction is the best method of extraction (Table 5 in Appendix A).

4. Optimization of spice and herb ultrasound extraction based on different settings

Different ultrasound setting – 20% or 100% amplitude for 5 min each was studied for the extraction of fenugreek ethanol extracts at different ratios. It was found that ultrasound extraction at 20% amplitude for 5 min is better method than 100% amplitude in inhibition of selected microorganisms (result table in Appendix A).

2.4.4 Screening of processed and unprocessed Indian spices and herbs based on antimicrobial properties

Processed and unprocessed Indian origin spices and herbs including- coriander, cumin, thyme, bay leaves and cinnamon was compared against six tested pathogenic microorganisms. Table 2.4 and Table 2.5 shows the results of effect of water and ethanol extracts of processed and unprocessed Indian spices and herbs on tested microorganisms. Water extracts using stirring and ultrasound method shows little or no effect on inhibition as compared to ethanol extraction using stirring and ultrasound method. Ultrasound-assisted extraction method using ethanol was found to most efficient in unprocessed Indian origin thyme followed by bay leaves, cinnamon than any other method for inhibition.

Table 2.4 - Screening of water extracts processed vs unprocessed Indian spices and herbs based on antimicrobial properties

Selected microorganisms		Coriander		Cumin		Thyme		Bay leaves		Cinnamon		Water
		Processed	Unprocessed	Processed	Unprocessed	Processed	Unprocessed	Processed	Unprocessed	Processed	Unprocessed	
Bacillus cereus	**Str**	0 ± 0	0 ± 0	0 ± 0	0 ± 0	5.5 ± 0.57	4 ± 0	3.5 ± 0.57	4 ± 0	5.5 ± 0.57	4 ± 0	0 ± 0
	Ultr	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	3 ± 0	4.5 ± 0.57	4 ± 0	5.5 ± 0.57	0 ± 0
Listeria monocytogenes	**Str**	0 ± 0	0 ± 0	3.5 ± 0.57	7 ± 0*	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	4 ± 0	0 ± 0
	Ultr	4.5 ± 0.57	0 ± 0	0 ± 0	0 ± 0	4 ± 0	0 ± 0	4 ± 0	0 ± 0	4 ± 0	0 ± 0	0 ± 0
Kocuria rhizophilia	**Str**	0 ± 0	0 ± 0	7.5 ± 0.57*	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	4 ± 0	1 ± 0	0 ± 0
	Ultr	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	1.5 ± 0.57	0 ± 0
E.coli	**Str**	3.5 ± 0.57	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	Ultr	4 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Salmonella typhi	**Str**	3.5 ± 0.57	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	Ultr	3.5 ± 0.57	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Pseudomonas aeruginosa	**Str**	4.5 ± 0.57	4 ± 0	3 ± 0	4 ± 0	0 ± 0	5 ± 0	3.5 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	Ultr	0 ± 0	3 ± 0	0 ± 0	4 ± 0	0 ± 0	5 ± 0	3 ± 0	0 ± 0	3 ± 0	0 ± 0	0 ± 0

Diameter of zone of inhibition (ZoI) (in mm), values represent mean of four replicates ± SD, excluding diameter of disc 6mm from readings.

Table 2.5 - Screening of ethanol extracts of processed vs unprocessed Indian spices and herbs based on antimicrobial properties

Selected microorganisms		Coriander		Cumin		Thyme		Bay leaves		Cinnamon		Ethanol
		Processed	Unprocessed	Processed	Unprocessed	Processed	Unprocessed	Processed	Unprocessed	Processed	Unprocessed	
Bacillus cereus	**Str Eth**	9 ± 0	9 ± 0	12 ± 0	11 ± 0	14 ± 0	13 ± 0	**16.5 ± 0.5***	**15 ± 0***	**15.5 ± 0.57***	14 ± 0	
	Ultr Et	9 ± 0	11 ± 0	9 ± 0	13 ± 0	12 ± 0	**20 ± 1.15****	9 ± 0	**17 ± 0***	**15 ± 0***	**16 ± 1.15***	9
Listeria monocytogenes	**Str Eth**	7.5 ± 0.57	8 ± 0	**13 ± 0***	**12 ± 0***	9.5 ± 0.57	**12.5 ± 0.57***	**13 ± 0***	9 ± 0	**15.5 ± 0.57***	9 ± 0	
	Ultr Eth	11 ± 0	8 ± 0	6 ± 0	10 ± 0	6 ± 0	**17 ± 0****	6 ± 0	**12 ± 0***	6 ± 0	9 ± 0	6
Kocuria rhizophilia	**Str Eth**	6 ± 0	6 ± 0	9 ± 0	**13.5 ± 0.57***	6 ± 0	**14.5 ± 0.57***	9 ± 0	9 ± 0	**12 ± 0***	9 ± 0	
	Ultr Eth	6 ± 0	7.5 ± 0.57	9 ± 0	**15 ± 0***	11 ± 0	**15.5 ± 0.57***	9 ± 0	9 ± 0	3 ± 0	9 ± 0	6
E.coli	**Str Eth**	12 ± 0	14 ± 0	13 ± 0	9 ± 0	9 ± 0	12 ± 0	13 ± 0	9 ± 0	11 ± 0	9 ± 0	
	Ultr Eth	**16 ± 0***	13 ± 0	14 ± 0	13 ± 0	12 ± 0	13 ± 0	13 ± 0	9 ± 0	13 ± 0	9 ± 0	9
Salmonella typhi	**Str Eth**	12 ± 0	14 ± 0	9 ± 0	12 ± 0	9 ± 0	14 ± 0	14 ± 0	12 ± 0	14 ± 0	9 ± 0	
	Ultr Eth	12 ± 0	11.5 ± 0.57	12 ± 0	14 ± 0	12 ± 0	12 ± 0	13 ± 0	12 ± 0	12 ± 0	9 ± 0	9
Pseudomonas aeruginosa	**Str Eth**	9 ± 0	9 ± 0	6 ± 0	**12 ± 0***	4 ± 0	9 ± 0	7 ± 0	**12 ± 0***	9 ± 0	**14 ± 0****	
	Ultr Eth	9 ± 0	**11.5 ± 0.57***	7 ± 0	**13 ± 0***	9 ± 0	**14 ± 0****	9 ± 0	**12 ± 0***	4 ± 0	**12 ± 0***	4

Diameter of zone of inhibition (ZoI) (in mm), values represent mean of four replicates ± SD, excluding diameter of disc 6mm from readings. Negative control Ethanol. Ethanol alone response against respective microorganisms are included in the readings. Significance * - intermediate antimicrobial agent, ** - susceptible antimicrobial and effective as antibiotic. (Str Eth – stirring ethanol, Ultr Eh – Ultrasound ethanol).

	Bacillus	*Listeria*	*Kocuria*	*E.coli*	*Salmonella*	*Pseudomonas*
Erythromycin	24	18	34	6	6	14
Ampicillin	4	18	34	9	14	0

2.4.5 Screening of 35 spices and herbs based on antimicrobial properties

For final screening, thirty-five unprocessed Indian origin spices and herbs were compared for the selection of most efficient spices and herbs in Table 2.6 and 2.7. Each spice extract compared within type of extraction method used (stirring or ultrasound-assisted extraction), type of solvent used (water or ethanol), type of microorganism (Gram-positive or Gram-negative) and with positive (antibiotic disc) and negative controls (water or ethanol alone).

Water extracts showed no effect on inhibition of pathogenic microorganisms (Table 2.6). Ethanol extracts extracted using stirring and ultrasound-assisted extraction process was able to inhibit growth of tested microorganisms (Table 2.7). However, some ethanol extracts show no effect in inhibition, inhibitory effect was noticed which was due to ethanol alone. In most of the spices extracts, ethanol extracts prepared using ultrasound-assisted extraction method was found to most efficient in inhibition of *Bacillus, Listeria* and *Pseudomonas* than stirring ethanol extraction method (Table 2.7).

The results of effect of ethanol extracts on *E.coli* multi resistant strain (Leeds MR C822687) showed that ethanol extract of stirring method is more efficient than ultrasound extract of Indian cumin (5mm ZoI). Ethanol extracts of ultrasound-assisted method was effective in inhibition of *E.coli* MR strain, in Indian thyme (>5mm ZoI) and bay leaves (5mm ZoI). Interestingly, antibiotic sensitivity was not observed for this strain of *E.coli* in Erythromycin and ampicillin.

Table 2.6- Screening of 35 unprocessed Indian spices and herbs water extracts based on antimicrobial properties

Selected spices and herbs	*Bacillus cereus*		*Listeria monocytogenes*		*Kocuria rhizophilia*		*E.coli*		*Salmonella typhi*		*Pseudomonas aeruginosa*	
	Str	**Ultr**	**Str**	**Ultr**	**Str**	**Ultr**	**Str**	**Ultr**	**Str**	**Ultr**	**Str**	**Ultr**
Ajwain	4 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Asafoetida (Hing)	4 ± 0	4 ± 0	4 ± 0	4 ± 0	3.5 ± 0.57	4 ± 0	4 ± 0	0 ± 0	0 ± 0	0 ± 0	4 ± 0	4 ± 0
Bay leaves	4 ± 0	4.5 ± 0.57	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Black pepper	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Black salt	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Brown Cardamom	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Brown mustard	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	4.5 ± 0.57
Cinnamon	4 ± 0	5.5 ± 0.57	4 ± 0	0 ± 0	1 ± 0	1.5 ± 0.57	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Cloves	3.5 ± 0.57	0.5 ± 0.57	3 ± 0	3 ± 0	4 ± 0	3 ± 0	3 ± 0	3 ± 0	1 ± 0	0 ± 0	3 ± 0	3 ± 0
Coriander	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	4 ± 0	3 ± 0
Cumin	0 ± 0	0 ± 0	7 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	4 ± 0	4 ± 0
Curry leaves	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Dry ginger powder	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	4 ± 0
Fennel	0 ± 0	1 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Fenugreek	0 ± 0	0 ± 0	0 ± 0	2 ± 0	0 ± 0	0 ± 0	0 ± 0	3 ± 0	0 ± 0	3 ± 0	0 ± 0	0 ± 0
Ginger (paste)	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Green cardamom	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	4 ± 0
Holy basil	0 ± 0	1 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	4 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Indian Gooseberry	0 ± 0	0 ± 0	6 ± 0	5 ± 0	0 ± 0	0 ± 0	6 ± 0	6 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Inkut (Harad)	0 ± 0	0 ± 0	6 ± 0	0 ± 0	4 ± 0	3 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
long Pepper	0 ± 0	0 ± 0	0 ± 0	0 ± 0	4 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Mace	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

Table 2.6 continued... Screening of 35 unprocessed Indian spices and herbs water extracts based on antimicrobial properties

Mango powder (Amchoor)	0 ± 0	0 ± 0	0 ± 0	4 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	5.5 ± 0.57
Mint leaves	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Mustard (Black)	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Nigella seeds (Kalonji)	3 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Nutmeg	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Pomegranate seeds	0 ± 0	0 ± 0	4 ± 0	3.5 ± 0.57	3 ± 0	3 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	3 ± 0	3 ± 0
Poppy seeds	0 ± 0	0 ± 0	0 ± 0	0 ± 0	6 ± 0	4 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Red chilli powder	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Saffron	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Sesame seeds	3 ± 0	2.25 ± 0.28	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Star anise	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Tamarind	2 ± 0	1.75 ± 0.28	0 ± 0	4.5 ± 0.57	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Turmeric	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

Diameter of zone of inhibition (ZoI) (in mm), values represent mean of four replicates ± SD, excluding diameter of disc 6mm from readings.

Table 2.7- Screening of 35 unprocessed Indian spices and herbs ethanol extracts based on antimicrobial properties

Selected spices and herbs	*Bacillus cereus*		*Listeria monocytogenes*		*Kocuria rhizophilia*		*E.coli*		*Salmonella typhi*		*Pseudomonas aeruginosa*	
	Str Eth	**Ultr Eth**	**Str Eth**	**Ultr Eth**	**Str Eth**	**Ultr Eth**	**Str Eth**	**Ultr Eth**	**Str Eth**	**Ultr Eth**	**Str Eth**	**Ultr Eth**
Ajwain	13 ± 0	**20 ± 0****	**12.5 ± 0.57***	**17 ± 1.15****	**14.5 ± 0.57****	**15.5 ± 0.57****	12 ± 0	13 ± 0	14 ± 0	12 ± 0	9 ± 0	**14 ± 0****
Asafoetida (Hing)	12 ± 0	13 ± 1.15	11 ± 1.15	9.5 ± 0.57	**13 ± 1.15***	**13 ± 1.15***	13 ± 0	13.5 ± 0.57	11.25 ± 0.95	9 ± 0	9 ± 0	**10 ± 1.15***
Bay leaves	**15 ± 0***	**17 ± 0***	9 ± 0	**12 ± 0***	9 ± 0	9 ± 0	9 ± 0	9 ± 0	12 ± 0	12 ± 0	**12 ± 0***	**12 ± 0***
Black pepper	9 ± 0	9 ± 0	6 ± 0	6 ± 0	6 ± 0	6 ± 0	9 ± 0	9 ± 0	9 ± 0	9 ± 0	9 ± 0	9 ± 0
Black salt	9 ± 0	9 ± 0	11 ± 0	9.5 ± 0.57	9 ± 0	9 ± 0	12 ± 0	12 ± 0	11.5 ± 0.57	12 ± 0	9 ± 0	9 ± 0
Brown Cardamom	9 ± 0	9 ± 0	**12 ± 0***	10 ± 0	7.5 ± 0.57	6 ± 0	14 ± 0	13 ± 1.15	10 ± 0	10 ± 0	**13 ± 1.15***	**11.25 ± 0.5***
Brown mustard	9 ± 0	9 ± 0	6 ± 0	6 ± 0	6 ± 0	6 ± 0	12 ± 0	12 ± 0	9 ± 0	9 ± 0	9.5 ± 0.57	10.5 ± 0.57*
Cinnamon	14 ± 0	**16 ± 1.15***	9 ± 0	9 ± 0	9 ± 0	9 ± 0	9 ± 0	11 ± 0	9 ± 0	9 ± 0	**14 ± 0****	**12 ± 0***
Cloves	14 ± 0	14 ± 0	9 ± 0	9 ± 0	10 ± 0	9 ± 0	**15 ± 0***	14 ± 0	9 ± 0	9 ± 0	13.5 ± 0.57	13 ± 0
Coriander	9 ± 0	11 ± 0	8 ± 0	8 ± 0	6 ± 0	7.5 ± 0.57	14 ± 0	13 ± 0	14 ± 0	11.5 ± 0.57	9 ± 0	**11.5 ± 0.57***
Cumin	11 ± 0	13 ± 0	**12 ± 0***	10 ± 0	**13.5 ± 0.57***	**15 ± 0***	9 ± 0	13 ± 0	12 ± 0	14 ± 0	**12 ± 0***	**13 ± 0***
Curry leaves	9 ± 0	9 ± 0	9 ± 0	9 ± 0	8.5 ± 0.57	9 ± 0	9 ± 0	9 ± 0	10.5 ± 0.57	10 ± 0	9.5 ± 0.57	**10.5 ± 0.57***
Dry ginger powder	9 ± 0	9 ± 0	6 ± 0	9 ± 0	6 ± 0	6 ± 0	9 ± 0	12 ± 0	9 ± 0	9 ± 0	**10 ± 1.15***	**10 ± 0***

Table 2.7 continued… Screening of 35 unprocessed Indian spices and herbs ethanol extracts based on antimicrobial properties

Fennel	9 ± 0	9 ± 0	9 ± 0	9 ± 0	6 ± 0	7 ± 0	11.5 ± 0.57	13 ± 1.15	9 ± 0	11 ± 0	5 ± 0	6.5 ± 0.57
Fenugreek	9 ± 0	9 ± 0	**12 ± 0***	**15 ± 0***	**12 ± 0***	**12 ± 0***	12 ± 0	12 ± 0	12 ± 0	12 ± 0	7 ± 0	4 ± 0
Ginger (paste)	11 ± 0	9 ± 0	10 ± 0	9 ± 0	11 ± 0	10 ± 0	12 ± 0	12 ± 0	9 ± 0	9 ± 0	7.5 ± 0.57	7 ± 0
Green cardamom	9 ± 0	9 ± 0	9 ± 0	9.5 ± 0.57	6 ± 0	6 ± 0	14 ± 0	14 ± 0	9 ± 0	10 ± 0	**14 ± 0****	**14 ± 0****
Holy basil	9 ± 0	9 ± 0	**12 ± 0***	11 ± 0	**14 ± 0***	**12 ± 0***	14 ± 0	10 ± 0	11 ± 0	10 ± 0	9 ± 0	**10 ± 1.15***
Indian Gooseberry	**16 ± 0***	**16 ± 0***	11.5 ± 0.57	11 ± 0	**12 ± 0***	**12 ± 0***	9 ± 0	9 ± 0	9 ± 0	9 ± 0	**12.5 ± 0.57***	**12 ± 0***
Inkut (Harad)	9 ± 0	9 ± 0	9 ± 0	9 ± 0	10 ± 0	6 ± 0	9 ± 0	9 ± 0	12 ± 0	12 ± 0	**12 ± 0***	**12 ± 0***
long Pepper	9 ± 0	9 ± 0	6 ± 0	6 ± 0	9 ± 0	9 ± 0	12 ± 0	12 ± 0	10.5 ± 0.57	10 ± 0	9 ± 0	9 ± 0
Mace	14 ± 0	9 ± 0	**18.25 ± 0.5****	9 ± 0	6 ± 0	6 ± 0	11.5 ± 0.57	12.5 ± 0.57	9 ± 0	9 ± 0	**14 ± 0****	**14 ± 0****
Mango powder (Amchoor)	9 ± 0	9 ± 0	**12 ± 0***	**13.75 ± 0.5***	11 ± 0	**12 ± 0***	12 ± 0	12 ± 0	11 ± 0	11.5 ± 0.57	9 ± 0	**12 ± 0***
Mint leaves	9 ± 0	9 ± 0	6 ± 0	6 ± 0	6 ± 0	6 ± 0	9 ± 0	11 ± 0	9 ± 0	9 ± 0	4 ± 0	4 ± 0
Mustard (Black)	9 ± 0	9 ± 0	6 ± 0	6 ± 0	7 ± 0	8 ± 0	10 ± 0	10 ± 0	10 ± 0	11 ± 0	7 ± 0	7 ± 0
Nigella seeds (Kalonji)	9 ± 0	9 ± 0	6 ± 0	7 ± 0	6 ± 0	7 ± 0	9 ± 0	9 ± 0	9 ± 0	9 ± 0	8.5 ± 0.57	8 ± 0
Nutmeg	**15.5 ± 0.57***	**15 ± 0***	**18.5 ± 0.57****	**16.5 ± 0.57****	**16 ± 0****	**15 ± 1.15****	**15 ± 0***	14 ± 0	12.5 ± 0.57	12 ± 0	**12 ± 0***	**10 ± 0***
Pomegranate seeds	9 ± 0	9 ± 0	**14.75 ± 0.95***	**13 ± 1.15***	9.5 ± 0.57	9 ± 0	**15 ± 1.15***	13 ± 1.15	12.5 ± 0.57	12 ± 0	**10.5 ± 0.57***	**10.5 ± 0.57***
Poppy seeds	9 ± 0	9 ± 0	6 ± 0	10 ± 1.15	**12 ± 0***	11 ± 1.15	12 ± 0	12 ± 0	13 ± 1.15	14 ± 0	**12 ± 0***	**11.5 ± 0.57***

Table 2.7 continued… Screening of 35 unprocessed Indian spices and herbs ethanol extracts based on antimicrobial properties

Red chilli powder	**16 ± 0***	**15 ± 1.15***	11.5 ± 0.57	10.5 ± 0.57	9.5 ± 0.57	9 ± 0	14 ± 0	14 ± 0	12 ± 0	12 ± 0	9 ± 0	9 ± 0
Saffron	9 ± 0	9 ± 0	9 ± 0	9 ± 0	7.5 ± 0.57	7 ± 0	10.5 ± 0.57	10 ± 0	9 ± 0	9 ± 0	7 ± 1.15	6 ± 0
Sesame seeds	9 ± 0	9 ± 0	9 ± 0	10.25 ± 0.95	9 ± 0	9 ± 0	11.5 ± 0.57	12.5 ± 0.57	11.5 ± 0.57	11.5 ± 0.57	**11.5 ± 0.57***	**12.5 ± 0.57***
Star anise	9 ± 0	9 ± 0	9.5 ± 0.57	9.5 ± 0.57	9 ± 0	7 ± 0	12.5 ± 0.57	13.5 ± 0.57	9 ± 0	9 ± 0	**14 ± 0****	**10.5 ± 0.57***
Tamarind	9 ± 0	9 ± 0	**12 ± 0***	10 ± 0	10 ± 1.15	10 ± 1.15	13 ± 1.15	11.5 ± 0.57	12 ± 0	11.5 ± 0.57	**10 ± 0***	**11 ± 1.15***
Turmeric	9.5 ± 0.57	9 ± 0	9 ± 0	9 ± 0	9.5 ± 0.57	9 ± 0	12 ± 0	12 ± 0	9 ± 0	9 ± 0	8 ± 0	8 ± 0

Diameter of zone of inhibition (ZoI) (in mm), values represent mean of four replicates ± SD, excluding diameter of disc 6mm from readings. Negative control Ethanol. Ethanol alone response against respective microorganisms are included in the readings. Significance * - intermediate antimicrobial agent, ** - susceptible antimicrobial and effective as antibiotic. (Str Eth – stirring ethanol, Ultr Eh – Ultrasound ethanol).

	Bacillus	*Listeria*	*Kocuria*	*E.coli*	*Salmonella*	*Pseudomonas*
Erythromycin	24	18	34	6	6	14
Ampicillin	4	18	34	9	14	0

2.5 Discussion

From microbial profile analysis, it was observed that in processed grounded spices and herbs TVC count was higher than unprocessed Indian spices and herbs, which was primary procured and obtained from wholegrain market without any processing and packaging (Table 2.2). No *Enterobacteriaceae* and *Listeria* growth was noticed in both sample types of processed and unprocessed Indian spices and herbs, which indicates initial hygienic conditions of samples (Table 2.2). It was assumed that unprocessed Indian raw spices and herbs were more efficient than processed samples in terms of purity of raw samples. Processed spices were processed and packed which increased its chance to compromise with hygienic conditions. Whereas unprocessed spices and herbs were directly collected from fields and chance of handling contamination is low. Although these spices have dry stalk, straws and dirt which doesn't show much effect on initial microbial profile.

In the current study, it was found that when ethanol was combined with ultrasound-assisted extraction process of unprocessed thyme extracts showed effective inhibitory activity compare to erythromycin (15 mcg) and ampicillin (10 mcg) against *Bacillus cereus, Listeria monocytogenes, E.coli* and *Pseudomonas aeruginosa* (Table 2.3). Similar to these result, application of thyme extract showed stronger antimicrobial activity compare to erythromycin (15 mcg) which was observed by Toroglu, (2007) in plant extracts. Ethanol combined with ultrasound-assisted extraction of processed coriander and unprocessed Indian cumin showed effective antimicrobial activity compare to erythromycin (15 mcg) and ampicillin (10 mcg) against *E.coli* and *Salmonella typhimurium* respectively. Surprisingly, *E.coli* multi resistant strain showed resistance to antibiotic such as erythromycin and ampicillin. However, ethanol extracts of unprocessed Indian cumin, thyme and bay leaves was found to be moderately efficient in inhibition based on antimicrobial analysis.

Antimicrobial sensitivity of *Kocuria rhizophilia* to erythromycin and ampicillin was highest (34mm ZoI) (Table 2.3). Spices such as thyme and cumin are susceptible in inhibition of *K. rhizohilia* whereas ethanol extracts of thyme (9mm ZoI) and cumin (8mm ZoI) for *K. rhizophilia* were not effective as positive controls - erythromycin (15 µg) and ampicillin (10 µg). *K. rhizophila* which has not previously been identified as a pathogen but was isolated from chicken treated with oxalic acid (Anang *et al*., 2006), has shown resistant to many antibiotic

and very limited literature on *Kocuria* species are cited and therefore of interest. To understand more about this pathogen, selected spices extracts were tested, and further antimicrobial properties were examined in chapter 3. Unprocessed Indian bay leaves and cinnamon ethanolic extracts also showed antimicrobial activity against *Bacillus cereus, Listeria monocytogenes* and *Pseudomonas aeruginosa* compare to erythromycin and ampicillin. Although ethanol itself showed some antimicrobial activity against these pathogens (Table 2.5). However, these ethanol spice extracts were more effective than ethanol alone.

Optimization of extraction process- Ratio of 1:10 of spice or herb extract to water or ethanol was found to be most effective against pathogens (result tables provided in Appendix A). In most of the studies 1:10 and 1:20 ratio of sample to water or ethanol and absolute ethanol concentration was used (Henie *et al*., 2009; Witkowska *et al.*, 2013). Therefore, from result figure 2.3, 1:10 ratio of spices or herbs to water or ethanol was used to examine antimicrobial activity of further extracts.

The type of solvent used for extraction of spices and herbs is another factor that have a great impact on antimicrobial property. All ethanol extracted spices and herbs indicated a significant antimicrobial activity for the extracts (Table 2.7). The antimicrobial property of water extracts of spices and herbs showed that water extracts demonstrated little or no significant antimicrobial effect on the tested foodborne pathogens (Table 2.6). Generally, extraction of spices and herbs using ethanol extraction showed greater antimicrobial activity as compared to water extracts. This is due to the fact that, different chemical compounds were extracted using non-polar solvents their high level of solubility in solvents such as ethanol, methanol or hexane (Cowan, 1999). Previous studies support these finding in which water extracts showed little or no effect against tested pathogens as compared to effective results obtained with ethanol or other non-polar solvents. Henie *et al.* (2009), found guava leaf extracts from methanol were effective against *S. typhimurium, K. rhizophila* and *L. monocytogenes.* According to Weerakkody *et al*. (2010), results of extraction of *A. galangal, R.* Officinalis and *O. vulgare* with hexane showed greater antimicrobial activities than extraction with ethanol and water. Zhang *et al.* (2009) found that ethanol extracts of clove and rosemary showed significant inhibitory activities against *Listeria monocytogenes, E.coli a*nd *P. fluorescens*. Solvents such as hexane, methanol could be effective for extraction than

ethanol or water because non-polar solvent has a high preferential interaction with non-polar chemical compounds, leading to the extraction of more chemical compounds that could be responsible for antimicrobial properties. In the present study, antimicrobial properties were evaluated using water and ethanol extracts of spices and herbs, which limits to obtain the bioactive ingredients against tested pathogens. However, above-mentioned results suggested a further investigation with other organic solvent such as extraction using hexane could provide the exponential growth inhibition findings. However, these solvents has their own disadvantages to be used in food.

In the present study, water extracts of cinnamon and mustard displayed weak or no antimicrobial activities against *E.coli, Salmonella, Listeria* and *Kocuria* (Table 2.4). Contrastingly, study by Sofia *et al.* (2007), reported good inhibitory effect of water extracts of mustard, cinnamon, garlic and clove against *E.coli* and *S. aureus*. Interestingly, in the present study, water extracts of some spices such as processed coriander, cumin, thyme and cinnamon obtained using stirring extraction method, showed intermediate susceptible (5mm ZoI) antimicrobial activity against *Bacillus, Listeria* and *Kocuria* (Table 2.4). Therefore, these results suggest that some water-soluble compounds such as polyphenolic compounds showed some moderate antimicrobial effect and were present in these extracts.

In the present study, ultrasound-assisted ethanol extracts of thyme, cumin, bay leaves and cinnamon showed effective antimicrobial properties against the tested pathogens compare to other extraction methods such as use of stirring and a separating funnel (Table 2.5). Similar to the present study, Deng (2015), found that ultrasound extracted leaf extracts of *S. Canadensis*, showed more antibacterial effect compared to other extraction method. A study by Witkowska *et al* (2010) showed that, ethanol extracts using universal shaker gives efficient inhibition results over water extracts of some spices and herbs. In the present study, extraction using separating funnel, was found to be not effective against extraction of spices and herbs extracts. Using this method, water extraction was not possible as it blocked the separating funnel. Therefore, the samples were not analysed. Ethanol extracts yielded some extracted sample however, when antimicrobial activity of the yielded sample was analysed, no effective results were obtained. It was concluded that, this method was not effective for

extraction of spices and herbs. Contrastingly, Henie *et al*. (2009) observed effective results in guava leaves that was extracted using fractionated column using hexane and methanol. In this study, different plant material (Guava leaves), different ratio of plant to solvent, different extraction method was used that is why this is difficult to compare directly with the present study.

Spices and herbs such as cumin, thyme, bay leaves, cinnamon and coriander have shown an effective antimicrobial efficiency against a broad range of foodborne pathogens such as *Listeria monocytogenes, Bacillus cereus, E.coli, Salmonella typhimurium, Pseudomonas* sp and *Kocuria rhizophilia* (Table 2.7). Previous studies supported the findings in which the tested spices and herbs showed an effective antimicrobial potency against Gram-positive and Gram-negative bacterial species (Burt, 2004; Tajkarimi *et al.*, 2010). In our study, ultrasound-assisted ethanol extracts of almost all spices and herbs against Gram-negative *E.coli* and *Salmonella typhimurium* was less effectiveness compare to other Gram-positive organisms. Similarly Deng (2015), found that the ultrasound extracted leaf extracts of the *S. Canadensis*, showed more antibacterial effect compared to ethanolic extraction and found Gram-negative *E. coli*, and *Salmonella* spp. (< 6mm ZoI) were less susceptible to plant extracts than the Gram-positive *S. aureus*, and *L. monocytogenes* (> 7.5mm ZoI). Moreover, results showing Gram-positive bacteria are always more susceptible (Burt, 2004) and results of some studies were found with greater antimicrobial efficacy (ZoI >5mm) of *Eucalyptus staigerana* against Gram-negative *E.coli* compared to Gram-positive *L. monocytogenes* (Weerakkody et al., 2010).

A study by Raj et al. (2008) reported similar results, of thyme and *Syzygium gardneri* were effective against *Bacillus cereus* and *Pseudomonas fluorescens* respectively. Another study revealed that 0.5-1% concentration of thyme oil showed an enhanced inhibition against *L. monocytogenes* (Davidson and Naidu, 2000). In a study by Ceylan and Fung (2004) cumin was showed to be effective against *Bacillus cereus, L. monocytogenes, Salmonella enteridis* and *P. flurorescens.* Similar to the present results, cinnamon, cloves and cumin showed strongest antimicrobial effects against *E.coli, Pseudomonas s*p with inhibition zones between <10 and >30 mm by the disc diffusion method (Agaoglu *et al*., 2007). Thyme was most effective against *Bacillus cereus* and *Pseudomonas aeruginosa* (Gutierrez *et al*., 2008a). Similar to the present study, antimicrobial properties can differ significantly within the same spice or herb species

as the proportions of individual components in essential oils of plants are affected by genotype (Arrebola *et al.,* 1994; Witkowska., 2013). Justification to these statements could be observed in comparative results of the same species of processed and unprocessed Indian spices and herbs, where difference can be observed within the same spice or herb species. Contrastingly*,* in a study by Zhang *et al.* (2016), cinnamon EO exerted potent inhibitory effects against *E.coli* with ZoI values of 19.2mm. A study by Turgis *et al*. (2009) reported cellular structure destruction of *E.*coli by mustard EO at its minimal inhibitory concentration (MIC). However, in the present study, antimicrobial properties of crude extracts of cinnamon and mustard were found to be ineffective on *E.coli.*

Noteworthy differences were found between the tested spices and herbs antimicrobial efficiency against each tested foodborne pathogen in the present study (Table 2.4 to 2.7). It cannot be ruled out that some bacterial strains are more susceptible for one spices and/or herb compared to others. Mostly, ethanol extracts of all the tested spices and herbs extracts showed a greater antimicrobial potency against Gram-positive compared to Gram-negative organisms except *Pseudomonas aeruginosa* (Table 2.7). Generally, Gram-negative bacteria such as *Escherichia coli* and *Salmonella enteritidis* are less sensitive to antimicrobials because of presence of lipopolysaccharide layer on outer membrane of this group, which restricts diffusion of phenolic components present in spice extracts (Martinez *et al*., 2015). Alternatively, Gram-positive bacteria such as *Staphylococcus aureus*, *Listeria monocytogenes* and *Bacillus cereus* are usually more susceptible to bioactive components of spices and herbs due to the direct interaction of the cell membrane with these lipophilic components (Martinez *et al*., 2015).

Many studies have focused mainly on antimicrobial effects of spices and herbs extracts. Although these data are useful, this information is difficult to compare directly due to number of factors such as geographic regions, different climatic factors, composition of soil, the plant part used, and the age and season when the plant material is collected (Witkowska *et al.*, 2013; Burt, 2004). Furthermore, drying process and storage time can alter, the essential oil composition quantitatively, and qualitatively (Burt, 2004). Even though the antimicrobial properties of essential oils are well reported, the mechanisms of their actions are not yet fully

understood (Burt, 2004). In the present study, antimicrobial activity were performed by the conventional disc diffusion method, which allows the test sample interaction with the agar media, leading to a diffusion. It is supposed that various other factors such as sample and bacterial cell interaction mechanisms, sample diffusion into bacterial media during laboratory analysis, and time of contact influences spices and herbs antimicrobial activity (Tajkarimi *et al.,* 2010). Thus, the factors, which are associated with the spices and herbs antimicrobial activity needs further investigation. It was observed that tested spices and herbs extracts in this study showed a moderate to high antimicrobial potency against six tested foodborne pathogens. Moreover, as indicated from the supportive literature, spices and herbs extracts significantly enhances antimicrobial potency when ultrasound-assisted ethanol extraction was used.

2.6 Conclusion

This study showed that tested processed and unprocessed Indian spices and herbs possess moderate to high antimicrobial efficiency against six tested foodborne pathogens. Noteworthy difference were shown between ethanol and water extracts of tested spices and herbs extract's efficacies. The result of initial study indicated that the four unprocessed spices and herbs (ajwain, cumin, bay leaves, and cinnamon) out of the tested processed and unprocessed Indian spices and herbs extracts possessed an effective antimicrobial potency against Gram-positive *Bacillus cereus*, *Listeria monocytogenes and Kocuria rhizophilia* and Gram-negative *Pseudomonas aeruginosa.* Coriander, bay leaves and cinnamon showed minimal antimicrobial efficacy against all six tested pathogens. The greatest antimicrobial potency was observed for thyme and cumin against the tested pathogens. Unprocessed Indian thyme showed the greatest ZoI (20 mm) and was found most efficient compared to all the tested spices and herbs extracts. In final screening of thirty-five tested spices and herbs, ultrasound-assisted ethanol extracts of ajwain followed by nutmeg, and cumin showed potential antimicrobial effect against all tested microorganisms.

The antimicrobial potency for all spices were found to be higher when ethanol extraction was combined with ultrasound-assisted extraction than stirring method. Water extracts of thyme, bay leaves and cinnamon showed little effect in inhibition except processed coriander and cumin, which were more effective than other spices. Ratio 1:10 was most effective than any

other ratio and absolute ethanol concentration (98%) was found to be most effective. Ethanol extracts of cumin and thyme were effective on *E.coli* multi resistance strain. Out of all tested spices and herbs, six spices - ajwain, cumin, clove, bay leaves, nutmeg and Indian gooseberry (amla) selected on the basis of present antimicrobial results. Hence, these six spices were selected for further evaluation of antimicrobial properties and analysis of bioactive component responsible to the antimicrobial activity.

Chapter 3

Further study on antimicrobial potential of selected spices and herbs

3.1 Introduction

In chapter 2, ultrasound-assisted extraction (UAE) was evaluated for its effectiveness in for extracting bioactive chemical compounds from spices and herbs, and extracts showed effective antimicrobial results in inhibition of pathogenic foodborne microorganisms. UAE is highly reproducible, adaptable, potential technology (Asbahani *et al*, 2015). The studies showed some main advantages of ultrasound-assisted extraction method are shorter extraction time, less solvent use, effective increased yield and lower extraction temperature (Asbahani *et al*., 2015; Chemat *et al*., 2017). The mechanism of ultrasonic enhanced extraction produces chemical, physical, and mechanical effects which result in disruption of bacterial cell walls, enabling release of bioactive compounds and enhancing transport of solvent into plant cells (Kadam *et al*., 2015; Chemat *et al*., 2017). In this study, UAE method was used for extraction of bioactive components of spice and herbs and antimicrobial potential and responsible mode of action was studied.

Spices and herbs extracts are used by the food manufacturers as antimicrobial agents for extending the shelf life of foods. Essential oils extracted from plant-based material contain variety of chemical components. Some of these exert antimicrobial effect such as chemical compounds present in clove, cinnamon, coriander, rosemary, oregano (Burt, 2004; Tajkarimi *et al*., 2010). The essential oil containing chemical components such as carvacrol, cinnemaldehyde, eugenol and thymol are identified as the main chemical components responsible for antimicrobial activity (Burt, 2004; Tajkarimi *et al*., 2010; Weerakody *et al*., 2010). A number of studies shown that there is correlation between presence of total phenolic components and antimicrobial efficacy of spices and herbs extracts (Burt, 2004; Tajkarimi *et al*., 2010; Witkowska *et al*., 2013). These bioactive (phenolic) components in spices extracts act by degradation of bacterial cell wall, disruption of cell membrane and leakage of cellular components on exposure with bacterial cell (Witkowska *et al*., 2013). It is therefore, relevant to investigate the potential of spices extracts to inhibit the growth of foodborne pathogenic microorganisms and determine their mechanism of action.

In chapter 2, each spices was individually compared within type of extraction method used (stirring or ultrasound-assisted extraction), type of solvent used (water or ethanol), type of microorganism (Gram-positive or Gram-negative) and with positive (antibiotic disc) and negative controls (water or ethanol alone). In final screening of all tested spices and herbs, only ajwain, nutmeg, cumin, bay leaves, clove, and Indian gooseberry showed potential antimicrobial effect against all tested microorganisms. Hence, these six spices were selected for further analysis of their antimicrobial potential and bioactive compound content. In this chapter, these six selected spices and herbs were compared with each other on type of extraction method, type of solvent for antimicrobial potency within each tested microorganism. The six selected spices were further analysis for minimum inhibition concentration (MIC) and minimum bacterial concentration (MBC). The MIC defines as the lowest concentration of antimicrobial agent that completely inhibits growth of the organism in wells. The MBC is defined as the lowest concentration of antimicrobial agent needed to kill 99.9% of the final inoculum after incubation for 24hr under a standardized set of conditions described in document (CLSI, 2012). The effect of spice extract (ajwain and nutmeg) and some pure bioactive compounds such as thymol, p-cymene and carvacrol on morphological and structural changes of selected microorganisms was also studied in order to understand the possible mechanism of action of spice extracts on the inhibition of pathogen.

During antimicrobial screening of spices and herbs, one of the most potent antimicrobial herbs identified via screening was originally thought to be thyme (*Thymus vulgaris*). However, following a recent collection of fresh samples in India, and subsequent literature review and chemical analysis, it has since been discovered that this potent material is in fact a lesser known material called “Ajwain” (*Trachyspermum ammi*, AKA Indian thyme). True thyme and ajwain plants are similar in appearance, flavour and aroma, but are from distinct and separate families. Whilst ajwain has been used both as a seasoning ingredient in Indian cuisine, and as part of Indian traditional herbal medicine for centuries, very little systematic research has been performed concerning its chemical profile and antimicrobial properties. In the light of this recent discovery, it is needed to confirm the chemical profile of this novel food ingredient, and how this influences its potent antimicrobial activity. This work will also aim to confirm the posited role of thymol as the key determinant of antimicrobial activity in Ajwain and

establish this compound as a quality marker relevant to food manufacturers wishing to decrease use of synthetic preservatives.

3.2 Aims and objective

Aim – To investigate antimicrobial potential and possible mechanism of action of selected spices and herbs on selected microorganisms.

Objectives-

- To compare the antimicrobial activities of the selected Indian spices and herb extracts against pathogenic microbes, including *Bacillus cereus, Listeria monocytogenes, Kocuria rhizophila, Escherichia coli, Salmonella typhi,* and *Pseudomonas aeruginosa* using agar disc diffusion method.
- To determine and compare the minimum inhibition concentration (MIC) of selected Indian spices and herb extracts obtained using stirring and ultrasound-assisted extraction (UAE) methods against tested foodborne pathogenic microorganisms.
- To determine and compare the minimum bacterial concentration (MBC) of selected Indian spices and herb extracts obtained using stirring and UAE methods against tested foodborne pathogenic microorganisms.
- To investigate microscopic morphology of tested microorganisms treated with spice extracts using scanning electron microscopy (SEM).

3.3 Methodology

3.3.1 Sample preparation

Ajwain, nutmeg, cumin, clove, bay leaves and Indian gooseberry spices used are same as mentioned in Chapter 2.3.1. The spice extract was prepared by adding 1:10 ratio of spice to solvent ratio (ethanol), the mixture was vortex for 30 sec to ensure the contents were mixed together properly. The extraction was prepared by stirring method (24hr extraction method) and ultrasound-assisted extraction method, which is used at 20 KHz, 400W power, cycle 0, 20% amplitude for 5 min. The total content was collected and centrifuged at 9000 rpm for 10 min and supernatants were filtered through Whatman filter No 4, while the residues were used for a second ultrasound extraction with 100ml of ethanol. After the second extraction, the filtrates were evaporated at 40^{o}C to evaporate ethanol from the extract. The extracts were analysis for antimicrobial properties.

3.3.2 Determination of minimum Inhibition concentration (MIC)

The MIC determinations was prepared by two-fold serial dilutions of spice extracts in Mueller Hilton (MH) broth dispensed in smaller volumes using 96-well micro-titration plate (microdilution) (Figure 3.1). Then, well is inoculated with tested microorganism inoculum prepared in the same medium after dilution of standardized microbial suspension adjusted to 0.5 McFarland scale (Refer to Figure 2.1 in Chapter 2). A positive control contained MH broth and tested microorganism and a negative control contained tested spice extract was maintained. After well-mixing, the 96-well micro-titration plate were incubated at 37^{o}C for 24 hours. The experimental methodology to perform accurately the microdilution is schematized in Figure 3.1 (Balouiri *et al*., 2016). After 24 hr, the wells were visually examined for the lowest concentration of spice extract that showed inhibition of tested microorganism growth (indicated by a clear solution, no visible growth of microorganism). The concentration in the lowest serial dilution of the spice extract at which growth did not occur on MH broth was recorded as MIC. Ethanol extracts of ajwain, nutmeg, cumin, clove, bay leaves and Indian gooseberry spices studied for MIC. Water extracts were not effective (results shows <5mm ZoI in previous chapter 2) against tested microorganisms. Hence, water extracts were not analysed for MIC.

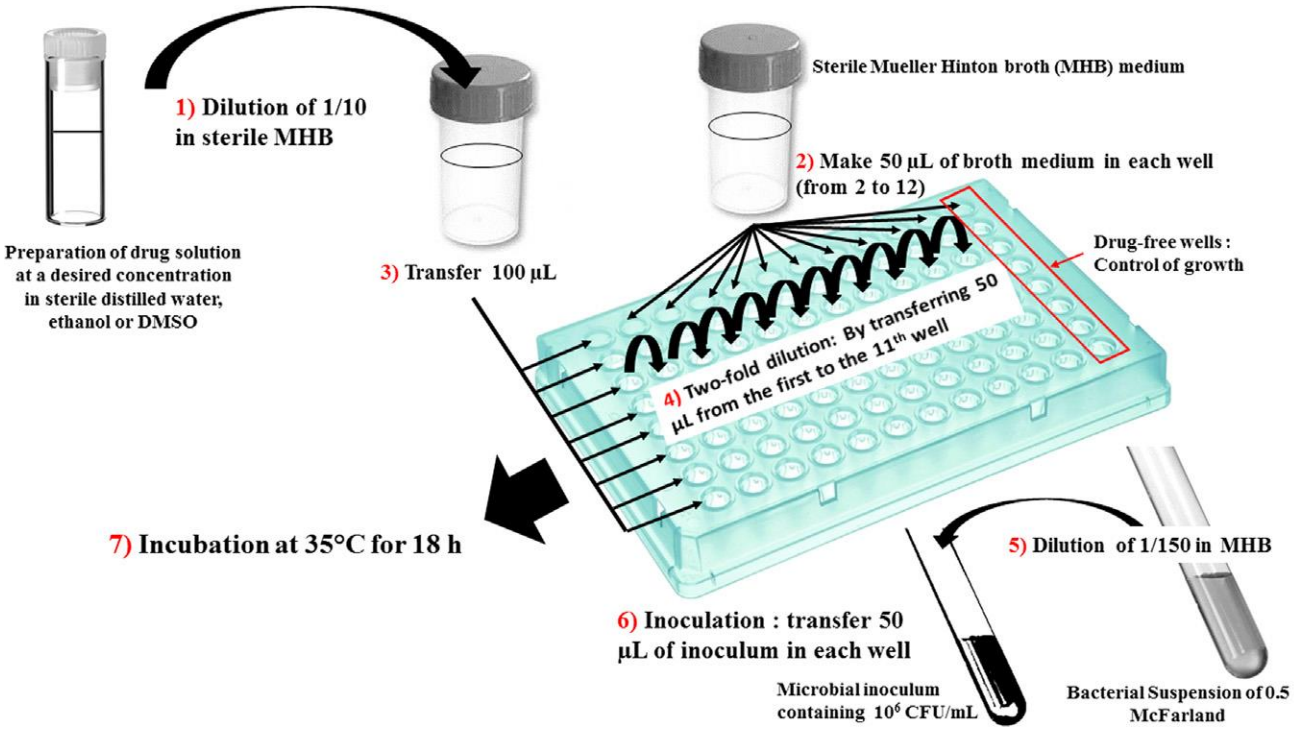


Figure 3.1- MH broth 96-well micro-titration plate for antimicrobial testing as recommended by CLSI protocol (Balouiri *et al*., 2016)

3.3.3 Determination of minimum bacterial concentration (MBC)

The minimum bacterial concentration (MBC) was determined by taking suspension from the 96 well microtiter plate well with the concentration greater than MIC and spotting it on MHA plates. MBC was determined by inoculating twenty microliters (20µl) of sub-culture from the MIC well that showed no growth (negative microbial growth) on MH agar plates and incubated at 37^{o}C for 24 hr to determine the number of surviving cells (CFU/ml). After incubation, for 24 hours, the highest dilution that yielded no single colony on the MH plate (solid medium) was recorded as MBC (Balouiri *et al*., 2016).

3.3.4 Effect of spice extract on morphology and cellular structure of selected microorganisms

Scanning electron microscopy (SEM) was performed to observe the morphological changes to microorganisms in presence of spice extract at different intervals, using the method as described by Paul, (2011) with some modifications. SEM studies were performed on *Bacillus*

cereus, Kocuria rhizophila, E.coli and *Pseudomonas aeruginosa.* The bacteria culture were incubated in Mueller Hilton broth medium at 37^{o}C. The microbial suspension adjusted to 0.5 McFarland scale (Refer to Figure 2.1 in Chapter 2), approximately 10^{7} cfu/ml for 18hr. All tested bacterial samples treated with spice and herb extract samples (ultrasound-assisted ethanol extracts of ajwain and nutmeg) were incubated at 37^{o}C for 0hr, 3hr and 24hr. The bacteria cells were prepared in 2.5% glutaraldehyde and fixed in 0.1 M phosphate buffer solution (PBS, pH 7.4) at 4°C overnight. The sample were washed in 0.1 M PBS twice. The samples were dehydrated for 30 min each time in a sequential graded ethanol (20%, 40%, 60%, 80% and 100%). The samples were dried in a vacuum-assisted desiccator overnight (or until dry). The samples were dried using Polaron and were mounted onto aluminium pin stubs using adhesive carbon tabs and coated with a thin layer of gold (Au) metal using a sputter coater for 30 s at 800V, 5mA current. The stubs were then loaded into the SEM (Carl Zeiss Ltd, Supra 40VP, SmartSEM software) for imaging and analysis. The secondary electron detector was used to obtain images of the samples. The images were taken at 25,000x and 10,000x magnifications using an acceleration voltage of 2kV and a working distance of approximately 6mm. Spice extract untreated bacterial cells were considered as control cells. In the magnified image, few cells selected at random that were fully visible and not crossing over other cells. The length of bacterial cell size (n=4) was measured for comparison with other treated cells.

3.3.5 Experimental design and Statistical analysis

This study was designed to obtain the antimicrobial efficacy of selected Indian spices and herbs against six organisms. All the experiments were performed in duplicates. The data was analysed using Microsoft office Excel and results were expressed as mean ± standard deviation.

3.4 Results

3.4.1 Comparison of six selected spices and herbs on basis of antimicrobial analysis against all selected microorganisms

Table 3.1 – Effect of ajwain ethanol extracts against all tested microorganism

		Stirring ethanol extracts			Ultrasound ethanol extracts	
	ZoI (mm)	**MIC (µl/ml)**	**MBC (µl/ml)**	**ZoI (mm)**	**MIC (µl/ml)**	**MBC (µl/ml)**
Bacillus cereus	13 ± 0	3.12 ± 0	3.12 ± 0	**20 ± 0****	**0.78 ± 0**	**1.56 ± 0.39**
Listeria monocytogenes	**12.5 ± 0.57***	3.12 ± 0	3.12 ± 0	**17 ± 1.15****	1.56 ± 0.67	3.12 ± 0
Kocuria rhizophilia	**14.5 ± 0.57****	**1.56 ± 0**	**1.56 ± 0**	**15.5 ± 0.57****	1.56 ± 0.39	1.56 ± 0.39
E.coli	9 ± 0	nd	nd	13 ± 0	Nd	nd
Salmonella typhi	14 ± 0	nd	nd	12 ± 0	Nd	nd
Pseudomonas aeruginosa	9 ± 0	50 ± 0	nd	14 ± 0**	50 ± 0	nd

Diameter of zone of inhibition (ZoI) (measured in mm), values represent mean of four replicates ± SD, excluding diameter of disc 6mm from readings. Significance * - intermediate, ** - susceptible antimicrobial. Minimum inhibition concentration and minimum bacterial concentration for the spice extract against all tested microorganisms. MIC and MBC - µl/ml. values represent mean of four replicates ± SD. Nd – not detected in the samples.

Table 3.1 shows the results of effect of ajwain ethanol extract on different microorganisms. The lowest effective concentration of ajwain extract is 0.78 µl/ml against *Bacillus cereus*. The MIC and MBC values for *E.coli* and *Salmonella* was not detected in the samples. It was also confirmed from ZoI that ajwain extract is effective in inhibition of Gram-positive microorganisms. Although 50 µl/ml MIC value was found for *Pseudomonas*.

Table 3.2 – Effect of bay leaves ethanol extracts against all tested microorganism

	Stirring ethanol extracts			Ultrasound ethanol extracts		
	ZoI (mm)	**MIC (µl/ml)**	**MBC (µl/ml)**	**ZoI (mm)**	**MIC (µl/ml)**	**MBC (µl/ml)**
Bacillus cereus	**15 ± 0***	3.12 ± 0	3.12 ± 0	**17 ± 1.15***	**1.56 ± 0.67**	3.12 ± 0.67
Listeria monocytogenes	9 ± 0	nd	Nd	12 ± 0*	3.12 ± 0	3.12 ± 0
Kocuria rhizophilia	9 ± 0	nd	Nd	9 ± 0	Nd	nd
E.coli	9 ± 0	nd	Nd	9 ± 0	Nd	nd
Salmonella typhi	12 ± 0	nd	Nd	12 ± 0	Nd	nd
Pseudomonas aeruginosa	**12 ± 0***	3.12 ± 0	3.12 ± 0	**12 ± 0***	3.12 ± 0	3.12 ± 0

Diameter of zone of inhibition (ZoI) (measured in mm), values represent mean of four replicates ± SD, excluding diameter of disc 6mm from readings. Significance * - intermediate, ** - susceptible antimicrobial. Minimum inhibition concentration and minimum bacterial concentration for the spice extract against all tested microorganisms. MIC and MBC - µl/ml. values represent mean of four replicates ± SD. Nd – not detected in the samples.

Table 3.2 shows the results of effect of ethanol extracts of bay leaves on all tested microorganisms. The lowest effective concentration of bay leaves is 1.56 µl/ml against *Bacillus cereus*. The effective lowest concentration of bay leave extracts on *Listeria, Kocuria, E.coli,* and *Salmonella* was not detected in the samples.

Table 3.3 – Effect of clove ethanol extracts against all tested microorganism

		Stirring ethanol extracts			Ultrasound ethanol extracts	
	ZoI (mm)	**MIC (µl/ml)**	**MBC (µl/ml)**	**ZoI (mm)**	**MIC (µl/ml)**	**MBC (µl/ml)**
Bacillus cereus	14 ± 0	6.25 ± 0	6.25 ± 0	14 ± 0	3.12 ± 0	3.12 ± 0
Listeria monocytogenes	9 ± 0	nd	Nd	9 ± 0	Nd	nd
Kocuria rhizophilia	10 ± 0	nd	Nd	9 ± 0	Nd	nd
E.coli	**15 ± 0***	3.12 ± 0	3.12 ± 0	14 ± 0	3.12 ± 0.67	3.12 ± 0.67
Salmonella typhi	**16 ± 0***	3.12 ± 0	3.12 ± 0	15 ± 0*	3.12 ± 0.67	3.12 ± 0.67
Pseudomonas aeruginosa	9 ± 0	nd	Nd	9 ± 0	Nd	nd

Diameter of zone of inhibition (ZoI) (measured in mm), values represent mean of four replicates ± SD, excluding diameter of disc 6mm from readings. Significance * - intermediate, ** - susceptible antimicrobial. Minimum inhibition concentration and minimum bacterial concentration for the spice extract against all tested microorganisms. MIC and MBC - µl/ml. values represent mean of four replicates ± SD. Nd – not detected in the samples.

Table 3.3 shows the results of effect of ethanol extracts of clove on all tested microorganisms. The lowest effective concentration of clove is 3.12 µl/ml against *Bacillus cereus, E.coli* and *Salmonella typhi.* The lowest effective concentration of clove on *Listeria, Kocuria* and *Pseudomonas* was not detected in the samples. The result shows clove extract has potential to inhibit Gram-negative microorganisms.

Table 3.4 – Effect of cumin ethanol extracts against all tested microorganism

		Stirring ethanol extracts			Ultrasound ethanol extracts	
	ZoI (mm)	**MIC (µl/ml)**	**MBC (µl/ml)**	**ZoI (mm)**	**MIC (µl/ml)**	**MBC (µl/ml)**
Bacillus cereus	11 ± 0	50 ± 0	50 ± 0	13 ± 0	50 ± 0	50 ± 0
Listeria monocytogenes	**12 ± 0***	3.12 ± 0	3.12 ± 0	10 ± 0	6.25 ± 0	6.25 ± 0
Kocuria rhizophilia	**13.5 ± 0.57***	**1.56 ± 0.67**	3.12 ± 0	**15 ± 0***	**1.56 ± 0.67**	3.12 ± 0
E.coli	9 ± 0	Nd	nd	13 ± 0	50 ± 0	nd
Salmonella typhi	12 ± 0	Nd	nd	14 ± 0	50 ± 0	nd
Pseudomonas aeruginosa	**12 ± 0***	3.12 ± 0	3.12 ± 0	**13 ± 0***	3.12 ± 0	3.12 ± 0

Diameter of zone of inhibition (ZoI) (measured in mm), values represent mean of four replicates ± SD, excluding diameter of disc 6mm from readings. Significance * - intermediate, ** - susceptible antimicrobial. Minimum inhibition concentration and minimum bacterial concentration for the spice extract against all tested microorganisms. MIC and MBC - µl/ml. values represent mean of four replicates ± SD. Nd – not detected in the samples.

Table 3.4 shows the results of effect of ethanol extracts of cumin on tested microorganisms. The effective lowest concentration of cumin is 1.56 µl/ml for *Kocuria* inhibition, 3.12 µl/ml for *Listeria, Pseudomonas*. The lowest concentration of cumin on *E.coli* and *Salmonella* was not detected in the samples.

Table 3.5 – Effect of Indian gooseberry ethanol extracts against all tested microorganism

		Stirring ethanol extracts			Ultrasound ethanol extracts	
	ZoI (mm)	**MIC (µl/ml)**	**MBC (µl/ml)**	**ZoI (mm)**	**MIC (µl/ml)**	**MBC (µl/ml)**
Bacillus cereus	**16 ± 0***	3.12 ± 0	3.12 ± 0	**16 ± 0***	3.12 ± 0	3.12 ± 0
Listeria monocytogenes	11.5 ± 0.57	6.25 ± 0	6.25 ± 0	11 ± 0	3.12 ± 0	6.25 ± 1.56
Kocuria rhizophilia	**12 ± 0***	3.12 ± 0	3.12 ± 0	**12 ± 0***	3.12 ± 0	3.12 ± 0
E.coli	9 ± 0	nd	nd	9 ± 0	Nd	nd
Salmonella typhi	9 ± 0	nd	nd	9 ± 0	Nd	nd
Pseudomonas aeruginosa	**12.5 ± 0.57***	3.12 ± 0	3.12 ± 0	**12 ± 0***	3.12 ± 0	3.12 ± 0

Diameter of zone of inhibition (ZoI) (measured in mm), values represent mean of four replicates ± SD, excluding diameter of disc 6mm from readings. Significance * - intermediate, ** - susceptible antimicrobial. Minimum inhibition concentration and minimum bacterial concentration for the spice extract against all tested microorganisms. MIC and MBC - µl/ml. values represent mean of four replicates ± SD. Nd – not detected in the samples.

Table 3.5 shows the results of effect of ethanol extracts of Indian gooseberry on tested microorganisms. The lowest effective concentration of Indian gooseberry was 3.12 µl/ml. The effective lowest concentration of Indian gooseberry on *E.coli* and *Salmonella* was not detected in the samples.

Table 3.6 – Effect of nutmeg ethanol extracts against all tested microorganism

		Stirring ethanol extracts			Ultrasound ethanol extracts	
	ZoI (mm)	**MIC (µl/ml)**	**MBC (µl/ml)**	**ZoI (mm)**	**MIC (µl/ml)**	**MBC (µl/ml)**
Bacillus cereus	**15.5 ± 0.57***	1.56 ± 0.39	1.56 ± 0.39	**15 ± 0***	1.56 ± 0.39	3.12 ± 0
Listeria monocytogenes	**18.5 ± 0.57****	**0.78 ± 0**	**0.78 ± 0**	**16.5 ± 0.57****	**0.78 ± 0**	1.56 ± 0.39
Kocuria rhizophilia	**16 ± 0****	1.56 ± 0.39	1.56 ± 0.39	**15 ± 1.15****	1.56 ± 0.39	3.12 ± 0
E.coli	**15 ± 0***	6.25 ± 0	6.25 ± 0	14 ± 0	6.25 ± 0	6.25 ± 0
Salmonella typhi	12.5 ± 0.57	3.12 ± 0	6.25 ± 0	12 ± 0	3.12 ± 0	6.25 ± 0
Pseudomonas aeruginosa	**12 ± 0***	3.12 ± 0	6.25 ± 0	**10 ± 0***	3.12 ± 0	6.25 ± 0

Diameter of zone of inhibition (ZoI) (in mm), values represent mean of four replicates ± SD, excluding diameter of disc 6mm from readings. Significance * - intermediate, ** - susceptible antimicrobial. Minimum inhibition concentration and minimum bacterial concentration for the spice extract against all tested microorganisms. MIC and MBC - µl/ml. values represent mean of four replicates ± SD.

Table 3.6 shows the results of the ethanol extracts of nutmeg are highly effective in the inhibition of all tested microorganisms. The lowest effective concentration of nutmeg is 0.78 µl/ml for *Listeria* inhibition and 1.56 µl/ml for *Bacillus cereus*. The ethanol extracts of nutmeg are effective in the inhibition of all tested microorganisms.

3.4.2 Effect of spice extract on morphology and cellular structure of selected microorganism

1. **Effect of spice extracts on *Bacillus cereus***

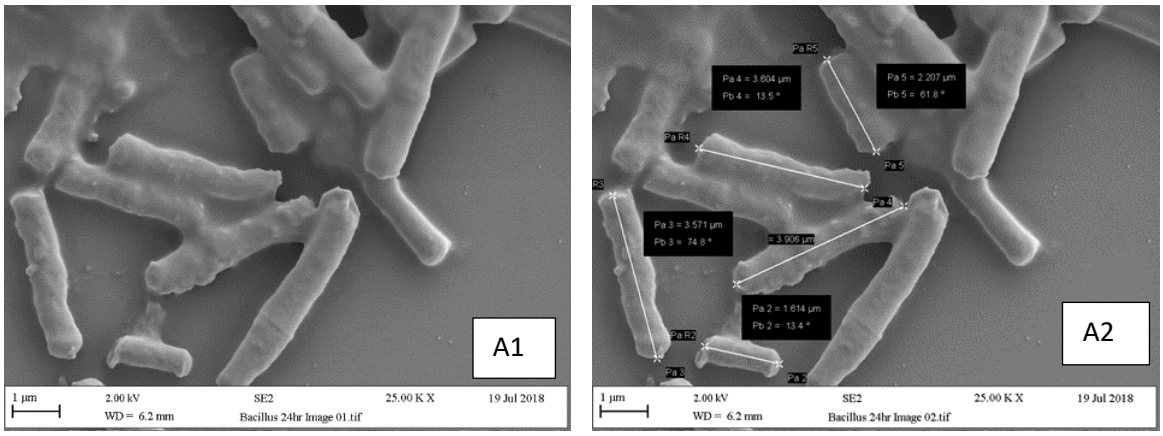


Figure 3.2 – *Bacillus cereus* without extract (Control). A1 shows the image of *Bacillus* at 25,000 X maginification and A2 shows the cell size were measured lengthwise.

The actual size of *Bacillus cereus* cells 2.02µm measured in this study and considered as control (without spice extract treatment). In Figure 3.2, A1 shows the image of *Bacillus* at 25,000 X maginification and A2 shows the cell size were measured lengthwise. The cell size was calculated lengthwise by taking mean of n=4 replicates as shown in figure 3.2b. When these *Bacillus* cells was exposed to ajwain or nutmeg ethanol extracts shows morphological changes in the bacterial cell – specially shrinkage of the cells, disruption of cellular structure as compared to control cells after 24-hour incubation (Figure 3.3 and 3.4). When these bacterial cells were treated with pure bioactive compounds such as thymol, p-cymene and carvacrol, pore formation and cell lysis was noticed (Figure 3.5). Bacterial cells absorb extract or pure chemical and cells were enlarged, result in breaking of cells and cell lysis after incubation.

Effect of ajwain extract on *Bacillus cereus*

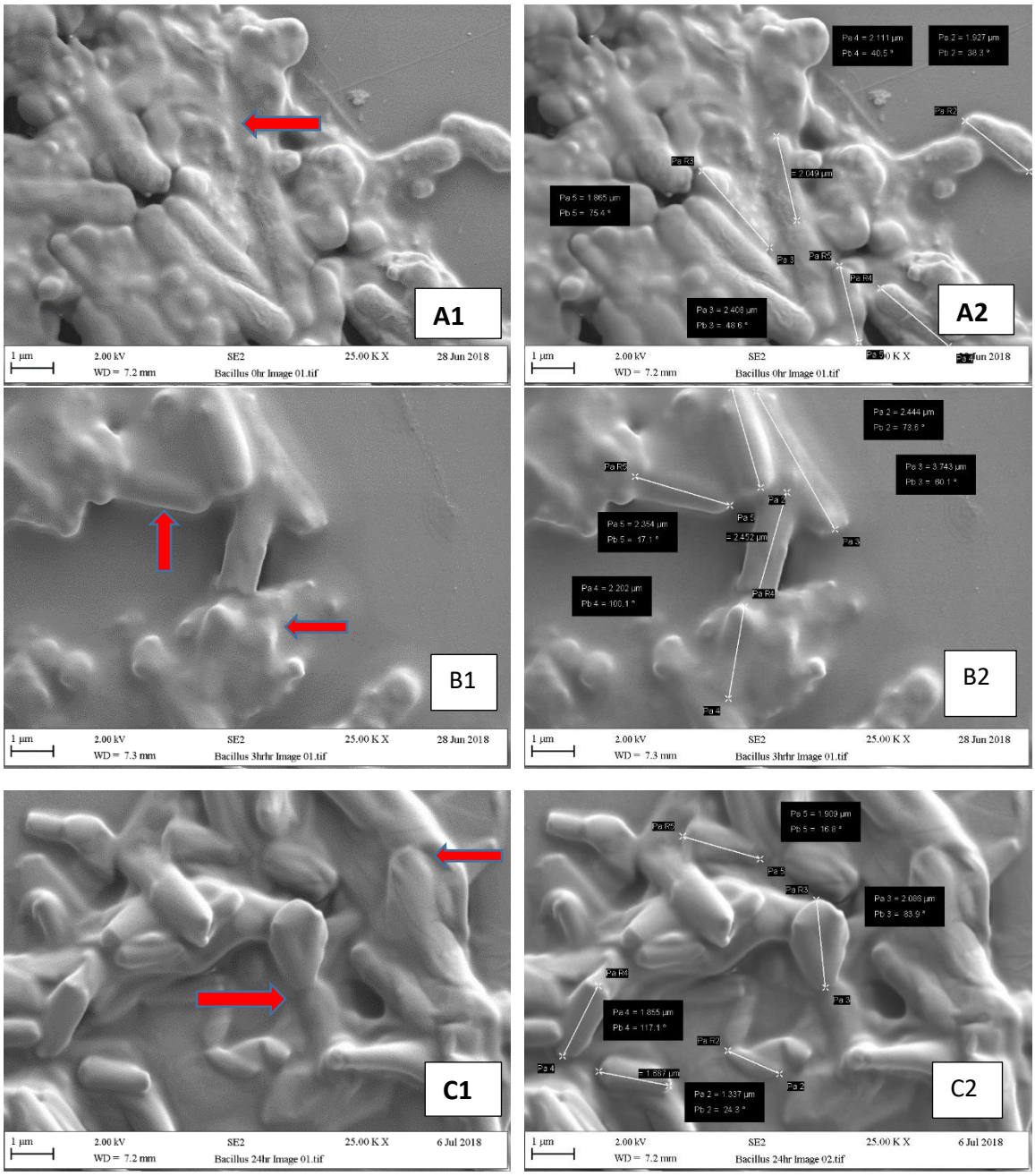


Figure 3.3 – Effect of ajwain extracts on morphology and cellular structure of *Bacillus cereus*. A1 shows the image of *Bacillus* at 25,000 X maginification and A2 shows the cell size were measured lengthwise a) *Bacillus cereus* with ajwain extract (0hr incubation), b) *Bacillus cereus* with ajwain extract (3hr incubation), and c) *Bacillus cereus* with ajwain extract (24hr incubation), red arrow shows loss of cell components.

Effect of nutmeg extract on *Bacillus cereus*

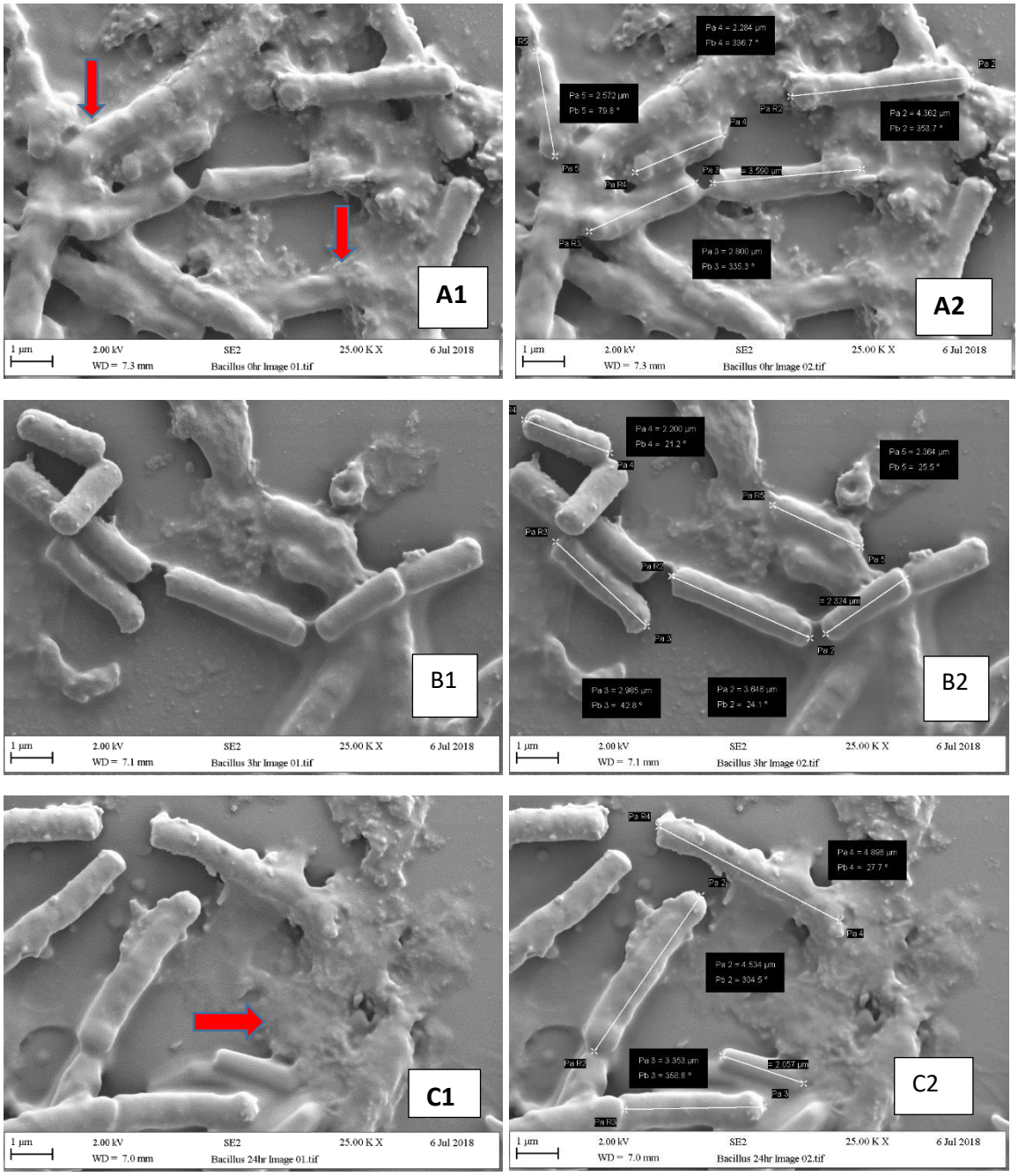


Figure 3.4 – Effect of nutmeg extracts on morphology and cellular structure of *Bacillus cereus*. A1 shows the image of *Bacillus* at 25,000 X maginification and A2 shows the cell size were measured lengthwise. a) *Bacillus cereus* with nutmeg extract (0hr incubation), b) *Bacillus cereus* with nutmeg extract (3hr incubation), and c) *Bacillus cereus* with nutmeg extract (24hr incubation), red arrow shows loss of cell component. Nutmeg extract alter the *Bacillus cereus* cell morphology as compared to control cell (Figure 3.2).

Effect of pure compounds on *Bacillus cereus*

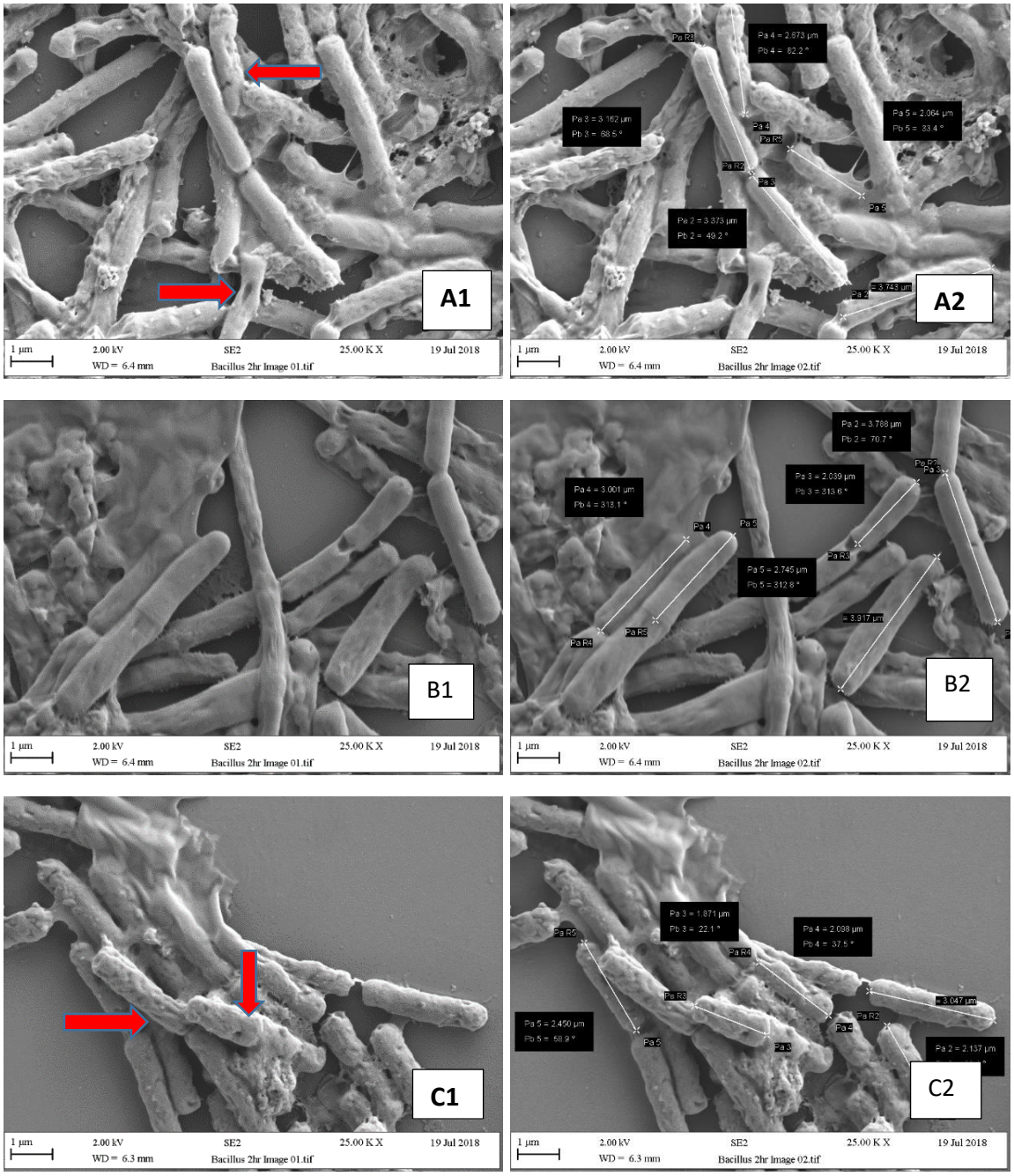


Figure 3.5 – Effect of pure compound extracts on morphology and cellular structure of *Bacillus cereus*. A1 shows the image of Bacillus at 25,000 X maginification and A2 shows the cell size were measured lengthwise. a) *Bacillus cereus* with thymol (2hr incubation), b) *Bacillus cereus* with p-cymene (2hr incubation), and c) *Bacillus cereus* with carvacrol (2hr incubation), red arrow shows pore formation in the cells.

2. Effect of spice extracts on *Kocuria*

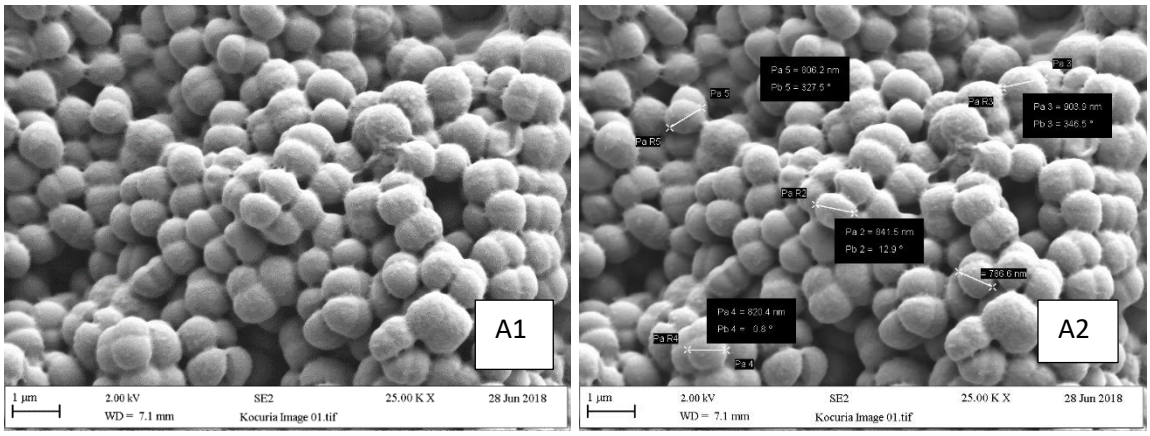


Figure 3.6 - *Kocuria* cells without extract (control). A1 shows the image of *Kocuria* at 25,000 X maginification and A2 shows the cell size were measured lengthwise.

The actual size of *Kocuria* cells 0.84 µm measured in this study and considered as control (without spice extract treatment). The cell size was calculated by taking mean of n=4 replicates shown in figure 3.6. A1 shows the image of Kocuria at 25,000 X maginification and A2 shows the cell size were measured lengthwise. When these cells was treated with ajwain or nutmeg extracts the bacterial cell alter in size (Figure 3.7 and 3.8). When these bacterial cells were treated with pure bioactive compounds such as thymol, p-cymene and carvacrol, shrinkage in bacterial size as compare to control cells was noticed (Figure 3.9).

Effect of ajwain extract on *Kocuria*

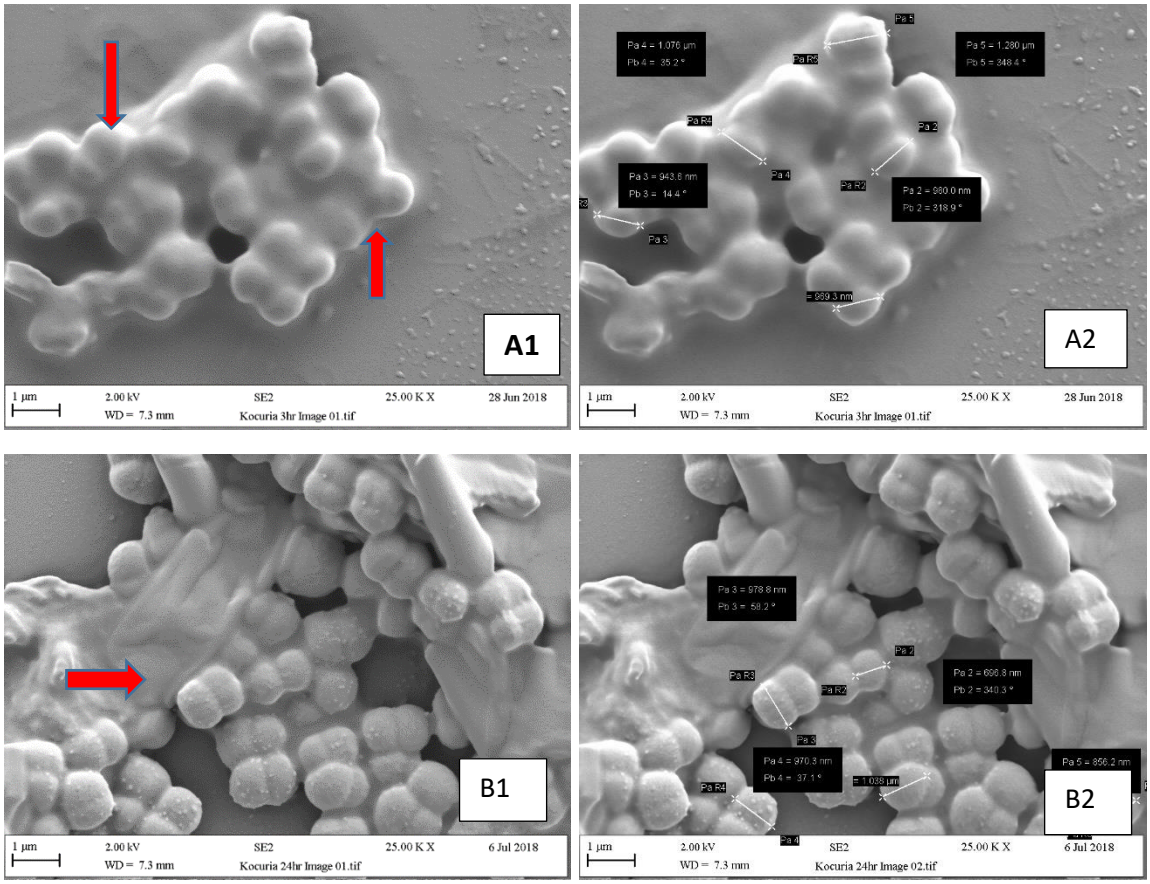


Figure 3.7 – Effect of ajwain extracts on morphology and cellular structure of *Kocuria*. A1 shows the image of *Kocuria* at 25,000 X maginification and A2 shows the cell size were measured lengthwise. a) *Kocuria* with ajwain extract (3hr incubation), and b) *Kocuria* with ajwain extract (24hr incubation), red arrow shows cellular disruption.

Effect of nutmeg extract on *Kocuria*

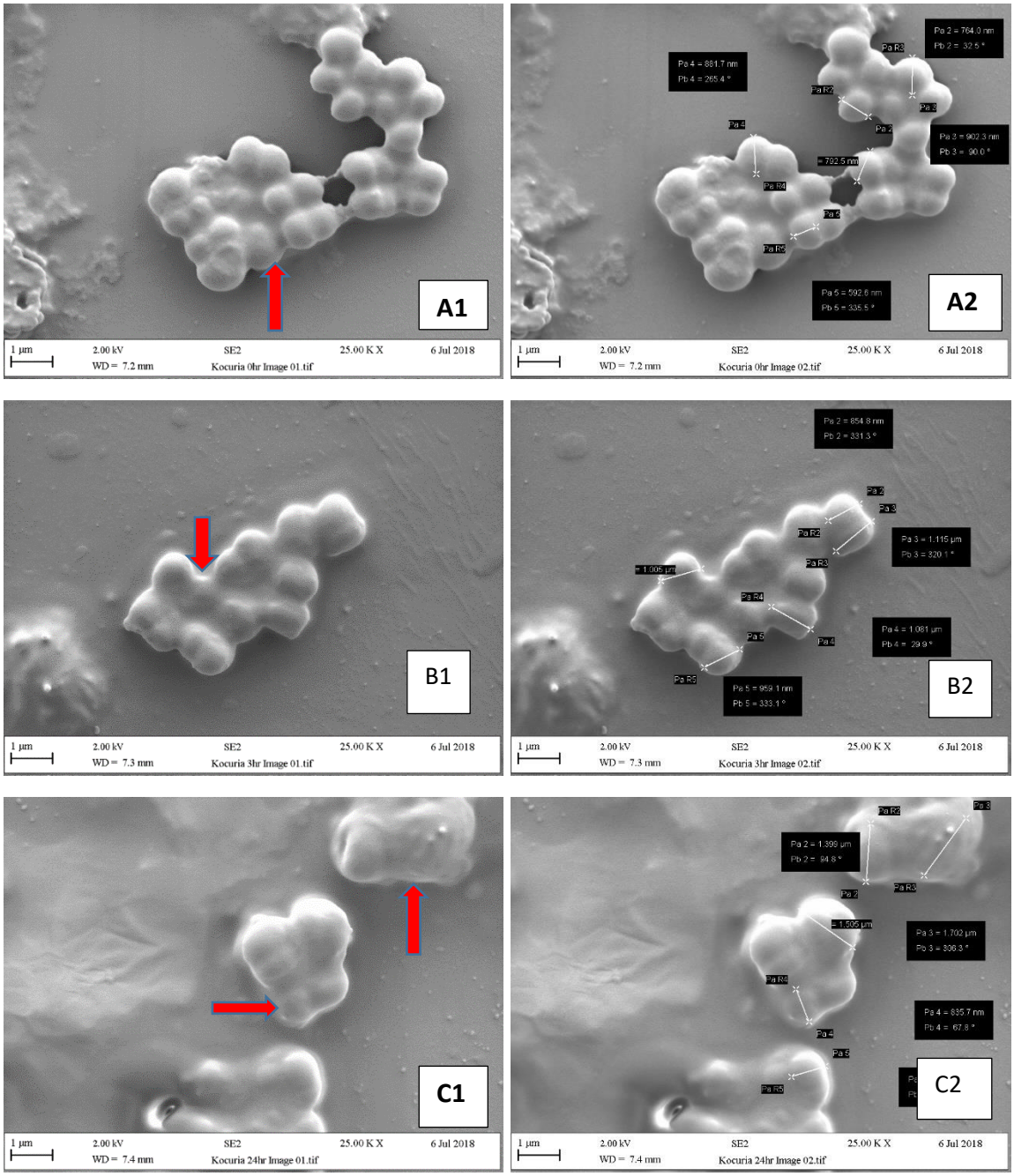


Figure 3.8 – Effect of nutmeg extracts on morphology and cellular structure of *Kocuria*. A1 shows the image of *Kocuria* at 25,000 X maginification and A2 shows the cell size were measured lengthwise. a) *Kocuria* with nutmeg extract (0hr incubation), b) *Kocuria* with nutmeg extract (3hr incubation), and c) *Kocuria* with nutmeg extract (24hr incubation), red arrow shows cellular disruption and loss of cell component.

Effect of pure compounds on *Kocuria*

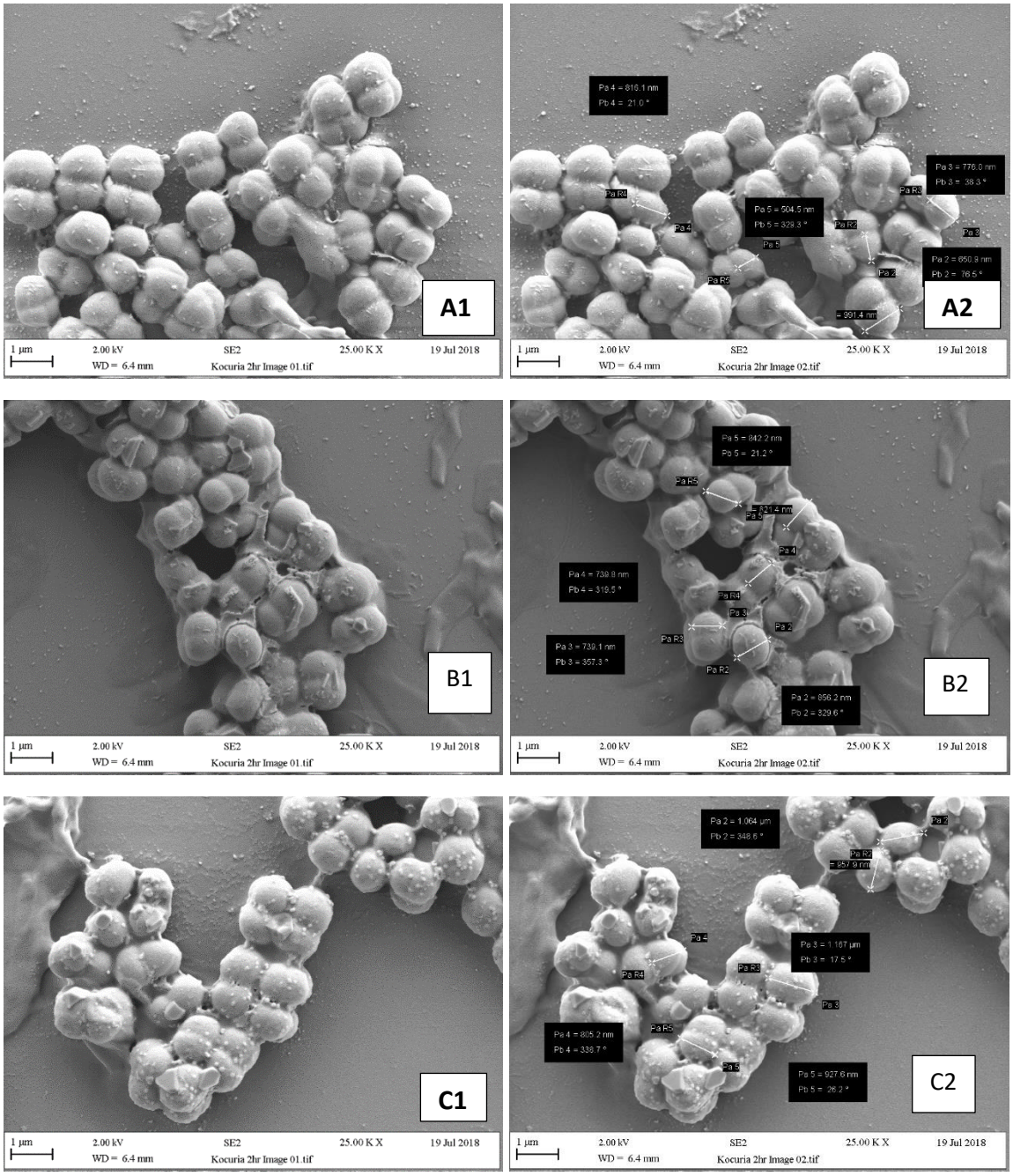


Figure 3.9 – Effect of pure compound extracts on morphology and cellular structure of *Kocuria*. A1 shows the image of *Kocuria* at 25,000 X maginification and A2 shows the cell size were measured lengthwise. a) *Kocuria* with Thymol (2hr incubation), b) *Kocuria* with p-cymene (2hr incubation), and c) *Kocuria* with Carvacrol (2hr incubation). Shrinkage of cells as compared to control cells was observed.

3. Effect of spice extracts on *E.coli*

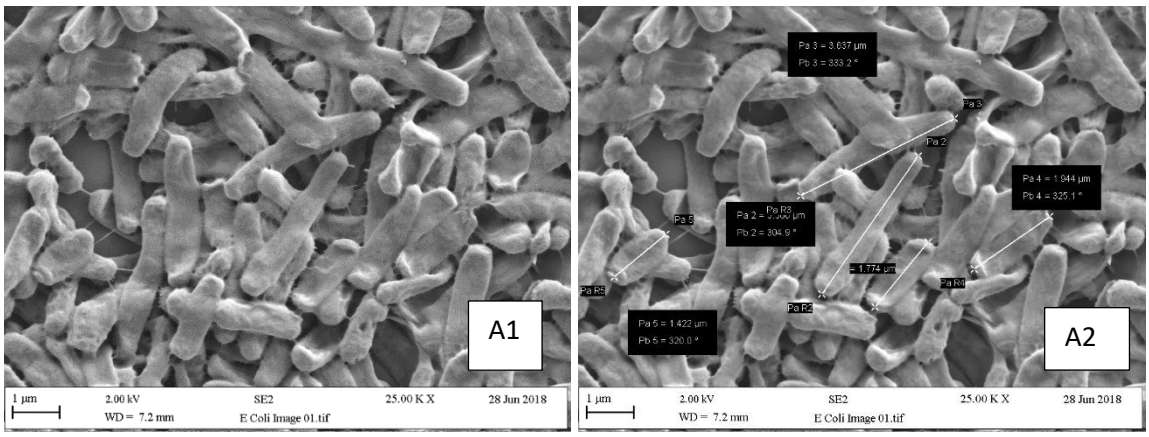


Figure 3.10 - *E.coli* cells without extract (control). A1 shows the image of *E.coli* at 25,000 X maginification and A2 shows the cell size were measured lengthwise.

The actual size of *E.coli* cells 2.57µm measured in this study and considered as control (without spice extract treatment). The cell size was calculated by taking mean of n=4 replicates shown in figure 3.10. A1 shows the image of *E.coli* at 25,000 X maginification and A2 shows the cell size were measured lengthwise. When these *E.coli* cells was exposed to ajwain or nutmeg extracts the bacterial cell alter in size (figure 3.11 and 3.12). When these bacterial cells were treated with pure bioactive compounds such as thymol, p-cymene and carvacrol, pore formation on cells was noticed, on exposure of thymol and carvacrol bioactive chemical compounds (figure 3.13).

Effect of ajwain extract on *E.coli*

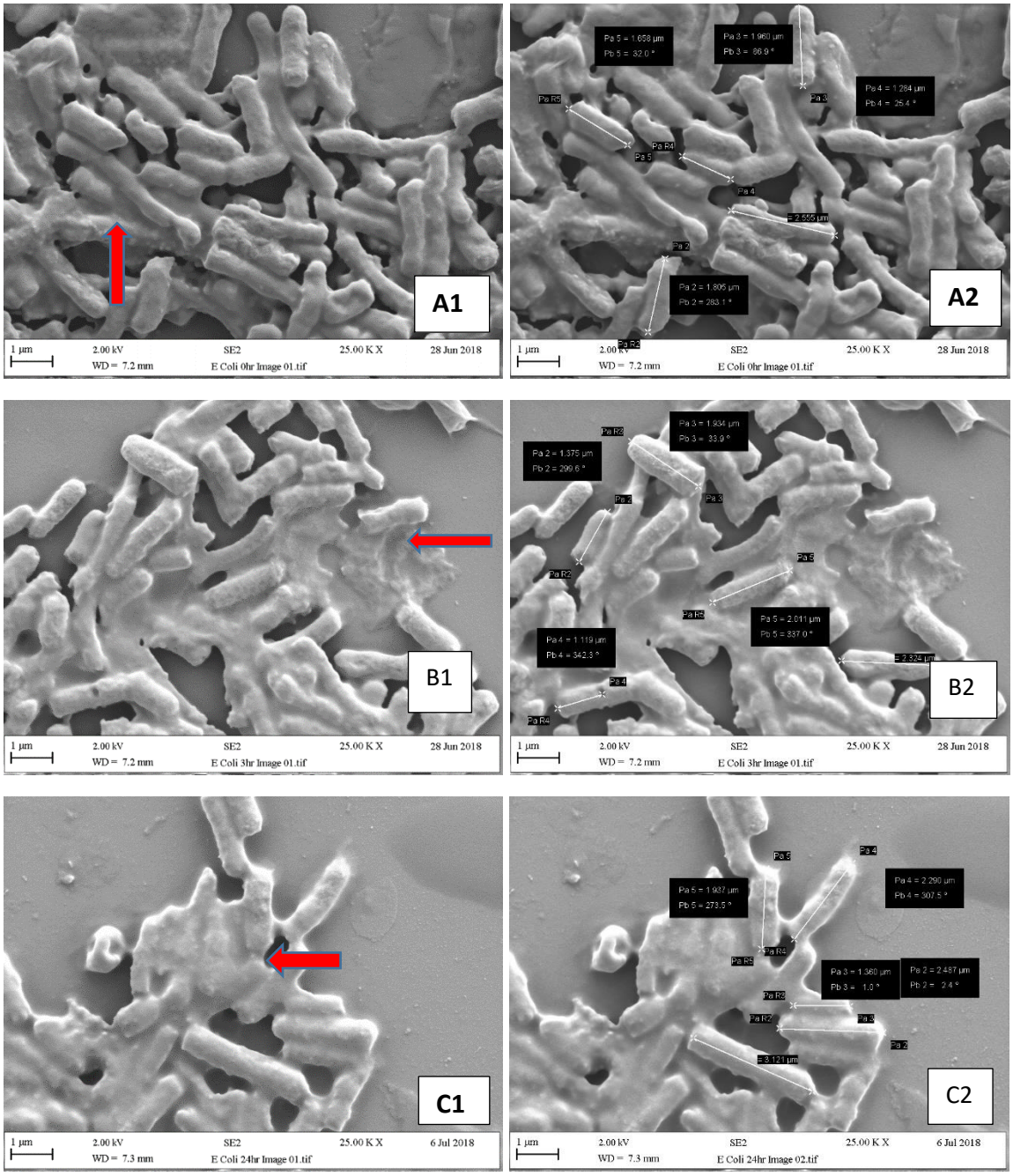


Figure 3.11 – Effect of ajwain extracts on morphology and cellular structure of *E.coli*. A1 shows the image of *E.coli* at 25,000 X maginification and A2 shows the cell size were measured lengthwise. a) *E.coli* with ajwain extract (0hr incubation), b) *E.coli* with ajwain extract (3hr incubation), and c) *E.coli* with ajwain extract (24hr incubation), red arrow show cellular disruption.

Effect of nutmeg extract on *E.coli*

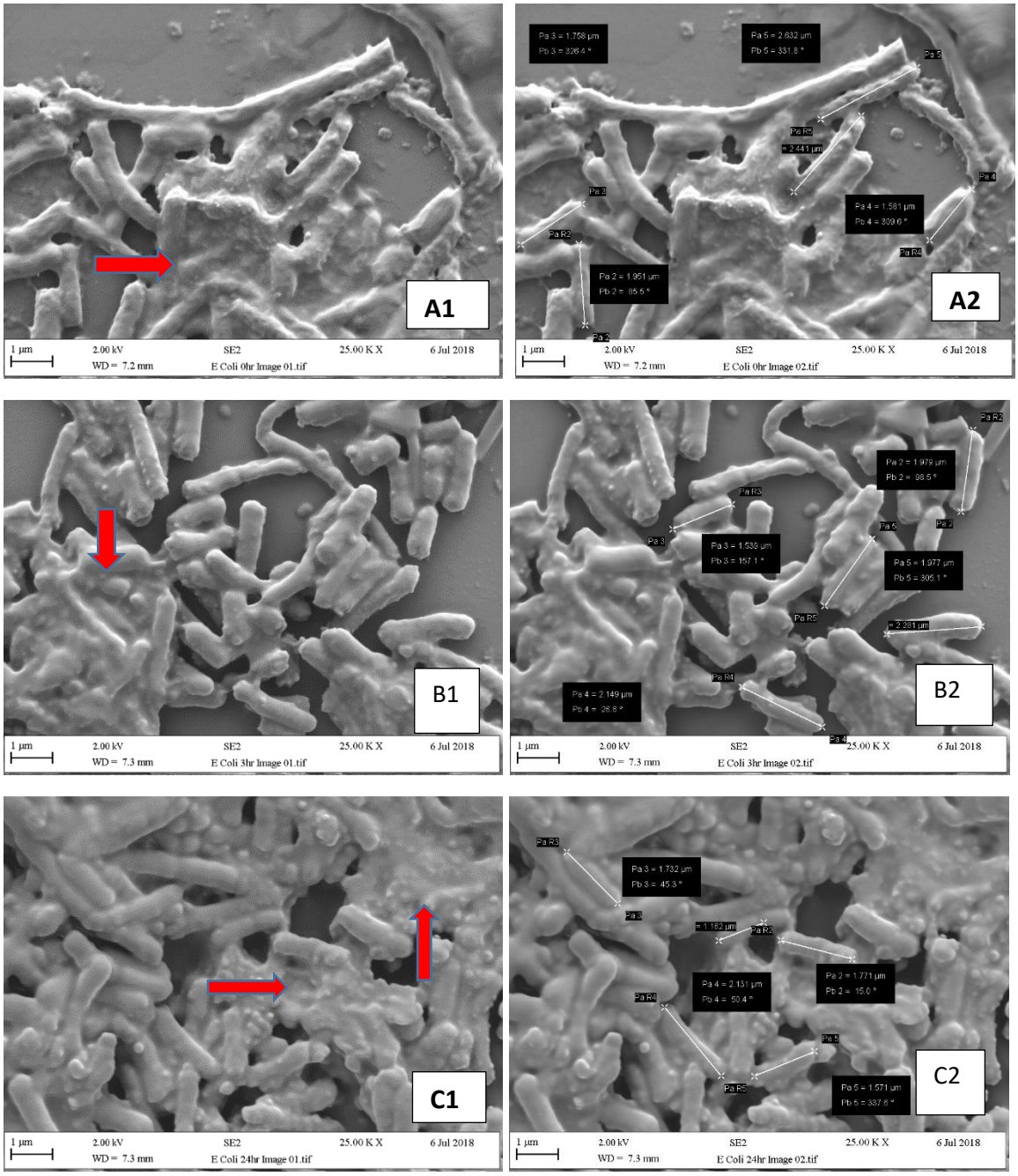


Figure 3.12 – Effect of nutmeg extracts on morphology and cellular structure of *E.coli*. A1 shows the image of *E.coli* at 25,000 X maginification and A2 shows the cell size were measured lengthwise. a) *E.coli* with nutmeg extract (0hr incubation), b) *E.coli* with nutmeg extract (3hr incubation), and c) *E.coli* with nutmeg extract (24hr incubation), red arrow shows cellular disruption.

Effect of pure compounds on *E.coli*

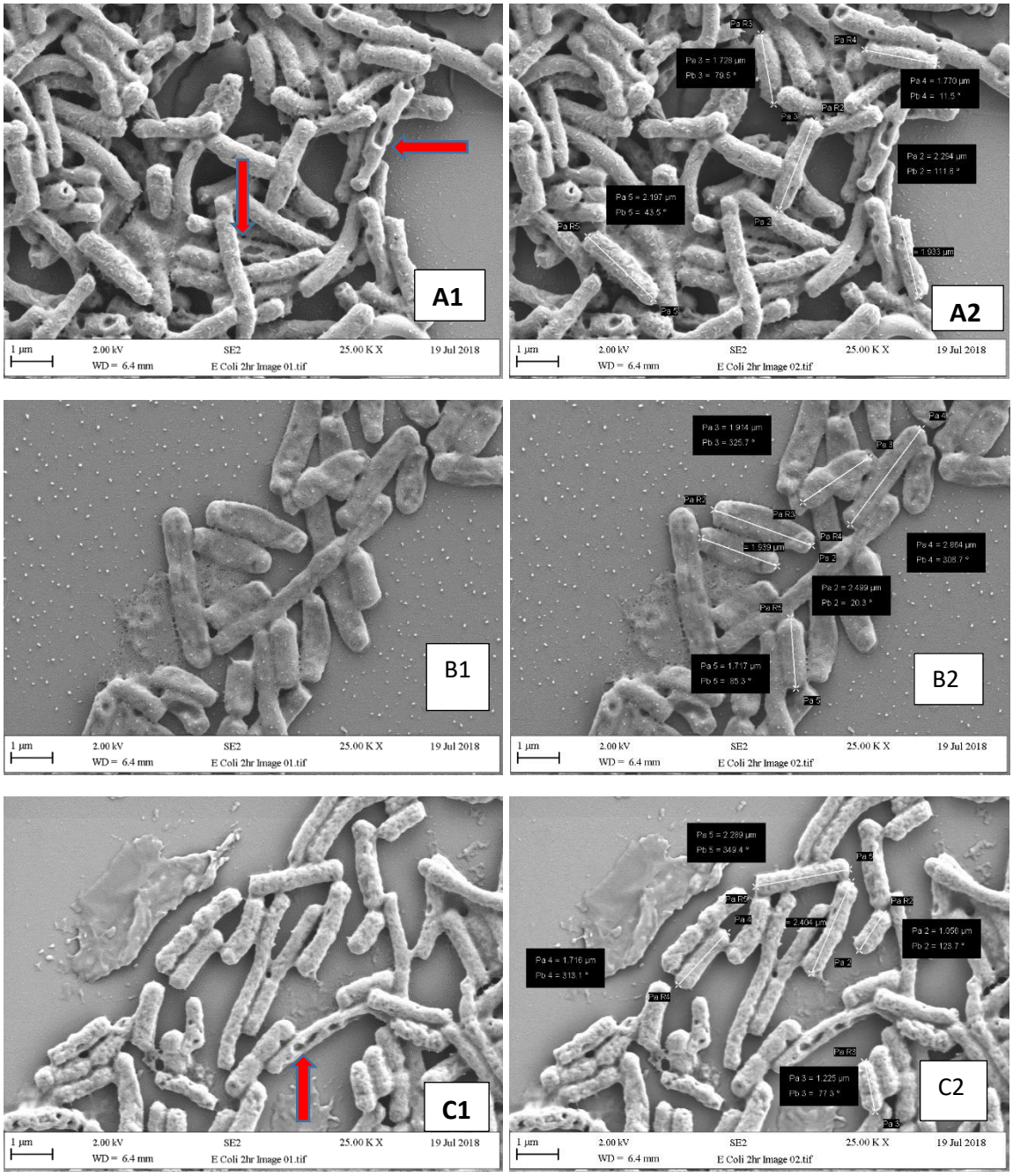


Figure 3.13 – Effect of pure compound extracts on morphology and cellular structure of *E.coli*. A1 shows the image of *E.coli* at 25,000 X maginification and A2 shows the cell size were measured lengthwise. a) *E.coli* with Thymol (2hr incubation), b) *E.coli* with p-cymene (2hr incubation), and c) *E.coli* with carvacrol (2hr incubation), red arrow show cell lysis and pore formation.

4. Effect of spice extracts on *Pseudomonas*

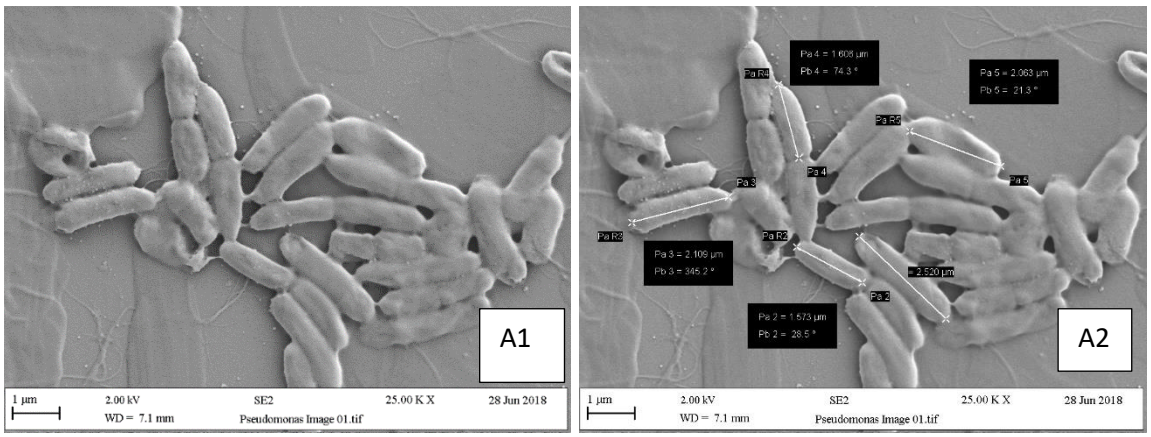


Figure 3.14 - *Pseudomonas* cells without extract (control). A1 shows the image of *Pseudomonas* at 25,000 X maginification and A2 shows the cell size were measured lengthwise.

The actual size of *Pseudomonas* cells 1.83µm, measured in this study and considered as control (without spice extract treatment). The cell size was calculated by taking mean of n=4 replicates shown in figure 3.14. A1 shows the image of *Pseudomonas* at 25,000 X maginification and A2 shows the cell size were measured lengthwise. When these cells were exposed to ajwain or nutmeg extracts the bacterial cell alter in size (figure 3.15 and 3.16). When these bacterial cells were treated with pure bioactive compounds such as thymol, p-cymene and carvacrol, swelling of cells and cellular alternation was noticed (Figure 3.17).

Effect of ajwain extract on *Pseudomonas*

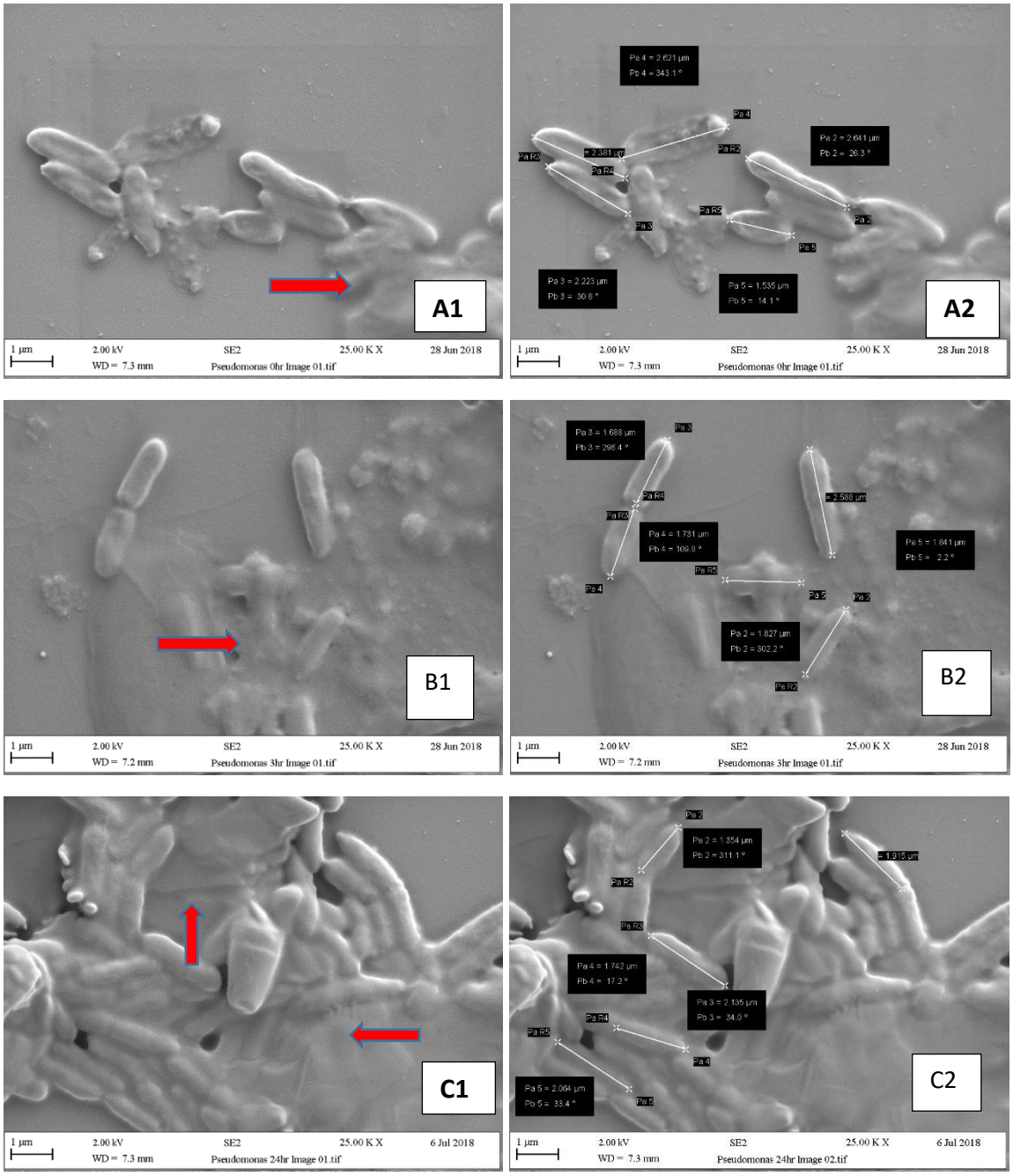


Figure 3.15 – Effect of ajwain extracts on morphology and cellular structure of *Pseudomonas*. A1 shows the image of *Pseudomonas* at 25,000 X maginification and A2 shows the cell size were measured lengthwise. a) *Pseudomonas* with ajwain extract (0hr incubation), b) *Pseudomonas* with ajwain extract (3hr incubation), and c) *Pseudomonas* with ajwain extract (24hr incubation), red arrow show loss of cellular component.

Effect of nutmeg extract on *Pseudomonas*

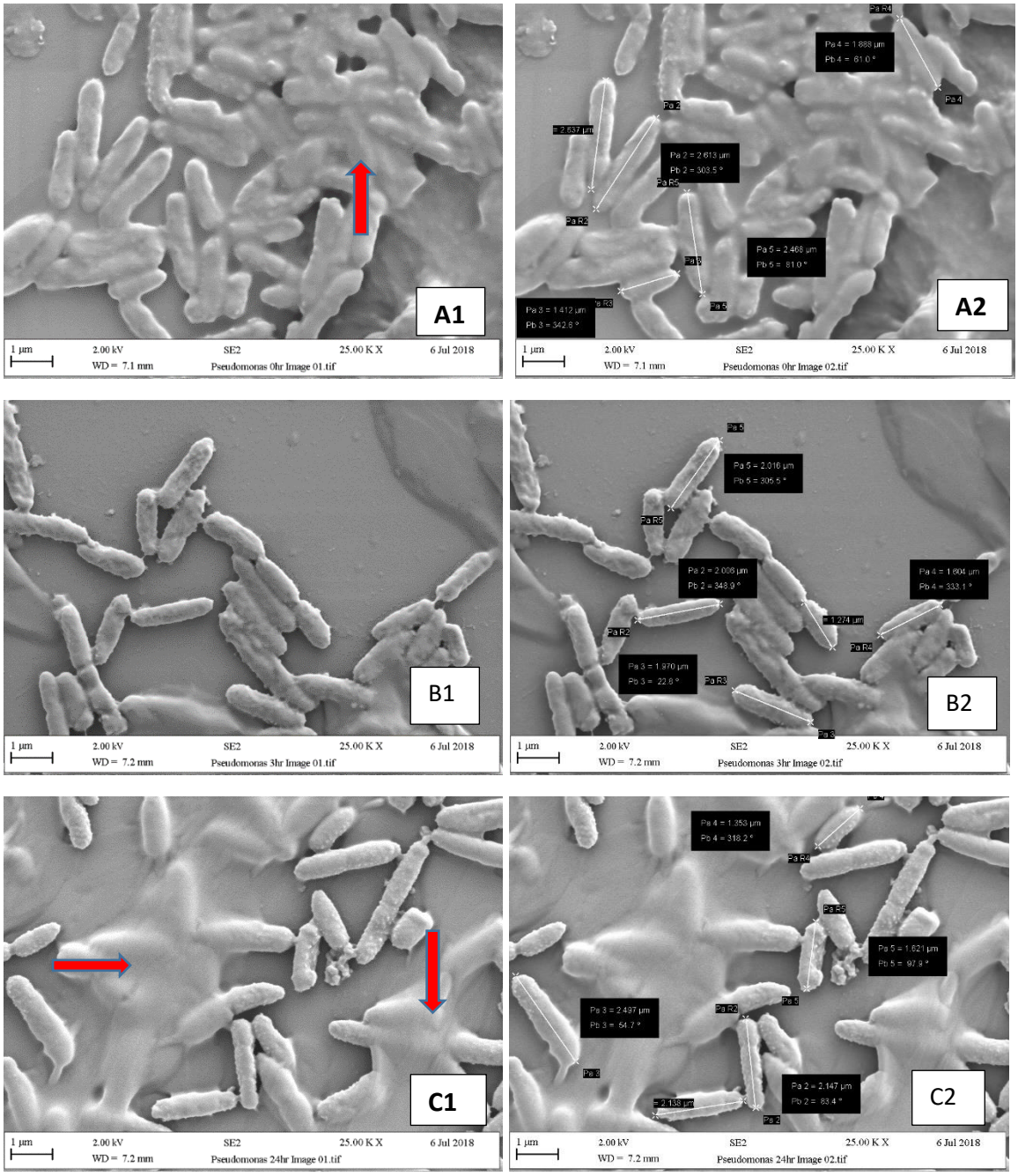


Figure 3.16 – Effect of nutmeg extracts on morphology and cellular structure of *Pseudomonas*. A1 shows the image of *Pseudomonas* at 25,000 X maginification and A2 shows the cell size were measured lengthwise. a) *Pseudomonas* with nutmeg extract (0hr incubation), b) *Pseudomonas* with nutmeg extract (3hr incubation), and c) *Pseudomonas* with nutmeg extract (24hr incubation), red arrows shows loss of cell components.

Effect of pure compounds on *Pseudomonas*

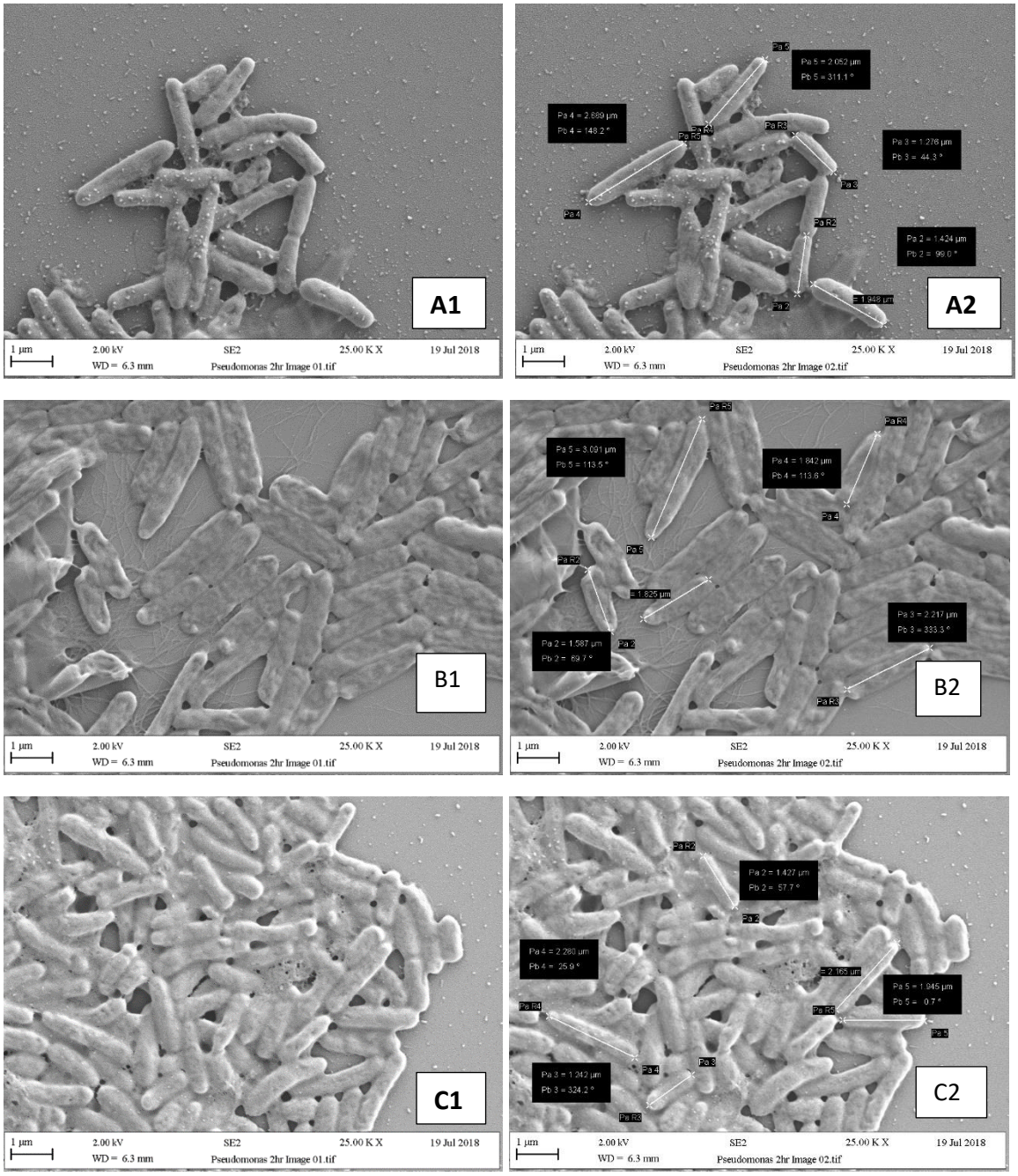


Figure 3.17 – Effect of pure compound extracts on morphology and cellular structure of *Pseudomonas*. A1 shows the image of *Pseudomonas* at 25,000 X maginification and A2 shows the cell size were measured lengthwise. a) *Pseudomonas* with Thymol (2hr incubation), b) *Pseudomonas* with p-cymene (2hr incubation), and c) *Pseudomonas* with carvacrol (2hr incubation). Swelling of the cells and aleration in cell size was noticed as compare to control cells.

3.5 Discussion

3.5.1 Comparative antimicrobial properties of selected spices and herbs

It can be observed that ethanol extracts of spices and herbs such as ajwain, nutmeg, bay leaves, clove, cumin and Indian gooseberry have shown an effective antimicrobial efficiency against a broad range of foodborne pathogens such as *Bacillus cereus, Listeria monocytogenes, Kocuria rhizophilia, E.coli, Salmonella typhimurium* and *Pseudomonas aeruginosa*. This study shown that ajwain and nutmeg possessed a moderate to high antimicrobial potency against the six tested foodborne pathogens. All ethanol extracts of spices and herbs displayed a significant antimicrobial activity. In chapter 2 it was observed that, the antimicrobial properties of water extracts of spices and herbs showed little or no significant antimicrobial effect on the tested foodborne pathogens. Previous studies supported the findings in which solvent extracts of the tested spices and herbs showed an effective antimicrobial potency against Gram-positive and Gram-negative microorganisms as compared to water extracts (Cowan, 1999, Henie *et al*., 2009, Weerakody *et al*., 2010 and Witkowska *et al*., 2013).

In the present study, agar disc diffusion assay showed effective inhibition of *Bacillus, E.coli, Salmonella* and *Pseudomonas* by tested spices and herbs. Inhibitory zone of 13 to 20mm highly effective for *Bacillus cereus*, 13mm for *E.coli*, 12mm for *Salmonella* and 14mm highly susceptible for *Pseudomonas* was observed by ajwain spice extract. Moreover, Inhibitory zone of 11-13mm for *Bacillus*, 9-13mm for *E.coli*, 14mm for *Salmonella* and 13mm for *Pseudomonas* was showed by Cumin. These results were contrasting with Mostafa *et al.,* (2018), who reported cumin and thyme were ineffective in inhibition of *Bacillus, E.coli Salmonella* and *Pseudomonas* except thyme 14.7mm for *Pseudomonas*. Another study by Mishra and Behal, (2010) reported effective antimicrobial inhibitory zone of 16-34mm for asafoetida, 12-18mm for cinnamon, 15-35mm for ginger and 13-21mm for cardamom extracts. In another study, cinnamon essential oil shows 19.2 mm zone of inhibition against *E.coli* (Zhang *et al*., 2016).

In the present study, it was observed that clove ethanol extracts showed effective inhibition of *Bacillus, E.coli*, and *Pseudomonas* with zones of inhibition of 14, 15 and 9 mm respectively.

Similar results were found in a study by Mostafa *et al*, (2018) against *B. cereus, E.coli, and Pseudomonas* inhibiting growth with zones of 14.6, 11.9 and 13.4 mm respectively. However, in the present study, clove ethanol extract were effective in inhibition of *Salmonella* with 15 mm zone of inhibition, these results were in contrast to those of Mostafa *et al*., (2018) which showed clove extract is not effective in inhibition of *Salmonella*. In the present study, ethanol extracts of clove, cumin and nutmeg were found to have potential antimicrobial action against *Salmonella*. Nanasombat, and Lohasupthawee (2005) found similar results in study and shows the antimicrobial capacity of several ethanolic spice extracts against *Salmonella*: clove, cumin and nutmeg.

3.5.2 Effect of spice extracts on MIC and MBC

In the present study, ajwain extract showed highly effective lowest concentration MIC value 0.78µg/ml and MBC 1.56µg/ml against *Bacillus cereus*. Paul *et al* (2011), shows the effective *T.ammi* (ajwain extract) MIC value 12.5µg/ml against *Bacillus subtilis*. In the present study, MIC value of cumin was found to be 50µg/ml for *Bacillus*, and not effective for *E.coli* and *Salmonella*. Similarly, Mostafa *et al*., 2018, showed that cumin was found to be not effective in inhibition of selected microorganisms and these results were contrasted with Dua *et al*., (2013) who reported effective range of MIC value 6.25 to 12.5 mg/ml for cumin. In another study, cinnamon essential oil shows MIC value 1mg/ml and MBC value 4mg/ml against *E.coli* (Zhang *et al.,* 2016).

In present study, all selected spices and herbs ethanol extracts were able to inhibit tested microorganisms except *E.coli* and *Salmonella*. However, clove and nutmeg spice were able to inhibit *E.coli* and *Salmonella* at MIC 3.12µg/ml. Similar to these results, study by Weerakody *et al*., 2010, demonstrated that MIC values of all tested spice and herb extracts obtained after 24hr showed <5mg/ml against all tested bacteria except *Salmonella* and *E.coli*. Whereas *G.quaesita* water extract inhibited *E.coli* growth at MIC 5mg/ml but there was no inhibition against *Salmonella* with exception of *R.officinalis* ethanol extract MIC of 2.5mg/ml.. Alternatively, In a study by Mostafa *et.al*. (2018), it was found that *S.aromaticum* (clove) extract was effective in inhibiting the growth of *Bacillus cereus, S. aureus, E.coli* and *P. aeruginosa* with a concentration of 10 mg/ml. In another study by Mishra and Behal, 2010,

tested spices extracts of asafoetida, ginger and cardamom were effective in a range between 12.5mg/ml to 3.12mg/ml except cinnamon extract which is effective against *E.coli* at MIC 25mg/ml.

Commonly, Gram-negative microorganisms such as *E.coli* and *Salmonella* are more resistant to plant oils due to presence of hydrophilic cell wall structure. Gram-negative microorganisms contain a lipopolysaccharide layer that blocks the penetration of plant oil (which is hydrophobic) and avoids the accumulation of extract oil in target cell membrane (Zaika, 1988; Gyawali *et al*., 2015). Davidson and Branen (2005) found similar suggestion in their study. Therefore, these results explain that why Gram-positive bacteria such as *S. aureus, Listeria monocytogenes* and *Bacillus* are more sensitive to plant extracts as compared to Gram-negative microorganisms (Gyawali *et al*., 2015).

3.5.3 Effect of spice extract on morphology and cellular structure of microorganism

In the present study, SEM images shows morphological changes in bacterial cells when exposed to ajwain and nutmeg ethanol extracts as compared to untreated bacterial cells (control). The effect of ajwain and nutmeg on *Bacillus cereus*, *Kocuria, E.coli* and *Pseudomonas* was an alteration in cell size – specially shrinkage of the cells as compared to control cells after 24 hour incubation. The possible reason could be the chemical compounds from these spices extract when comes in contact with bacterial cell, result in alteration and disruption of cell membrane and components of the bacterial cells. Similar to present study findings, a study by Weerakody *et al*., (2010), reported that major antibacterial components of plant essential oils could penetrate through cell wall and destroy the cytoplasmic membrane. Another study by Zhang *et.al*. (2016), SEM images showed the morphological alterations in the bacterial cell membrane brought about by cinnamon essential oil. Moreover, disrupted cell membrane structure and changes in bacteria cells may be due to cell membrane lysis and transformation on permeability of membrane. Therefore, the changes can lead to loss of inner cell content (Bajpai *et al.,* 2009). In a study by Paul *et.al.* (2011), SEM images showed the morphological changes of *Bacillus subtilis* showing pore formation and cell lysis at MIC concentration on treating with *T.ammi* (ajwain) essential oil.

Natural antimicrobial in plant-based compounds such as polyphenols, essential oil, act by rupture of the bacterial cell membrane, decay of proton motive force, ATP pool depletion, which may lead to essential biomolecules leakage. They can interfere with nucleic acid replication and/or transcription and protein synthesis (Pisoschi *et al*., 2018). Some of the possible modes of antimicrobial action on target microorganisms include – membrane disrupting components cause cell content leakage (Davidson, 1997). Organic acids present in plant species interfere with cell membrane permeability (Gyawali *et al.,* 2015). Goni *et al*., (2009) reported structural and functional damage to cell membrane due to production of essential oil. Antimicrobial content in plants can attack the cell membrane and disrupt the cell enzyme systems (Burt *et al*. 2007). These are the possible modes of mechanisms used to explain the antimicrobial effect of natural plant extracts.

In the present study, from SEM images of pure bioactive compounds it can be seen that bioactive compounds are responsible in inhibition of bacterial cells. The pure bioactive compounds such as thymol, p-cymene and carvacrol shows effective results in terms of disrupting bacterial cell membranes, loss of cellular components, cell lysis and pore formation in the bacterial cells. A study by Paul *et.al.* (2011), describes that in *T.ammi* (ajwain) essential oil, active compounds such as eugenol, thymol and carvacol are present in higher content and seem responsible for antimicrobial mode of action. It was also mentioned that, phenolic not only attack cell membrane but also interfere with membrane function such as electron transport, nutrient uptake, protein and nucleic acid synthesis and enzyme activity. However, the exact mode of antimicrobial action of specific natural components in plant extracts against target microorganisms are still unknown (Gyawali *et al*., 2015). Therefore, these results suggest further investigation of possible mode of action of plant extracts on pathogenic microorganisms.

3.6 Conclusion

The antimicrobial effect of spices and herbs are useful data, while this is difficult to compare with other studies due to different plant material use, origin from different geographical region, harvesting time, maturity of plant material, etc. (Burt, 2004; Witkowska *et al.*, 2013). Moreover, antimicrobial testing is difficult to compare in terms of different solvents,

concentration, extraction method, culture medium, microbial strain used, inoculum size, growth phase, etc. (Weerakkody *et al.,* 2010; Witkowska *et al*., 2013). Overall, antimicrobial test - zone of inhibition (ZoI) and MIC results of six selected spices and herbs extracts showed effectively strong antimicrobial activities against selected foodborne pathogens. Ethanol extracts displayed higher antimicrobial activities than water extracts. The type of solvent used to extract spices and herbs can have great impact on the antimicrobial efficacy. A non-polar solvent has a higher interaction with non-polar compounds present in spices and herbs, which leads to extraction of more bioactive compounds than water extracts (Weerakkody *et al.*, 2010). Ultrasound-assisted ethanol extracts showed higher efficacy than stirring method of extraction. Among all ultrasound-assisted ethanol extracts, ajwain followed by nutmeg, clove, bay leaves, Indian gooseberry and cumin against tested microorganisms showed effective antimicrobial properties. In general, Gram-positive microorganism such as *Bacillus, Listeria* and *Kocuria* are more susceptible to spices and herbs extracts than Gram-negative microorganisms such as *E.coli, Salmonella* and *Pseudomonas*.

Many previous research studies have reported that the plant bioactive compounds (phenolic compounds have antimicrobial activities on microorganism and responsible of their mode of action (But 2004; Tajkarimi *et al*., 2010; Weerakody *et al*., 2010; Zhang *et al*., 2016). Paul *et al*., (2011), mentioned that phenolic compounds namely terpenoids such as eugenol, thymol and carvacrol are responsible for antimicrobial mode of action. Most of the studies focused on effect of bioactive compounds on cell membrane whereas phenolics also affect permeability and release of cell content (Tajkarimi *et al*., 2010; Paul *et al*., 2011). Another finding by Tassou (2000), observes the leakage of cell content from *E.coli* and *S.auerus*, which shows correlation with phenolic concentration. Therefore, there is need to find possible bioactive compounds in spices and herbs extracts to justify the possible mode of antimicrobial action against microorganism.

Chapter 4

Bioactive compounds in ajwain and their potential antimicrobial action

4.1 Introduction

Numerous studies have shown that spices and herbs possess natural properties and exert antimicrobial activity by preventing growth of spoilage microorganisms (food preservation) and inhibiting the growth of pathogenic microorganisms i.e. food safety (Weerakkody *et al,* 2010; Tajkarimi *et al,* 2010). Spice and herb extracts (essential oils) are rich in phenolic compounds. Phenolic compounds are amongst several secondary metabolites in plants that can enhance their survival. Other phytochemical constituents include terpenes, monoterpenes and sesquiterpenes (Skendi *et al.,* 2017). Aglycone phenolic compounds have have been reported to cause structural and functional damage to microbial cells (Martinez *et al*, 2015). However, antimicrobial activity of spice and herb extracts on microbial inhibition may vary according to the type of spice and bioactive compounds present (Tajkarimi *et al.,* 2010). Most phenolic compounds found in number of points conjugated forms with sugars per aglycone, leading to diverse range of potential phenolic forms. This makes complexity in identification of the compounds. However, simple aglycone could be obtain from hydrolysis of glycosylated polyphenolic compounds (Pimpao *et al.,* 2013).

Non-polar phenolic compounds such as thymol, carvacrol have been reported in many studies as being responsible for antimicrobial properties (Tajkarimi *et* al, 2010; Burt, 2004). Some spices such as ajwain, nutmeg, cumin, clove and bay leaves has been previously investigated for its antimicrobial effect in previous chapter. Previous studies showed that major composition of ajwain extract include thymol, r-terpinene, p-cymene, along with traces of α- and β-pinenes, myrcene, 1,8-cineole, and carvacrol (Chahal *et al*., 2017). A study by Hyldgaard et al., (2012) reported that thymol is a major constituent of the chemical profile of ajwain and responsible for antimicrobial action. In other spices such as clove essential oil mainly constituent eugenol, β-caryophyllene and eugeyl acetate. Eugenol is reported to have antimicrobial properties (Omidbeygi *et al,* 2007). Similarly, major constituent of bay leaf essential oil includes 1, 8 cineol, eugenol, acetyl eugenol, methyl eugenol (Polovka *et al,* 2010). The major component responsible for antimicrobial effect of cumin oil has been

reported as cuminaldehyde (Chen *et al*, 2014). Active components of nutmeg include myristin, elemicin, eugenol and β-caryophyllene reported to exert antimicrobial properties (Periasamy *et al,* 2016).

Alternatively, a wide variety of polar-phenolic compounds of spices and herbs have also been reported to inhibit antimicrobial activity. According to Cowan (1999), phenolic acids such as cinnamic acid and caffeic acid are effective against bacteria and fungi, quinones have potentially antimicrobial effect, and flavonoids have ability to form complexes with proteins and cell wall and provide antimicrobial activity. Plant-based antimicrobial phenolic components such as saponin and flavonoids, thiosulfinates have extreme antimicrobial effects against Gram-negative microorganism (Tajkarimi *et al*, 2010).

The purpose of the current study was to determine non-polar and polar phytochemical compounds in spice and herb extract. Volatile compounds such as thymol, carvacrol present in spices and herbs extracts are mainly detected by Gas-Chromatography Mass-Spectrometry (GCMS). The purpose of use of GCMS is for the determination of particular monoterpenoid, non-polar phenolic compounds in spice and herb extracts (Skendi *et al.,* 2017). High-Performance Liquid Chromatography (HPLC), combined with diode array detection (DAD) is the most widely used separation technique to detect and quantify phytochemicals in plant extracts (Skendi *et al.,* 2017). The purpose of use of HPLC-DAD is for the analysis of different polar phytochemicals compounds such as phenolic acids, flavonoids and other polyphenols in the same extract. Analysis of spice extracts by HPLC-DAD revealed a great complexity and diversity of compounds, obstructing individual identification of compounds. To quantify polar polyphenols in the ajwain extract, a novel combination of enzymes for hydrolysis and quantification of aglycones by HPLC-DAD was studied.

The present study focused on determination of total phytochemicals such as non-polar and polar compounds in 10 different varieties of ajwain samples. The purpose of the study is to determine whether any individual compound(s) or groups of compounds are more responsible for antimicrobial behaviour than others. Therefore, the correlation graphs between individual/group of phytochemical compound(s) and antimicrobial activity against pathogenic microbes were studied. The aim of the present study was to practically

standardize the chemical profile of ajwain extract, find out possible mechanism of action against foodborne pathogenic microorganism, set markers of the quality to recommend it as natural preservative to enhance the shelf life of food products.

4.2 Aim – Identification of major phytochemical compounds specifically in ajwain that are potentially responsible for antimicrobial inhibition of pathogenic microorganisms.

Objectives -

- Identification of polar and non-polar compounds in ethanolic extracts of ajwain and other spices using HPLC and Gas-Chromatography Mass-Spectrophotometry (GCMS)
- Quantification of major chemical compounds in ten different ajwain samples
- Assess correlation between major compounds in ajwain extracts with antimicrobial properties of ajwain against selected pathogenic microorganisms

4.3 Methodology

4.3.1 Identification of non-polar bioactive compounds in spice extracts using Gas-Chromatography Mass-Spectrophotometer (GCMS)

1. Materials

For qualitative analysis by GCMS, spices and herbs such as ajwain, nutmeg, cumin, clove, bay leaves and cinnamon were purchased from whole grain market Faridkot, India. For Quantitative analysis of 10 different varieties of ajwain were purchased from different regions of India (Table 4.1).

2. Chemicals and standards

Ethanol and water used in this study was purchased from Fischer and of HPLC grade. Standard reference compounds such as thymol, α-pinene, β-pinene, ρ-cymene and ϒ-terpinene were purchased from Sigma-Aldrich.

3. Sample preparation

Ajwain spices seeds were blended using a small mill (IKA A11 basic) until they were homogeneous and sieve through 500µm sieved to obtain distribution of particles that all fall below 500µm size and stored in airtight containers at room temperature until used. However, the particle size distribution below 500µm was not determined in the current study. It has been demonstrated that, different particle size could affect efficiency of extraction.

4. Extract preparation for GCMS analysis

The spice extract was prepared by adding 1:10 ratio of spice to solvent (ethanol) w/v. The mixture was vortexed for 30 sec to ensure the contents were mixed together properly, then subjected to ultrasound-assisted extraction at 20 KHz, cycle 0, 400W power, 20% amplitude for 5 min. The total content was collected and centrifuged at 9000 rpm for 10 min and supernatants were filtered through Whatman filter No 4. An additional 100ml of ethanol was added to the pellet, then the extraction process repeated. After the second extraction, the filtrates were combined. Initial concentration of spice sample was 100mg/ml and this was diluted to 10mg/ml with ethanol and filtered through a 20 µm PTFE filter.

4.3.2 GCMS analysis of non-polar bioactive compounds

The method was performed as described by Kedia *et al.,* (2015) with some modifications. The chemical composition of ajwain ultrasound-assisted ethanolic extracts were determined using gas chromatography, a GC- (Agilent 190915-43301) fitted with a HP-5MS Ultra-Inert [30mm x 250µm x 0.25µm], fitted with HP column [0.2 mm internal diameter]. Helium carrier gas was used, the heater set to 280^{0}C and a column head pressure 7.06 psi. The oven temperature was set to 40^{0}C for 2 min then increased to 300^{0}C to a total run time of 11 min. Split injection (20:1) was performed with split flow 2ml/min. The initial identification of chemical components was done with comparison on the mass spectral information in a mass spectra library (Mass hunter/library/NIST14.L), and then confirmed by comparison to retention times and spectra of reference standards.

There were two modes of MS settings for identification of compounds – SCAN and SIM mode. The spice extracts were first analysed at full MS SCAN mode, qualitative analysis of samples were performed and compounds were identified from mass spectral information in a mass spectra library (in software application). In an individual sample run, mass spectra of all the compounds was obtained and reference compound peak were identified from ion size of each compound. Possibility of identification of the compound was based on molecular weight and comparison with software library and thereafter SIM mode run for the confirmation of the compound in the sample. The peak area of the compounds was too high in SCAN mode run, therefore, there is need to run SIM mode, to identify and calculate % concentration of the compound in the sample.

The selective analyses were done in MS SIM mode, which is quantitative analysis of the compounds in spice extract. Five trials were performed to optimize the SIM mode setting for quantitative analysis of the compounds. In the trial 1, ramp temp 300°C, run time 11min was used for the sample. In this method setting, few ion sizes were selected which assumes to be the ion size of the reference compounds. However, this method is not good at finding all the reference compounds in the sample. In trial 2, ramp temp adjusted from 30°C, 11 min run time, all ion sizes included (molecular weight of the compounds). Resulted in identification of all the reference compounds. In trial 3, ramp temp used was 15°C, run time 35min. In trial 4, ramp temp used was 22.5°C, run time 12.78min. In trial 5, ramp temp was 50°C, run time 8.87min. In all the trials either sample peaks were too high or unable to identify all require

reference compounds. Only trial 2 setting was found to be the best method for the identification of the compounds in a sample. Hence, used for identification of non-polar compounds in 10 different varities of ajwain samples.

Qualitative analyses were performed on different spice extracts of ajwain, nutmeg, cumin, clove, bay leaves, and cinnamon. For quantification, the study focused on ajwain as this was the most potent antimicrobial of the pre-screened spices yet had the least established chemical profile. The analyses were performed in triplicates on 10 different varieties of ajwain which were collected from different parts of India and was tested for the presence of number of bioactive compounds and concentration in all samples. The physical characteristics of ten different varieties of ajwain spice were listed in the Table 4.1.

Table 4.1– Different source of ajwain (Collected from different regions of India)

Ajwain sample number	Region and Common name	Physical characteristics of ajwain seeds (size, colour and flavour)	Pictures of ajwain samples
1	Gujrat (Natural plus)	Yellowish brown, medium size seeds, strong flavour	
2	Gujrat (Victory)	Greenish brown, big size seeds, mild flavour	

3	Maharashtra	Brown black colour seeds, medium size seeds, bitter taste	
4	Rajasthan (Karfansh)	Black seeds, small size, after taste and not strong flavour	
5	Rajasthan (Wall)	Greenish brown mixed with stalks and dust, big size	
6	Rajasthan (Kursani)	**Blackish round seeds (doesn't look like ajwain seeds but used as ajwain in that region)**	

7	Uttar Pradesh (UP)	Dried yellow, red seeds, big size, strong flavour	
8	Bihar	Dried yellow seeds, big size, strong flavour	
9	Punjab (Local market)	Dried greenish yellow, big size seeds, strong flavour	
10	Punjab (whole grain market)	Dried brown yellow seeds, medium size, strong flavour	

4.3.3 Preparation of standard curves for non-polar bioactive compounds

Reference standard compounds were prepared in stock solution (1ml/10ml ethanol concentration for each compound). The reference standards for – α-pinene, β pinene, ρ cymene, ϒ terpiene and thymol (>98% pure) were used to prepare stock solution for quantitative analysis. These compounds were mixed in equal quantity 1ml of each standard compound in 9 ml ethanol (HPLC grade) for each compound and then all were mixed together. These stock solutions were then serial diluted till 1µl/10ml ethanol (100ppm concentration) and then run via GCMS. Different dilution were used ranging 1000ppm, 900ppm, 800ppm, 700ppm, 600ppm, 500ppm, 400ppm, 300ppm, 200ppm, 100ppm, 75ppm, 50ppm, 25ppm, 10ppm, 1ppm. Standard calibration curves were made for pure compounds. The calibration curves were used to calculate concentration of sample peak area and compared with reference standards (control) peak area.

4.3.4 Identification of polar phenolic compounds in ajwain extract using HPLC

1. **Chemicals and standards**

Organic solvents were purchased from Fischer and were of HPLC grade. Gallic acid, 4-hydroxybenzic acid, protocatechuic acid, syringic acid, vanillic acid, ferulic acid, chlorogenic acid, rosmarinic acid, luteolin, apigenin, quercetin, kaempferol, rutin, catechin, epicatechin, epigallocatechin were purchased from Cambridge Biosciences. Trans-cinnamic acid, caffeic acid, p-coumaric acid, naringenin were purchased from Sigma-Aldrich. Cellulase (EC 3.2.1.4) was purchased from Sigma-Aldrich and hesperidinase (Rham 143) a thermostable α-L-rhamonosidase from *Thermomicrobia sp.* was purchased from Prokazyme, Iceland.

2. **Material and Sample preparation**

The HPLC analyse was performed on 10 different varieties of ajwain which were collected from different regions of India (Table 4.1). Ajwain spices seeds were blended using a mill (IKA A11 mill) until they were homogeneous and sieve through 500µm sieved to obtain distribution of particles that all fall below 500µm size and stored in airtight containers at room temperature until used.

3. Chemicals/reagent Preparation

Ethanol/water (1:1) with BHT (1mM BHT – molecular weight 220.35 g/mol was prepared. Daidzein with 0.1mM concentration – molecular weight 254.23 g/mol was prepared.

4. Enzymatic Hydrolysis of Glycosides

HPLC method was carried out according to Pimpao *et al.,* (2013). To perform enzymatic hydrolysis of glycosides, phytochemicals were extracted by homogenizing 0.3 g of blended ajwain spice with 2.7 mL of ethanol (absolute) containing 1 mM butylated hydroxytoluene (BHT) and 0.1 mM daidzein as internal standard. Samples were vortexed for 10 min, sonicated for 10 min, then vortexed again for 10 min, and then centrifuged at 3000g for 20 min at 4 °C. The supernatant were collected, the pellet was re-extracted according to the same procedure with 0.9 mL of the extraction solution (ethanol). Supernatants were combined, centrifuged as described, and filtered through a 0.2 µm PTFE filter.

One part of the extract (0.5 ml) was used directly for HPLC analysis of total phytochemicals. Another part of the extract was dried and reconstituted in water with 1mM ascorbic acid, to protect samples from oxidation. Samples for aglycone analysis were adjusted to pH 5 with 0.2M acetate buffer and incubated at 40^{o}C. Initially different enzyme concentrations and incubation times were compared in order to select the best conditions for the liberation of aglycone phenolics (Table 4.2).

Table 4.2 – Different enzyme concentrations and incubation times used for the liberation of aglycone phenolic compounds

Method	Enzyme concentration and condition	Total incubation time
1	100 µL of undiluted hesperidinase (0.52 U/100 µl), incubate for 16 hours at 40 °C followed by addition of 37mg cellulase enzyme incubate further for 4 hours	16hr Hesp + 4hr cellulase
2	100 µL of diluted hesperidinase (0.052 U/100 µl), incubate for 16 hours at 40 °C followed by addition of 37mg cellulase enzyme to the tubes, and incubate further for 4 hours.	16hr Hesp + 4hr cellulase
3	100 µL of undiluted hesperidinase (0.52 U/100 µl) together with 37mg cellulase enzyme to the tubes and incubated for 20 hours at 40^{0}C.	20hr Hesp + cellulase
4	100 µL of diluted hesperidinase (0.052 U/100 µl) together with 37mg cellulase enzyme to the tubes and incubated for 20 hours at 40^{0}C.	20hr Hesp + cellulase
5	100 µL of undiluted hesperidinase (0.52 U/100 µl) and incubate for 20 hours at 40 °C followed by addition of 37mg cellulase enzyme to the tubes and incubate further for 4 hours.	20hr Hesp + 4hr cellulase
6	100 µL of diluted hesperidinase (0.052 U/100 µl) and incubate for 20 hours at 40 °C followed by addition of 37mg cellulase enzyme to the tubes and incubate further for 4 hours.	20hr Hesp + 4hr cellulase
7	Add 100 µL of undiluted hesperidinase (0.52 U/100 µl) together with 37mg cellulase enzyme to the tubes and incubated for 24 hours at 40^{0}C.	24hr Hesp + cellulase
8	100 µL of diluted hesperidinase (0.052 U/100 µl) together with 37mg cellulase enzyme to the tubes and incubated for 24 hours at 40^{0}C.	24hr Hesp + cellulase

Hesperinidase (0.05 U/ml) – in acetate buffer pH 5 with 1 mg/ml Bovine Serum Albumin. Hesperidinase [1 unit (U) corresponds to 333 mg of protein and is defined as the amount

required to liberate 1.0 µmol of glucose from hesperidin per minute at pH 5 at 40 °C]. Cellulase (20 U/ml), the bottle contains 1.4 U/mg and the total volume of the sample is 2.6 ml. [(20 / 1.4) = 14.28 mg cellulase/ml 14.28 x 2.6 = 37 mg]. Cellulase 20 U/mL (1 U corresponds to 37 mg of protein and is defined as the amount required to liberate 1.0 µmol of glucose from cellulose in 1h at pH 5 at 40 °C).

After incubation all ajwain samples were extracted three times with ethyl acetate (Double the volume of the liquid in the tube for each extraction, 5 ml was used). The ethyl acetate phase (containing phenolic aglycones) was separated and evaporated under nitrogen, and samples were reconstituted with 2 mL of 0.1% (w/v) ascorbic acid solution in ethanol/water (1:1) and filtered through a 0.2 µm PTFE filter prior to analysis. The procedure was performed in triplicate for each ajwain sample. After selection of best enzyme concentration and incubation combination method, the procedure was performed in triplicates on 10 different sources of ajwain sample.

4.3.5 HPLC Analysis of polar polyphenolic compounds

HPLC analysis was conducted on a Atlantis T3, 3um, 2.1 x 100 mm equipped with Lab solution software, DGU – $20A_3$ prominence degasser, LC- 20AB prominence solvent delivery module, SIL-20AC $_{HT}$ prominence auto sampler, CBM-20A prominence communications BUS module, SPD- M30A diode array detector (DAD), and CTO-20A prominence column oven. The sample is so complex it is difficult to get adequate separation to identify all the compounds. Nonhydrolysed and enzyme treated compounds were identified and quantified based on their retention time and absorption spectra in comparison with standards. A sample volume of 5 µL was injected, and separations were achieved on the column, operated at 30 °C, with a 0.2 µm stainless steel in-line filter. The method was performed as described by Pimpao *et al,* (2013) with the exception of the mobile phase used at a flow rate of 0.26 mL/min. The mobile phase over a gradient of 100% solvent A (95% H_2O, 5% ACN with 0.2% v/v formic acid) for 10 min, reaching 15% B (95% ACN, 5% H_2O with 0.2% v/v formic acid) from 10 to 30 min. Solvent B increased to 25% at 50 min and to 100% B at 55 min where it was maintained for 5 min returning to 0% in 2 min. Chromatograms were recorded from diode array data collected between 200 and 400 nm. All standards were dissolved in ethanol/water (1:1) and used as

stock solutions. The calibration curves were used to calculate concentration of sample peak area and compared with reference standards (control) peak area.

4.3.6 Preparation of standard curves for polar phenolic compounds

Reference standards (>98% pure) were prepared in stock solution state strength, (all reference compounds mixed together in ethanol/water 1:1 ratio concentration). Including Gallic acid, 4-hydroxybenzic acid, protocatechuic acid, syringic acid, vanillic acid, ferulic acid, chlorogenic acid, rosmarinic acid, luteolin, apigenin, quercetin, kaempferol, rutin, catechin, epicatechin, epigallocatechin, trans-cinnamic acid, caffeic acid, p-coumaric acid, and naringenin. These stock solutions were then serial diluted and then run via HPLC. The calibration curves were used to calculate concentration of sample peak area and compared with reference standards (control) peak area.

4.3.7 Antimicrobial analysis of 10 distinct ajwain samples

Extracts of 10 different ajwain samples were tested for antimicrobial activity using an agar disc diffusion assay. Bacterial cultures of Gram-positive microorganisms such as - *Bacillus cereus* (ATCC 11778), *Listeria monocytogenes* (ATCC 7644), *Kocuria rhizophilia* (ATCC 9341), and Gram-negative microorganisms such as - *Escherichia coli* (ATCC 25922), *Salmonella enterica sv typhimurium* (ATCC 14028) and *Pseudomonas aeruginosa* (ATCC 27853), - used in the study were obtained from Remel Europe, Ltd. (Dartford, Kent, United Kingdom). Antimicrobial analysis of 10 different ajwain samples against tested foodborne pathogenic microorganisms was done. The method followed was same as explained in chapter 2.3.5. Then correlations graphs of antimicrobial analysis with non-polar bioactive compounds and with polar compounds were studied.

4.3.8 Statistical analysis and experimental design

This study was designed to obtain the potential bioactive phenolic compound responsible for antimicrobial properties of ajwain ethanolic extract against foodborne pathogenic microorganisms. All the experiments were performed in triplicates. The data was analysed using Microsoft office Excel and results were expressed as mean ± standard deviation.

4.4 Results

4.4.1 Bioactive compounds identified in different spice extracts using GCMS

The initial identification of chemical components in different spices and herbs were done with comparison on the mass spectral information in a mass spectra library, and full MS Scan mode was used (Table 4.3).

Table 4.3– Bioactive compounds identified in different spices and herbs ethanolic extract

Spice and herbs	Bioactive compounds identified in 100mg/ml extract (w/v)
Ajwain	α-pinene, camphene, β-pinene, β-mycrene, α-phellandrene, ρ-cymene, ϒ-terpinene, thymol, caryophyllene
Nutmeg	α-pinene, β-phellandrene (traces), β-pinene, β-myrcene, ϒ-terpinene, limonene, Terpine-4 ol, safrol, Methyleugenol, caryophyllene, trans-isoeugenol, isoelemicin
Cumin	α-pinene, β-pinene, ρ-cymene, ϒ-terpinene, Benzenealdehyde
Clove	Eugenol, caryophyllene, humulene, Phenol, 2-methoxy-4 (2 propenyl), acetate
Cinnamon	Cinnamaldehyde, caryophyllene
Bay Leaves	Eugenol

4.4.2 Identification of non-polar bioactive components in ajwain extract using GCMS

In ajwain ethanolic extract, α-pinene, camphene, β-pinene, β-mycrene, α-phellandrene, ρ-cymene, ϒ-terpinene, thymol, caryophyllene were qualitatively identified in 100mg/ml concentration of ajwain ethanolic ultrasound-assisted extract (Figure 4.1). The quantification is not possible at 100mg/ml concentration as intensity of major peaks were too high (high analyte concentrations can damage the filament in GCMS). However, quantification at 100mg/ml concentration was done on one ajwain sample for comparison with diluted 10mg/ml concentration sample (Table 4.4).

At 10mg/ml concentration of ajwain extract, thymol, p-cymene and r-terpinene were identified as a major compound along with α-pinene, β-pinene in traces (Figure 4.2a and b). These non-polar chemical compounds were selected for quantitative analysis in 10 different varieties of ajwain samples, as peaks for rest of the compounds, which was observed at 100mg/ml concentration, were not observed at this concentration.

Table 4.4– Chemical compounds identified at 100mg/ml concentration of ajwain extract

Chemical compound	**Molecular weight g/Mol**	**Retention time (RT min)**	**Peak area**	**% peak area**
α-pinene	136.23	4.93	4409514	0.796
Camphene	136.24	5.02	663495	0.120
β-pinene	136.23	5.22	5454083	0.985
β-mycrene	136.23	5.39	819775	0.148
ρ-cymene	134.22	5.51	83557921	15.104
ϒ-terpinene	136.23	5.75	134422538	24.266
Caryophyllene	204.36	6.43	5010156	0.904
Thymol	150.22	6.90	319502064	57.677

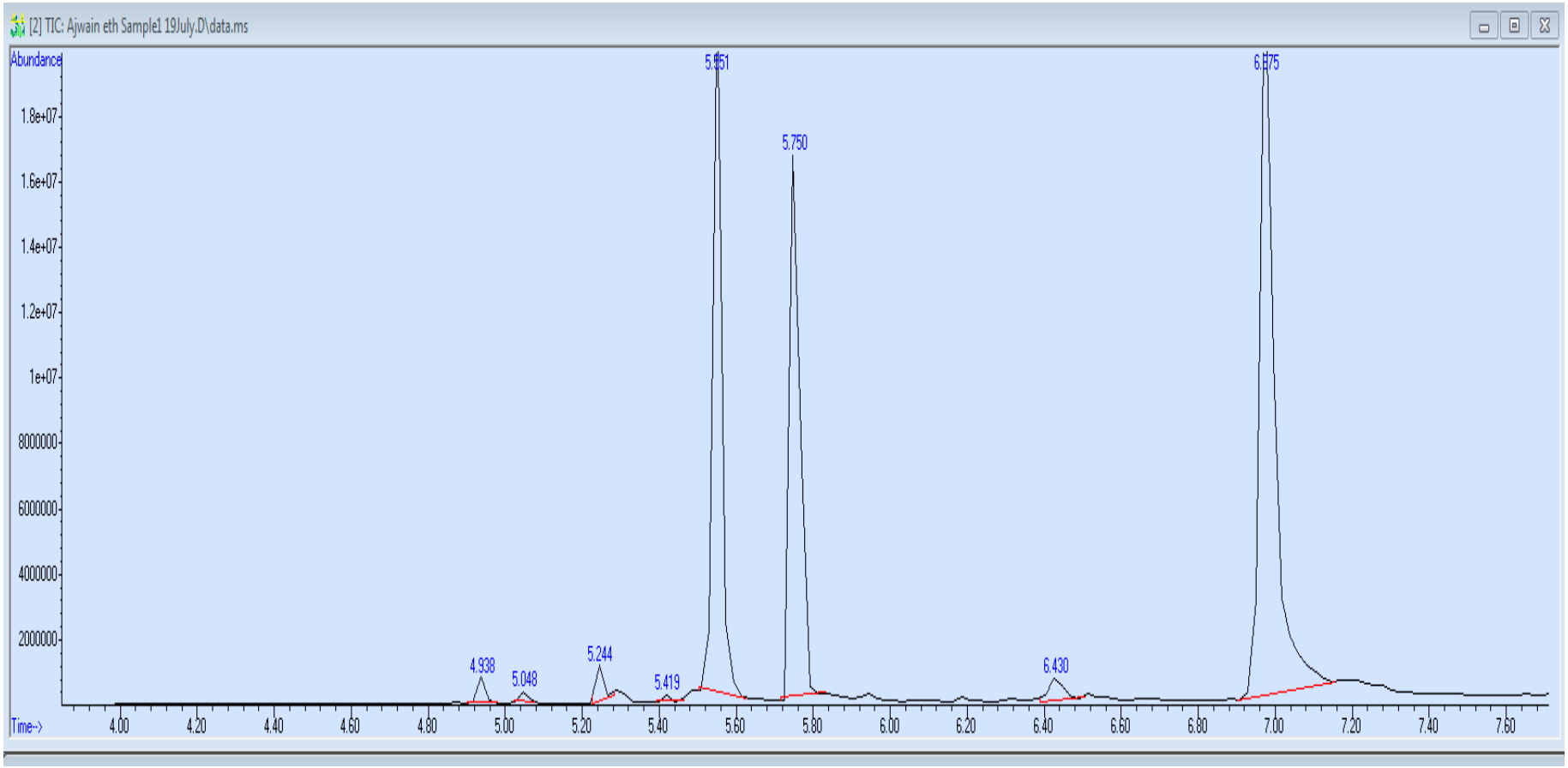


Figure 4.1 – Chromatograph for compounds identified in ajwain extract at 100mg/ml concentration

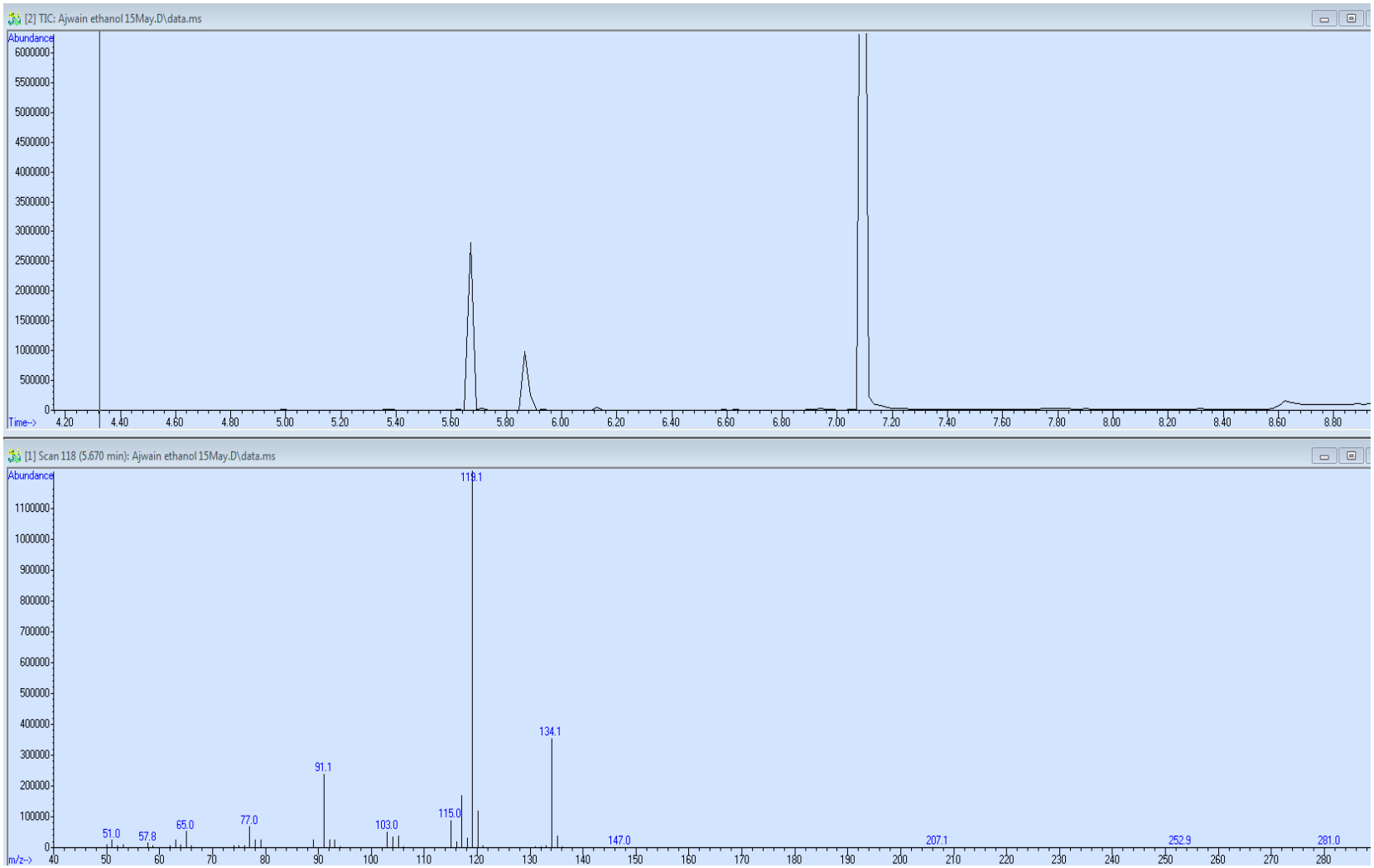


Figure 4.2a - Major compounds identified in ajwain extract at 10mg/ml concentration. ρ-cymene MW 134 peak at 5.65min (1st peak), gamma-terpinene MW 136 peak at 5.85 min (2nd peak), Thymol MW 150 peak at 7.10min (3rd peak). Ion spectra of thymol compound shows the ion size 119, found in SCAN mode. Similarly, other compounds ion size was identified, and all compounds were identified in SIM mode thereafter.

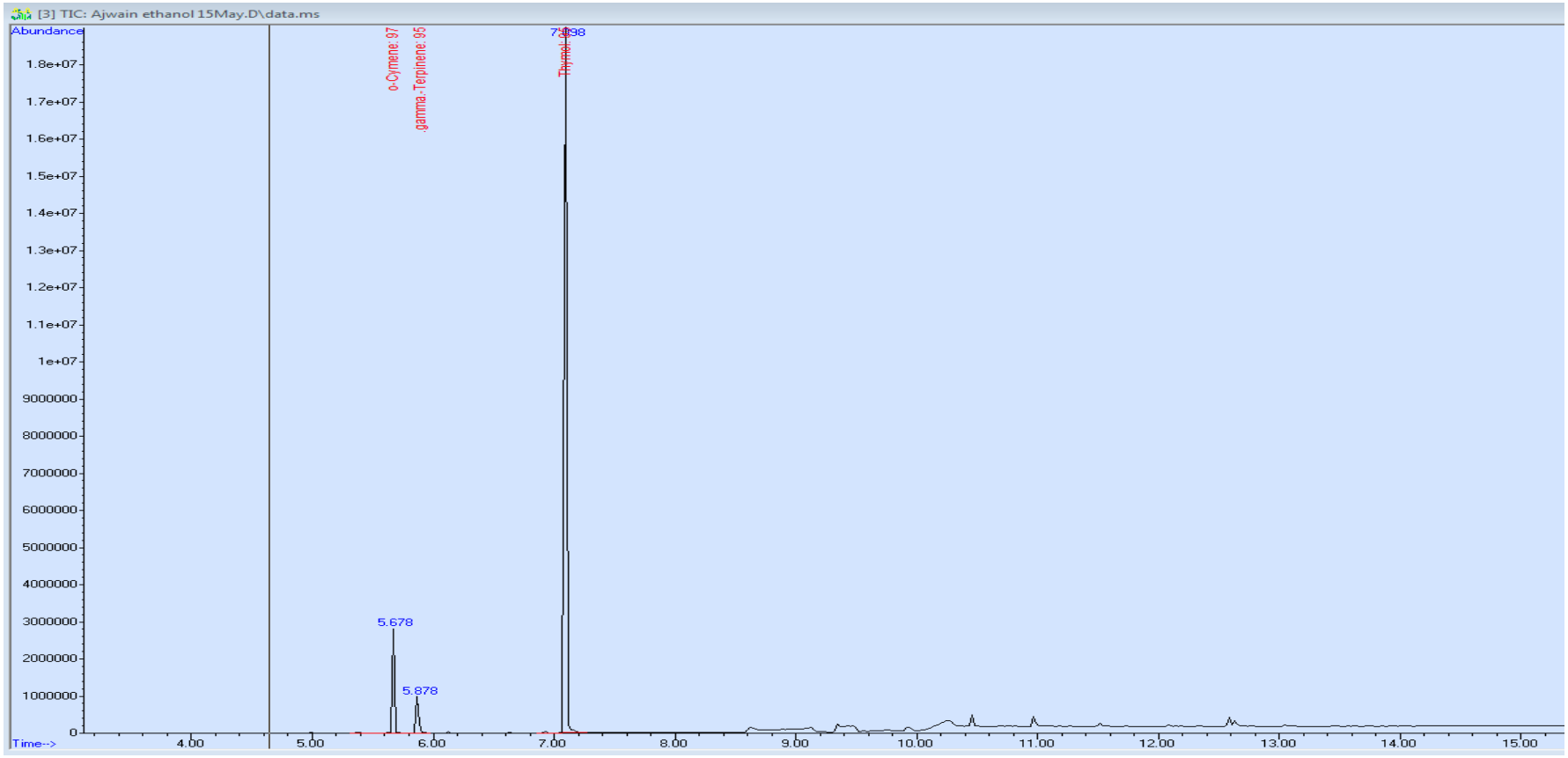


Figure 4.2b - Major compounds in ajwain extract at 10mg/ml concentration. ρ-cymene at 5.65min (1st peak), gamma-terpinene at 5.85 min (2nd peak), Thymol at 7.10min (3rd peak). This chromatograph shows the highest peak size in SCAN mode and need to do dilution of the extract to protect filament of the instrument (GCMS).

4.4.3 Standard curves used for non-polar bioactive compounds in GCMS analysis

Standard calibration curves were for pure non-polar bioactive compounds - α-pinene, β-pinene, ρ-cymene, ϒ-terpinene, and thymol were used as reference (Figure 4.3). The calibration curves were used to calculate concentration of sample peak area. The structure, IUPAC name and molecular weight of major bioactive compounds of ajwain extract showed in Table 4.5.

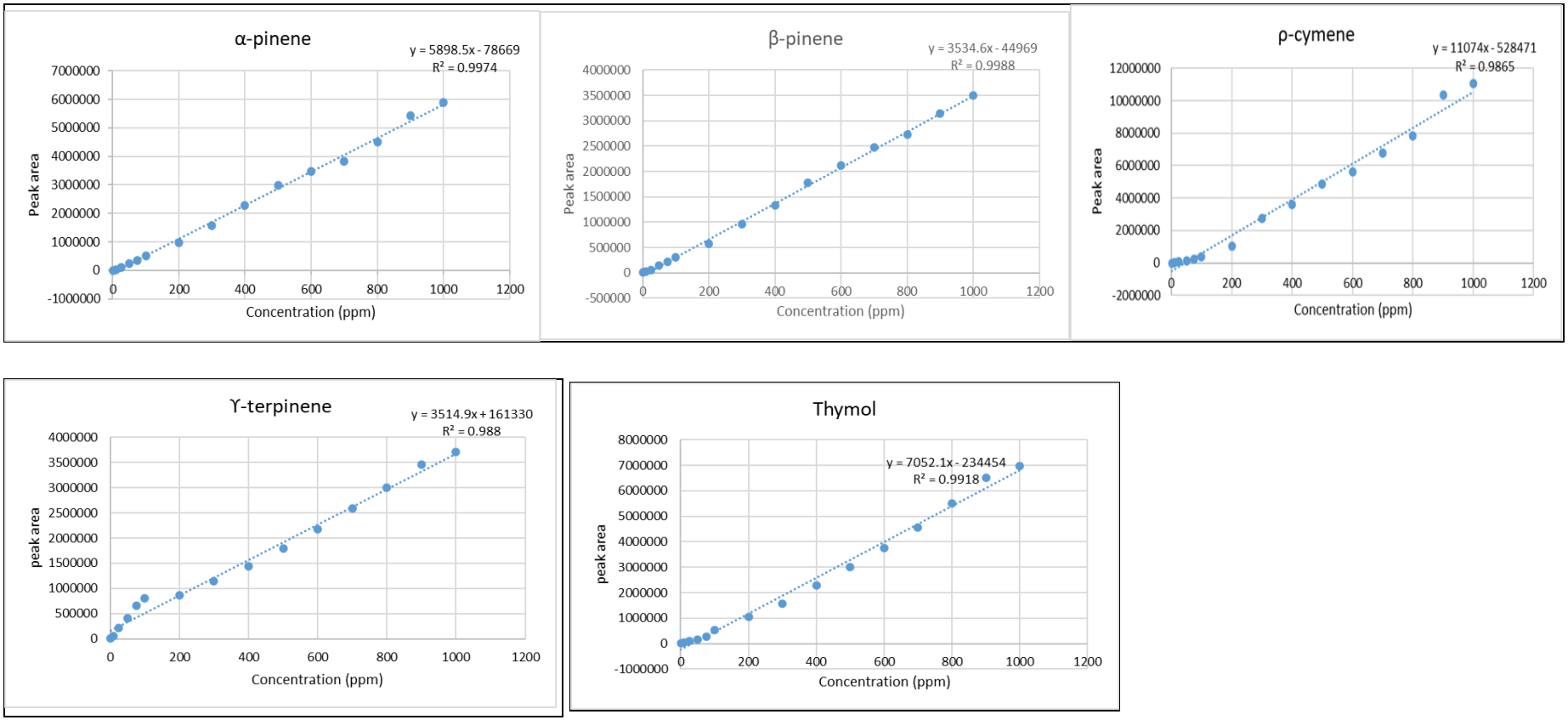
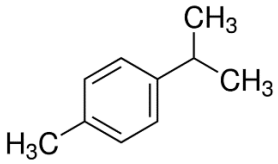
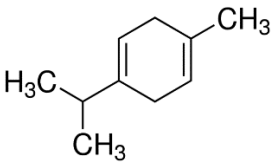
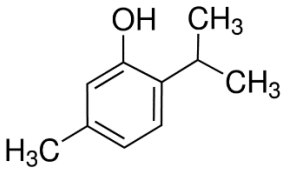


Figure 4.3- Standard curves for non-polar bioactive compounds used for quantification of ajwain extract

Table 4.5 – Structure, IUPAC name and molecular weight of major compounds of ajwain extract

Chemical compounds	Structure	IUPAC name	Molecular weight (g/mol)
ρ-cymene		1-Methyl-4-(propan-2yl) benzene Or 1-Isopropyl-4-methylbenzene, Or 4-Isopropyltoluene	134.22
γ-terpinene		4-Methyl-1-(1-methylethyl)-1,4-cyclohexadiene Or 1-Isopropyl-4-methyl-1,4-cyclohexadiene Or *p*-Mentha-1,4-diene	136.23
Thymol		5-Methyl-2-(propan-2-yl) phenol or 2-Isopropyl-5-methylphenol, Or 5-Methyl-2-(1-methylethyl) phenol, Or 5-Methyl-2-isopropylphenol	150.22

4.4.4 Quantification of non-polar bioactive compounds in different sources of ajwain

The concentration of non-polar chemical compounds present in 10 different sources of ajwain extracts with respect to pure compounds calibration curve were calculated and presented in Table 4.6.

The sample number 9 and 10 shows highest concentration of non-polar compounds. Surprisingly, ajwain sample 5 and 6 showed negligible amount of non-polar bioactive compounds. All ajwain samples were further analysed for polar phenolic compounds using HPLC.

Table 4.6- Concentration of non-polar chemical compounds in different sources of ajwain (solution concentration mg/100g ajwain sample, n=3 ± S.D.)

Chemical compounds	Ajwain samples									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
α-pinene	1.33 ± 0	0 ± 0	3.34 ± 7.2	5.28 ± 1.5	0 ± 0	0 ± 0	4.57 ± 2.3	3.47 ± 3.3	4.66 ± 6.7	**6.14 ± 2.9**
β-pinene	1.27 ± 0	13.79 ± 1.9	4.13 ± 1.9	14.49 ± 4.2	18.0 ± 3	0 ± 0	10.59 ± 5	14.90 ± 5.4	15.43 ± 8.9	**20.86 ± 5.6**
ρ-cymene	67.53 ± 2.1	38.02 ± 5.7	56.39 ± 5.2	75.25 ± 3	44.58 ± 7	0 ± 0	7.10 ± 2	51.90 ± 3.4	79.28 ± 1.7	**75.68 ± 5.1**
ϒ-terpinene	164.52 ± 6	87.64 ± 9.4	159.2 ± 2.9	**269.1 ± 1.17**	0 ± 0	0 ± 0	232.2 ± 5.9	128.43 ± 1.0	255.02 ± 3.2	225.87 ± 2.3
Thymol	302.25 ± 5.6	278.66 ± 3	274.0 ± 1.6	112.6 ± 3.7	0 ± 0	5.3 ± 2.9	301.97 ± 9	274.69 ± 1.6	315.75 ± 5.9	**337.09 ± 1.4**
Total Bioactive compounds	**536.92**	**418.13**	**497.19**	**476.76**	**62.58**	**5.39**	**556.49**	**473.41**	**670.16**	**665.67**

Average concentration of main non-polar ajwain constituents (concentration of the solution mg/100g ajwain sample, n=3 ± S.D) from 10 distinct varieties.

Values in bold text indicates the highest concentration of chemical compound in the sample.

4.4.5 Standard curves used for polar phenolic compounds in HPLC analysis

Standard calibration curves for pure polar phenolic compounds were used as reference. The calibration curves were used to calculate concentration of sample peak area and compared with reference standards (control) peak area. Calibration curves of peak area against concentration showed a good linear correlation with $r2 > 0.98$ (Figure 4.4). Calibration range as well as the wavelengths use to detect each compound are presented in Table 4.7.

Standard Curves

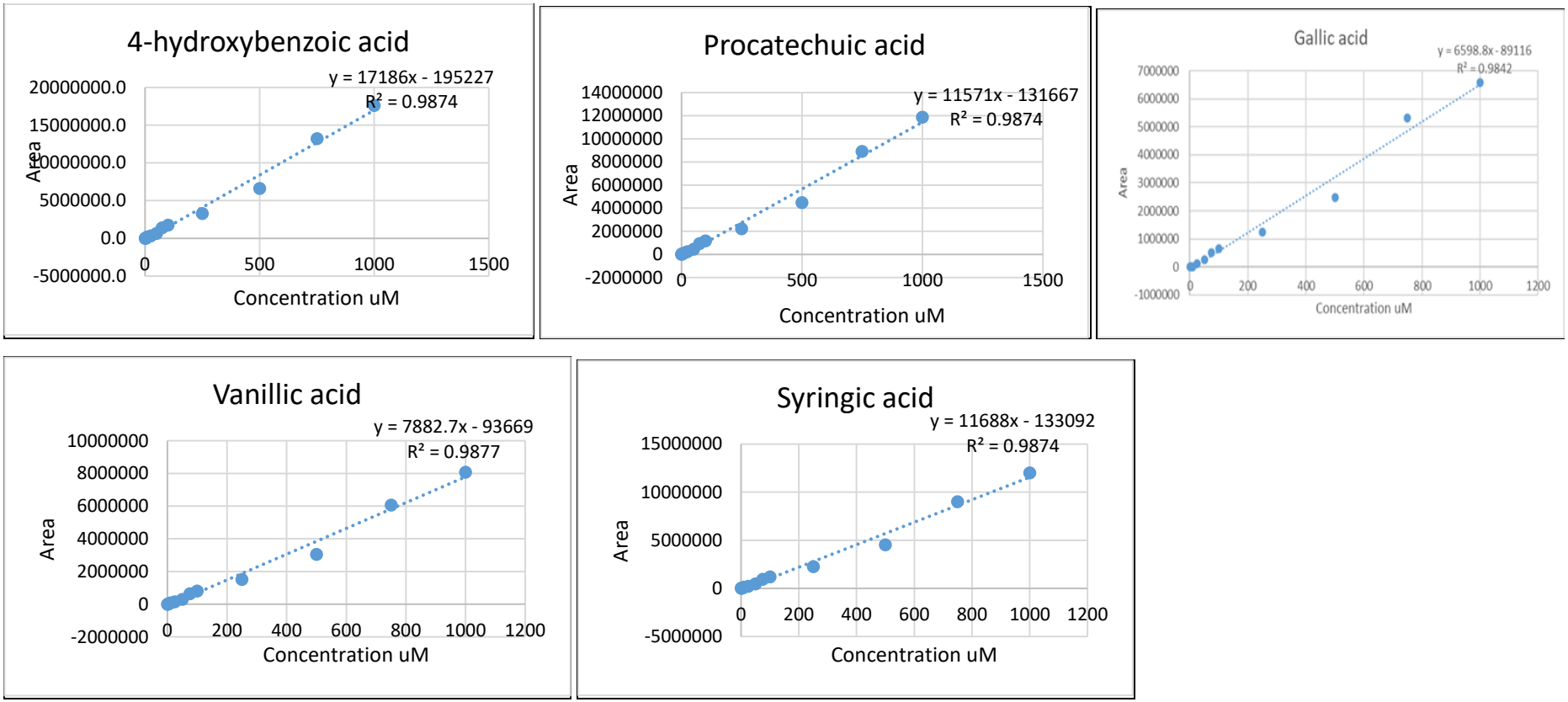


Figure 4.4 - Standard curves for polar phenolic compounds used for quantification of ajwain extracts. (Standard curve for rest of 15 compound were presented in appendix A).

Table 4.7 – Chromatography parameters of standards compounds used for peak identification

Standards	Molecular weight	Retention time (RT)	Wavelength (nm)	Calibration range (uM)	LOD (mg/ml diluted extract)	LOQ (mg /ml) undiluted extract
4-Hydroxy benzoic acid	138.12	12.145	254	1-1000	0.25	1
Procatechuic acid	154.12	6.553	260	1-1000	0.25	1
Gallic Acid	170.12	3.22	272	25-1000	0.25	25
Vanillic acid	168.14	17.802	272	1-1000	0.25	1
Syringic acid	198.17	20.977	272	0.5-1000	0.1	0.5
Epigallocatechin gallate	458.37	24.598	273	0.5-1000	0.1	0.5
Catechin	290.26	18.424	278	1-1000	0.25	1
Epicatechin	290.26	23.961	278	0.5-1000	0.1	0.5
Trans-cinnamic acid	148.15	41.976	280	0.5-1000	0.1	1
Naringenin	272.25	50.376	289	1-1000	0.25	0.5
p-Coumaric acid	164.04	25.426	302	0.5-1000	0.1	0.5
Chlorogenic acid	354.31	19.163	324	0.5-1000	0.1	0.5
Caffeic acid	180.16	19.546	324	0.5-1000	0.1	0.5
Ferulic acid	194.18	28.327	324	0.5-1000	0.1	0.5
Rosmarinic acid	360.31	38.132	324	0.5-1000	0.1	0.5
Apigenin	270.05	51.998	340	5-1000	1	5
Luteolin	286.24	45.330	348	0.5-1000	0.1	0.5
Rutin	610.52	31.596	354	0.5-1000	0.1	0.5
Quercetin	302.23	45.144	370	0.5-1000	0.1	0.5
Kaempferol	286.23	53.223	370	5-1000	1	5

LOD – Limit of detection, LOQ – limit of quantification

4.4.6 Identification of polar phenolic compounds using HPLC analysis

Phenolic reference standard compounds were diluted in a range and Figure 4.5 represent the HPLC-DAD chromatogram for all reference standard compounds at 1000µM concentration at 278nm wavelength. However, individual analytes were quantified at wavelengths of optimum response, with maxima having previously been established using spectral analysis of reference standards.

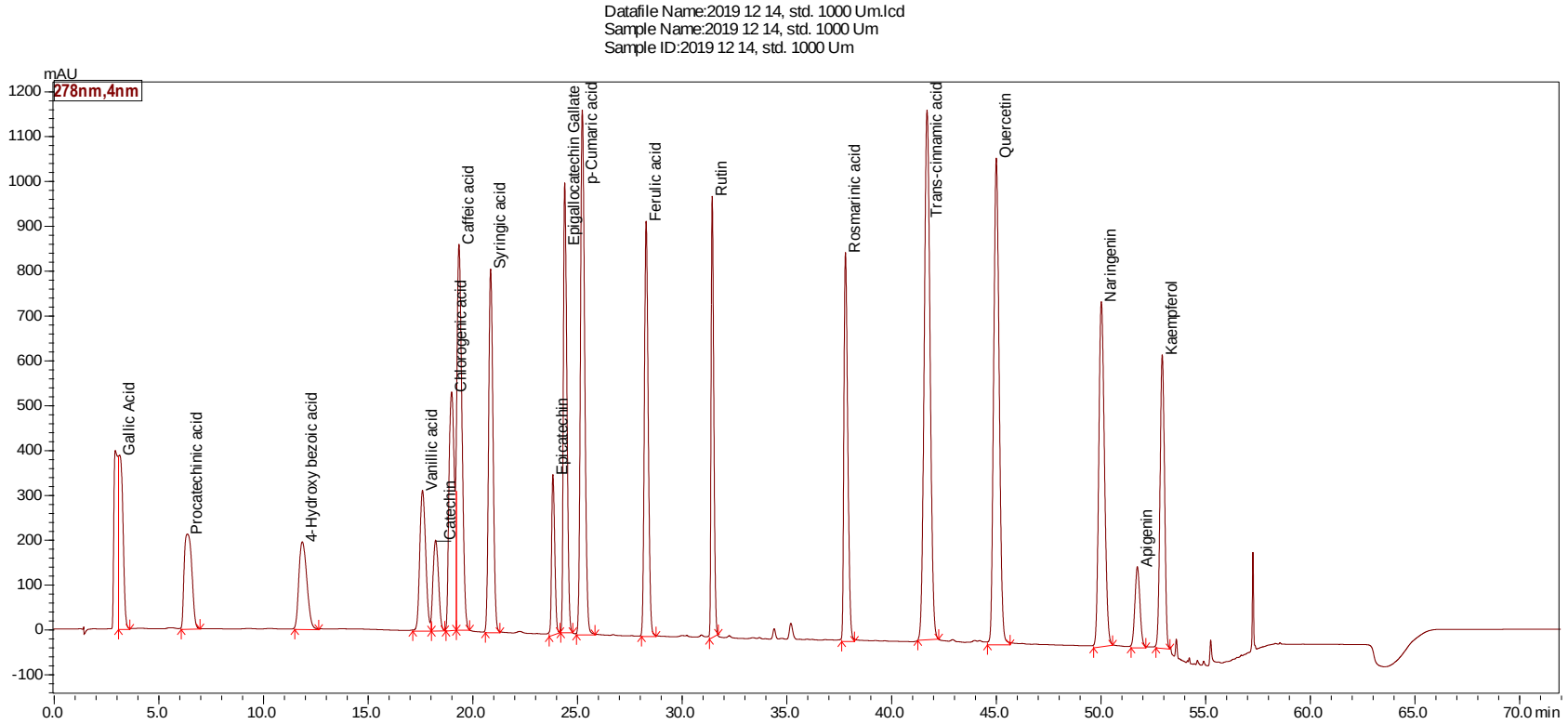


Figure 4.5 HPLC-DAD chromatograms of phenolic reference standard compounds. All reference standard compounds are shown at 278nm wavelength at 1000µM concentration. However, individual analytes were quantified at wavelengths of optimum response, with maxima having previously been established using spectral analysis of reference standards.

The best method of enzyme concentration and incubation time combination on ajwain enzyme treated samples was selected based on comparison with non-enzyme treated samples (Table 4.8). The best-selected enzyme treated method was method 7 (as out of 20 reference compounds, 12 individual compounds showed highest concentration in method 7 as compared to other method.

Table 4.8- Selection of best method for ajwain enzyme treated samples and compared with non-enzyme treated ajwain samples (mg/100g ajwain sample, n=3 ± S.D.)

	Non enzymatic ajwain sample	Method 1 0.5U Hesp 16hr + cellulase 4hr	Method 2 0.05U Hesp 16hr + cellualse 4hr	Method 3 0.5U Hesp + cellulase 20hr	Method 4 0.05UHesp + cellulase 20hr	Method 5 0.5U Hesp 20hr + cellulase 4hr	Method 6 0.05U Hesp 20hr + cellulase 4hr	Method 7 0.5U Hesp + cellulase 24hr	Method 8 0.05U Hesp + cellulase 24hr
4-Hydroxy benzoic acid	36.95 ± 0.62	41.18 ±18.8	46.61 ± 0.23	**65.98 ± 2.59**	65.47 ± 0.87	45.98 ± 0.47	46.10 ± 0.57	**65.29 ± 1.05**	60.92 ± 6.47
Procatechinic acid	122.38 ± 23.4	55.46 ± 2.8	56.34 ± 0.68	67.23 ± 3.01	67.52 ± 0.87	55.10 ± 0.60	56.01 ± 1.03	**67.54 ± 0.74**	63.14 ± 6.98
Gallic Acid	127.03 ± 3.85	25.48 ±1.8	24.60 ±1.16	26.47 ± 1.91	26.98 ± 0.47	25.42 ± 1.41	25.22 ± 1.03	**27.34 ± 0.21**	26.87 ± 0.68
Vanillic acid	35.54 ± 0.33	37.54 ± 14.8	45.37 ± 1.95	53.36 ± 3.17	**55.13 ± 0.57**	44.07 ± 1.36	45.02 ± 1.52	54.49 ± 0.17	49.28 ± 6.34
Syringic acid	22.3 ± 0.04	27.06 ± 6.4	31.60 ± 0.93	**37.55 ± 0.75**	36.67 ± 0.01	30.94 ± 1.47	31.14 ± 0.71	36.40 ± 0.73	34.27 ± 3.84
Epigallocatechin gallate	27.99 ± 0.01	52.30 ± 24.6	65.35 ± 0.49	75.10 ± 1.45	58.54 ± 30.02	64.96 ± 0.87	64.94 ± 0.43	**76.26 ± 0.68**	71.40 ± 9.25
Catechin	481.38 ± 11.8	174.21 ± 29.9	185.82 ± 5.3	**209.2 ± 9.6**	206.41 ± 13.5	165.55 ± 3.5	169.55 ± 7.9	193.54 ± 3.92	176.1 ± 34.2
Epicatechin	38.9 ± 1.0	668.13 ± 56	984.24 ± 18.4	361.08 ± 58	382.81 ± 62	973 ± 20.2	**1007.78 ± 31.1**	381.31 ± 62	284.65 ± 45
Trans-cinnamic acid	22.29 ± 0.06	38.85 ± 28.3	22.47 ± 0.5	28.10 ± 5.1	28.30 ± 5.3	22.62 ± 0.05	22.77 ± 0.07	28.60 ± 4.98	**65.90 ± 32**
Naringenin	20.97 ± 0.05	21.19 ± 0.25	20.99 ± 0.13	21.0 ± 0.12	21.01 ± 0.14	21.47 ± 0.13	21.62 ± 0.23	**21.85 ± 0.17**	21.44 ± 0.75
p-Coumaric acid	0.95 ± 0.01	2.97 ± 0.10	2.90 ± 0.03	3.56 ± 0.10	3.62 ± 0.02	2.88 ± 005	2.87 ± 0.02	**3.65 ± 0.05**	3.31 ± 0.65
Chlorogenic acid	58.42 ± 1.5	15.2 ± 0.0	15.2 ± 0.0	15.2 ± 0.0	15.2 ± 0.0	15.2 ± 0.0	15.2 ± 0.0	**15.2 ± 0.0**	15.2 ± 0.0
Caffeic acid	31.3 ± 0.2	198.3 ± 6.0	193.05 ± 2.8	**287.1 ± 7.6**	262.5 ± 1.5	196.9 ± 4.1	194.03 ± 0.52	263.3 ± 4.4	239.6 ± 4.3
Ferulic acid	26.75 ± 0.15	39.4 ± 0.7	38.4 ± 0.2	50.48 ± 1.38	50.28 ± 0.37	38.4 ± 0.34	38.1 ± 0.15	**50.74 ± 0.47**	47.65 ± 4.98
Rosmarinic acid	23.3 ± 0.2	25.3 ± 6.8	21.39 ± 0.02	22.37 ± 0.15	**22.84 ± 0.24**	21.2 ± 0.01	21.36 ± 0.02	22.15 ± 0.69	22.63 ± 0.85
Apigenin	167.5 ± 25.1	24.7 ± 1.3	24.99 ± 0.9	24.79 ± 0.79	25.5 ± 1.3	24.14 ± 0.33	24.52 ± 0.88	**38.2 ± 22**	24.3 ± 1.06
Luteolin	27.5 ± 0.1	96.2 ± 3.8	93.3 ± 0.76	95.2 ± 1.7	96.7 ± 2.6	95.8 ± 2.4	95.01 ± 1.8	**98.8 ± 1.3**	90.7 ± 15
Rutin	39.8 ± 0.7	25.6 ± 0.9	26.8 ± 0.9	22.25 ± 0.01	22.2 ± 0.01	25.8 ± 0.16	**27.2 ± 0.31**	22.2 ± 0.02	22.2 ± 0.04
Quercetin	25.9 ± 0.13	131.14 ± 5.8	127.2 ± 1.1	129.9 ± 2.7	132.2 ± 3.9	131.08 ± 3.7	129.69 ± 2.7	**135.3 ± 1.7**	123.3 ± 23
Kaempferol	16.3 ± 0.02	170 ± 13	167.1 ± 3.0	171.7 ± 3.2	176.7 ± 6.4	173.9 ± 4.2	173.1 ± 3.2	**179.06 ± 2.2**	162.98 ± 31.8

Average concentration of polar ajwain constituents (concentration of each compound in mg/100g ajwain sample, n=3 ± S.D).

Figure 4.6 represent HPLC-DAD chromatogram of non-enzyme treated ajwain (sample 1a) at 278nm wavelength. However, individual analytes were quantified at wavelengths of optimum response, with maxima having previously been established using spectral analysis of reference standards.

Analysis of non-enzymatic ajwain extracts by HPLC-DAD revealed a great complexity and diversity of compounds, obstructing individual identification of compounds (Table 4.9). The compounds identified based on their retention time and absorption spectra in comparison with reference standard compounds.

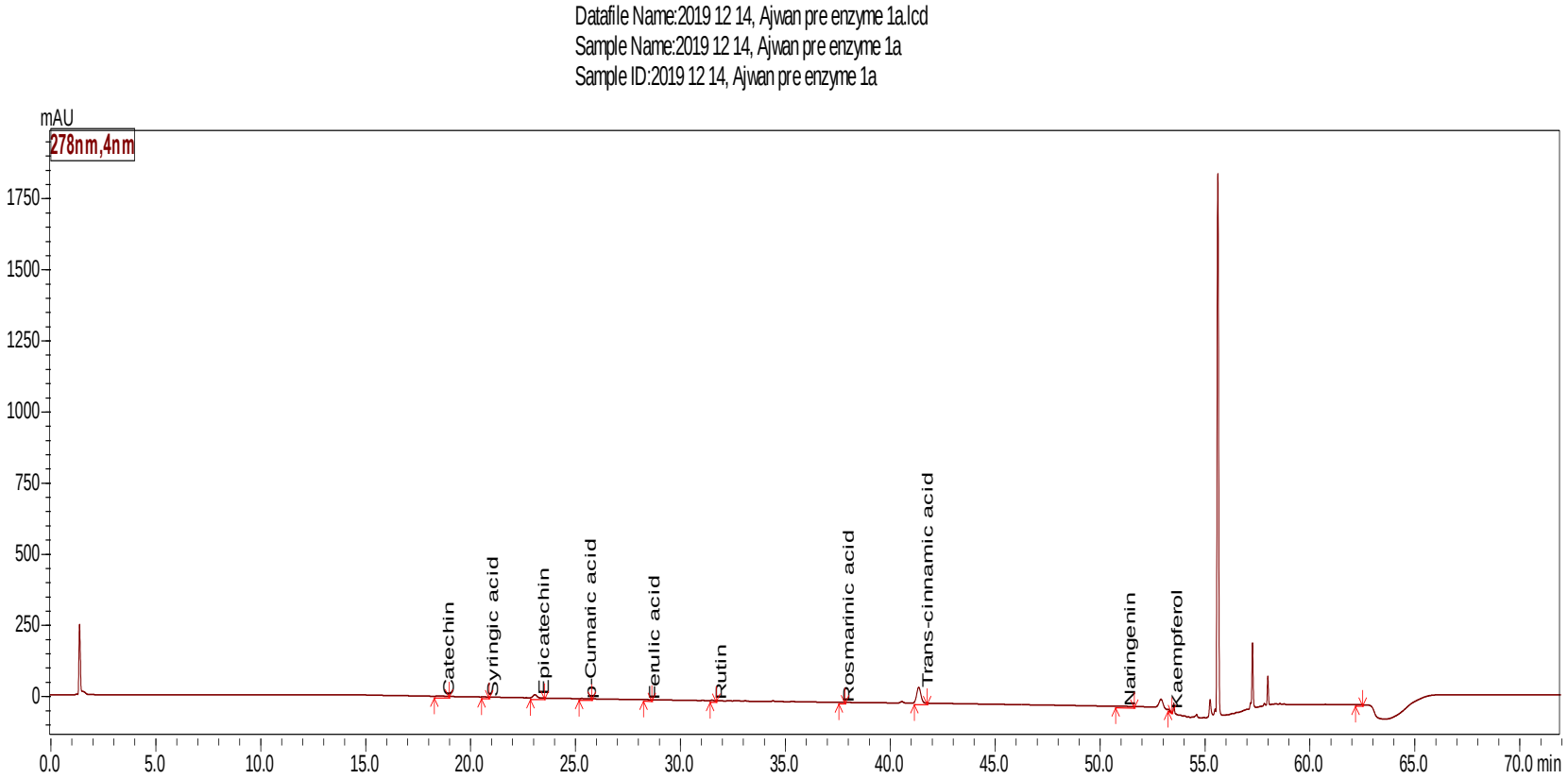


Figure 4.6 HPLC-DAD chromatograms of non-enzyme treated ajwain sample at 278nm wavelength.

Table 4.9 – Analysis of non-enzyme treated ajwain samples (mg/100g ajwain sample, n=3 ± S.D.)

	Non-enzyme treated ajwain samples									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
4-Hydroxy benzoic acid	20.9 ± 0.06	19.7 ± 0.0	21.2 ± 0.04	19.7 ± 0.0	**25.12 ± 0.2**	19.7 ± 0.0	24.5 ± 0.1	21.8 ± 0.2	23 ± 0.4	21.7 ± 0.08
Procatechinic acid	20.6 ± 0.7	19.7 ± 0.0	21.4 ± 0.3	19.7 ± 0.0	**26.1 ± 0.5**	19.7 ± 0.0	24.8 ± 0.1	21.7 ± 0.03	24.6 ± 0.8	20.1 ± 0.7
Gallic Acid	23.4 ± 0.0	23.4 ± 0.0	23.4 ±0.0	23.4 ±0.0	23.4 ± 0.0	23.4 ± 0.0	23.4 ± 0.0	23.4 ± 0.0	23.4 ± 0.0	23.4 ± 0.0
Vanillic acid	25 ± 0.5	27.2 ± 0.5	26.6 ± 0.7	20.5 ± 0.0	**38 ± 0.8**	20.5 ± 0.0	24 ± 0.1	27.6 ± 6.1	24.1 ± 0.3	30 ± 0.2
Syringic acid	20 ± 0.02	21.8 ± 0.12	20.9 ± 0.6	21 ± 0.3	21.3 ± 0.01	19.7 ± 0.0	24.1 ± 0.1	20.8 ± 0.01	23.6 ± 0.4	20.9 ± 0.1
Epigallocatechin Gallate	24.2 ± 0.09	23.5 ± 0.0	23.7 ± 0.3	23.3 ± 0.0	**26.8 ± 0.1**	26.5 ± 0.01	23.6 ± 0.0	23.8 ± 0.0	23.9 ± 0.0	23.9 ± 0.0
Catechin	37 ± 1.0	41.9 ± 1.4	40.8 ± 1.8	23.8 ± 0.0	68.3 ± 2.3	23.8 ± 0.0	87.5 ± 1.5	54.6 ± 0.6	**101.8 ± 13.7**	50.3 ± 0.6
Epicatechin	**92.7 ± 1.7**	22.3 ± 0.0	44.8 ± 4	32.4 ± 2.3	22 ± 1.7	34 ± 0.0	22 ± 0.0	21.9 ± 0.03	22.8 ± 0.3	22.6 ± 0.03
Trans-cinnamic acid	41 ± 3.2	**77 ± 3.8**	57.1 ± 3.0	67.7 ± 1.1	66.1 ± 1.2	71.9 ± 0.7	73.9 ± 0.7	38.8 ± 2.9	73.1 ± 0.2	73.6 ± 0.9
Naringenin	22 ± 1.2	20.8 ± 0.04	21.8 ± 1.1	**44.6 ± 2.1**	20.9 ± 0.0	20.6 ± 0.0	22.1 ± 0.2	20.9 ± 0.1	20.6 ± 0.13	21 ± 0.14
p-Coumaric acid	23.6 ± 0.04	23.1 ± 0.02	23.7 ± 0.12	22.9 ± 0.1	**37.7 ± 0.86**	24.6 ± 0.0	24.4 ± 0.06	23.8 ± 0.01	25.1 ± 0.8	25.2 ± 0.05
Chlorogenic acid	15.6 ± 0.0	21.2 ± 0.15	15.6 ± 0.06	15.2 ± 0.0	**32.7 ± 0.6**	15.2 ± 0.0	15.5 ± 0.01	20 ± 0.01	16.4 ± 0.2	20.8 ± 0.17
Caffeic acid	349.2 ± 0.3	344.9 ± 0.0	349.8 ± 0.2	350.5 ± 0.8	344.9 ± 0.0	344.9 ± 0.0	355.2 ± 0.7	361.3 ± 0.6	353.3 ± 0.8	**363.2 ± 2.3**
Ferulic acid	22.1 ± 0.0	21.9 ± 0.0	22.1 ± 0.0	21.8 ± 0.16	**47 ± 1.2**	21.7 ± 0.0	22.7 ± 0.01	22.5 ± 0.02	22.9 ± 0.1	23.8 ± 0.02
Rosmarinic acid	21.2 ± 0.02	21.2 ± 0.01	21.3 ± 0.06	23.3 ± 0.5	**52.6 ± 1.5**	20.9 ± 0.0	21.4 ± 0.01	21.2 ± 0.01	21.8 ± 0.1	21.2 ± 0.01
Apigenin	210 ± 5.8	222 ± 8.9	246.7 ± 8	138.9 ± 2.7	**435.9 ± 7.7**	22.9 ± 0.9	244 ± 5.4	254.4 ± 1.4	226.4 ± 1.3	224 ± 1.3
Luteolin	24.4 ± 0.09	24.9 ± 0.8	24.3 ± 0.1	**32.7 ± 1.4**	24.5 ± 0.03	23.9 ± 0.0	25.6 ± 0.0	24.6 ± 0.04	24.6 ± 0.07	24.2 ± 0.01
Rutin	25.2 ± 0.01	36.7 ± 0.3	25.7 ± 0.2	23.2 ± 0.2	24.5 ± 0.12	22.2 ± 0.04	30.48 ± 0.18	35.3 ± 0.47	31.1 ± 1.5	**35.6 ± 0.2**
Quercetin	23.2 ± 0.08	23.8 ± 1.3	23.1 ± 0.2	**35.9 ± 2.2**	23.5 ± 0.04	22.6 ± 0.0	25.2 ± 0.02	23.6 ± 0.03	23.5 ± 0.12	22.9 ± 0.01
Kaempferol	31.7 ± 4.9	30.9 ± 1.6	45.7 ± 3.5	20 ± 6.0	**67.4 ± 1.5**	16.2 ± 0.16	35.4 ± 3.0	29.6 ± 0.6	28.7 ± 1.3	26 ± 1.3

Average concentration of polar ajwain constituents in non-enzyme treated samples (concentration of each compound in mg/100g ajwain sample, n=3 ± S.D) from 10 distinct varieties.

Figure 4.7 represent HPLC-DAD chromatogram of enzyme treated ajwain (sample 1a) at 278nm wavelength. However, individual analytes were quantified at wavelengths of optimum response, with maxima having previously been established using spectral analysis of reference standards.

All 10 distinct ajwain samples were enzyme treated according to selected method (number 7) and quantified (Table 4.10).

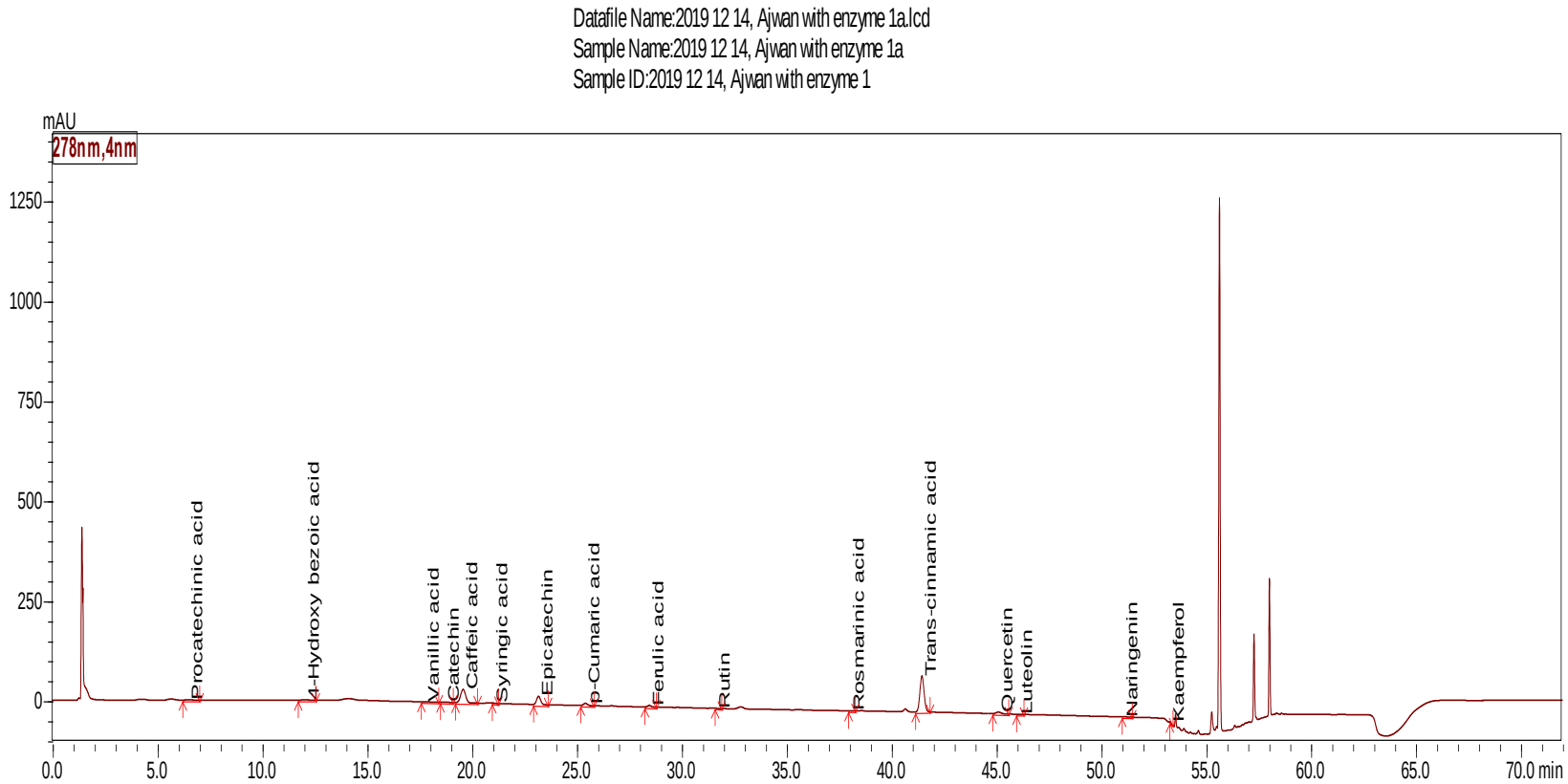


Figure 4.7 HPLC-DAD chromatograms of enzyme treated ajwain sample at 278nm wavelength.

Table 4.10 – Analysis of enzyme treated ajwain samples (mg/100g ajwain sample, n=3 ± S.D.)

Polar Phenolic compounds	Ajwain enzyme treated samples									
	1	2	3	4	5	6	7	8	9	10
4-Hydroxy benzoic acid	24.9 ± 0.2	23.6 ± 0.3	23.3 ± 2.5	21.7 ± 0.1	31.2 ± 0.6	20.7 ± 0.2	33.1 ± 0.5	26.6 ± 0.3	30.8 ± 1.4	25.3 ± 1.2
Procatechinic acid	23.6 ± 0.1	22.6 ± 0.2	22 ± 2.1	22.5 ± 1.2	29.1 ± 0.8	20.2 ± 0.9	28.2 ± 0.4	24.4 ± 0.13	27.3 ± 1.3	22.7 ± 0.6
Gallic Acid	23.4 ± 0.0	23.4 ± 0.0	23.4 ± 0.0	23.4 ± 0.0	23.4 ± 0.0	23.4 ± 0.0	23.4 ± 0.0	23.4 ± 0.0	23.7 ± 0.2	23.4 ± 0.0
Vanillic acid	24.7 ± 0.3	22.6 ± 0.2	22.7 ± 2.0	20.5 ± 0.0	23.8 ± 0.2	20.5 ± 0.0	29.3 ± 0.4	23.9 ± 0.07	30.4 ± 1.1	24.4 ± 1.2
Syringic acid	21.7 ± 0.2	21.5 ± 0.1	20.8 ± 0.9	23.1 ± 0.08	20.6 ± 0.1	19.7 ± 0.0	28.8 ± 0.4	22.5 ± 0.03	28.8 ± 1.3	22.1 ± 0.9
Trans-cinnamic acid	111.2 ± 2.9	105.4 ± 4.8	73.9 ± 3.3	78.6 ± 4.8	22.7 ± 0.2	108.4 ± 1.8	116.9 ± 1.7	111.3 ± 2.3	123 ± 3.0	85 ± 1.8
Chlorogenic acid	15.2 ± 0.0	15.2 ± 0.0	15.2 ± 0.0	15.2 ± 0.0	19.1 ± 0.4	15.2 ± 0.0	15.2 ± 0.0	15.2 ± 0.0	15.2 ± 0.0	15.2 ± 0.0
Caffeic acid	1208 ± 23	1559.9 ± 10.5	809.6 ± 35	732.4 ± 13.4	1152.9 ± 47.9	347.3 ± 4.1	1513 ± 54	1743.3 ± 17	1650.3 ± 14.8	1180.8 ± 28.8
Ferulic acid	25.4 ± 1.5	25.8 ± 0.4	23.3 ± 1.1	24.5 ± 0.11	151.3 ± 7.6	21.6 ± 0.14	26.8 ± 0.1	27 ± 0.16	28.3 ± 0.9	28.9 ± 2.0
Rosmarinic acid	21 ± 0.2	20.9 ± 0.0	21.1 ± 0.2	23.2 ± 1.3	33.4 ± 2.0	21.1 ± 0.05	20.9 ± 0.12	20.9 ± 0.08	21.2 ± 0.01	20.9 ± 0.12
p-Coumaric acid	28.4 ± 1.8	29 ± 0.8	25.4 ± 1.9	25.1 ± 0.07	27.8 ± 2.0	22.9 ± 0.08	28.5 ± 0.2	29 ± 0.17	31.6 ± 2.1	30.2 ± 2.1
Total phenolic acids	**1527.5**	**1879**	**1081.1**	**1010.2**	**1535.4**	**641**	**1864.1**	**2067.5**	**2010.6**	**1478.9**
Epigallocatechin gallate	29.5 ± 2.1	30.2 ± 0.9	26.2 ± 2.3	25.9 ± 0.1	23.8 ± 0.08	23.5 ± 0.1	29.7 ± 0.4	30.1 ± 0.2	32.9 ± 2.1	31.5 ± 2.3
Catechin	30.5 ± 0.7	30.1 ± 0.5	27.6 ± 3.5	23.8 ± 0.0	30.8 ± 1.2	23.8 ± 0.0	50.9 ± 1.3	35.9 ± 0.5	60.2 ± 7.9	30.3 ±3.0
Epicatechin	155.7 ± 7.4	162.2 ± 1.2	102.8 ± 6.6	43.9 ± 0.3	22.3 ± 0.3	21.7 ± 0.6	233.4 ± 2.9	151.7 ± 1.2	348.4 ± 5.5	152.3 ± 4.8
Naringenin	21 ± 0.6	21.4 ± 0.5	20.3 ± 0.0	20.8 ± 0.8	22.2 ± 0.1	20.8 ± 0.1	21.2 ± 0.17	21.3 ± 0.07	21.6 ± 0.1	21.2 ± 0.8
Apigenin	43.3 ± 1.5	71.4 ± 11	34.3 ± 12	26.1 ± 1.5	273.3 ± 31	23.6 ±0.5	96.8 ± 3.7	90.7 ± 3.3	115.5 ± 20.8	70.12 ± 23
Luteolin	28.3 ± 0.9	35.4 ± 1.1	26.3 ± 1.7	26.6 ± 0.08	76.3 ± 5.4	23.9 ± 0.0	35.5 ± 0.6	36.1 ± 0.3	35.4 ± 2.3	31.4 ± 3.2
Rutin	22.3 ± 0.1	22.4 ± 0.1	22.5 ± 0.5	24 ± 3.2	25.5 ± 0.7	22 ± 0.0	22.3 ± 0.02	22.2 ± 0.02	22.2 ± 0.1	22.2 ± 0.09
Quercetin	29 ± 1.3	39.8 ± 1.7	26 ± 2.6	25.6 ± 0.05	101.4 ± 8.3	22.8 ± 0.1	39.7 ± 0.9	40.7 ± 0.5	39.6 ± 3.4	22.9 ± 4.8
Kaempferol	31.8 ± 1.5	41.5 ± 5.1	23.2 ± 6.3	16.5 ± 0.7	25.7 ± 1.7	16.4 ± 0.08	49.4 ± 1.6	46.2 ± 1.8	56.7 ± 8.6	38.7 ± 9.0
Total flavonoids	**391.4**	**454.4**	**309.2**	**233.2**	**601.3**	**198.5**	**578.9**	**474.9**	**732.5**	**420.6**
Total phytochemicals	**1918.9**	**2324.4**	**1390.3**	**1243.4**	**2136.7**	**839.5**	**2443**	**2542.4**	**2743.1**	**1899.5**

Average concentration of polar ajwain constituents in enzyme treated samples (concentration in mg/100g ajwain sample, n=3 ± S.D) from 10 distinct varieties.

4.4.7 Correlation between antimicrobial properties and non-polar bioactive compounds

The major non-polar bioactive compounds identified by GCMS in ajwain samples were thymol, p-cymene, and r-terpinene. In the correlation analysis, the bioactive compounds concentration determined were compared with antimicrobial properties of ajwain samples against foodborne pathogenic microorganisms. Out of all major non-polar compunds identified, thymol was found in most abundant compound and has been previously reported to have antimicrobial effect selected for correlation with antimicrobial properties. The results obtained in correlation analysis of thymol concentration and antimicrobial properties are presented in Table 4.11.

Table 4.11- Correlation between antimicrobial properties (ZoI) and thymol of ajwain samples

Ajwain samples	mg thymol/100g ajwain powder	Antimicrobial properties (Zone of Inhibition in mm)					
		Bacillus	***Listeria***	***Kocuria***	***E.coli***	***Salmonella***	***Pseudomonas***
1	302.26	18	14	14	14	12	9
2	278.67	9	14	16	9	9	9
3	274.08	9	12	14	9	9	14
4	112.61	9	6	6	9	9	9
5	0	12	12	15	12	9	9
6	5.39	9	6	6	10	9	4
7	301.98	12	6	6	14	14	9
8	274.69	9	6	6	12	12	9
9	315.76	15	14	14	14	14	14
10	337.09	12	15	14	12	14	12

Zone of Inhibition measured in mm. Disc diameter 6mm excluded from the readings. Mean of n=3 replicates ± S.D. (0 – Not detected in the sample).

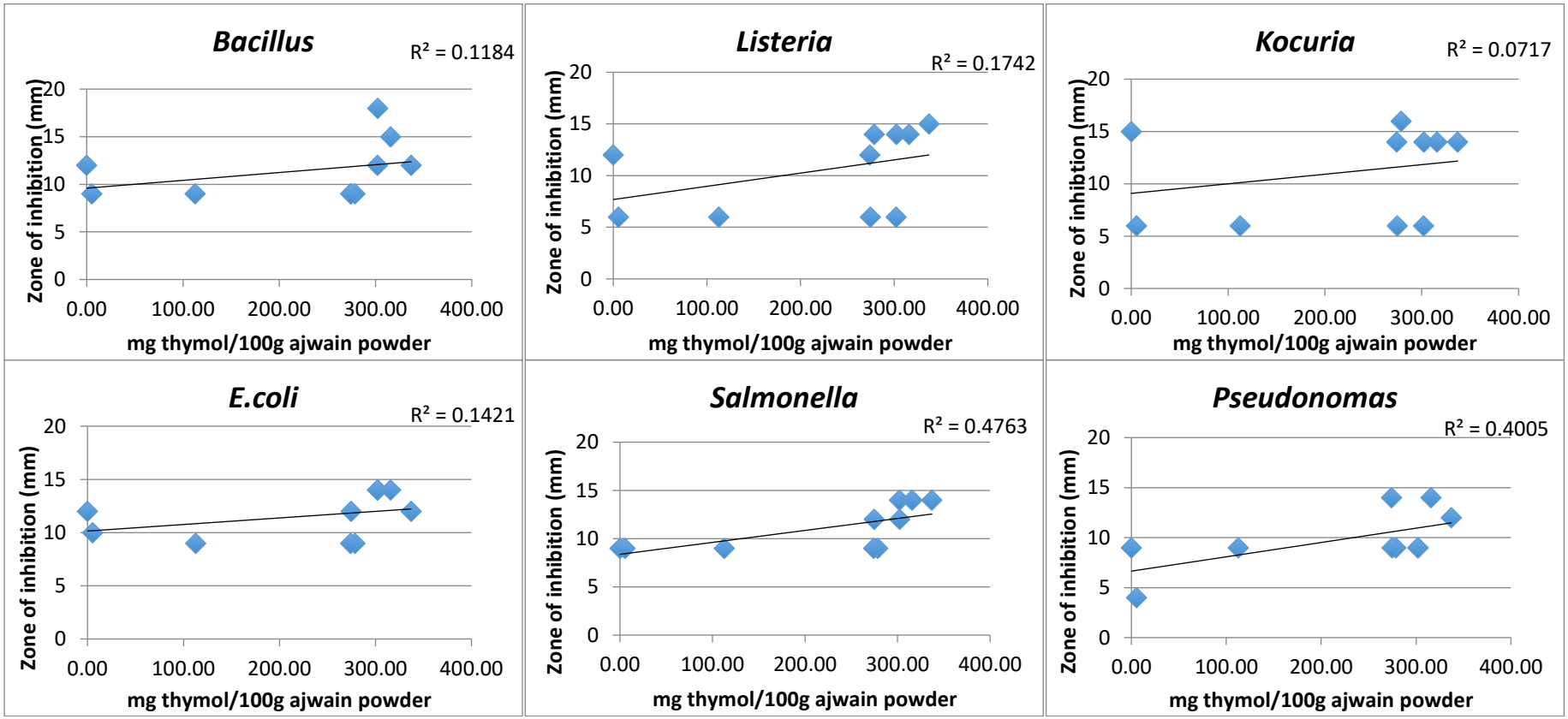


Figure 4.8 – Effect of thymol of ajwain ethanolic extracts on antimicrobial properties of ajwain extracts against different microorganisms

The correlation coefficient (R^2) was determined for each correlation. The highest correlation was found for *Salmonella* and *Pseudomonas* as compared to other microorganisms. When thymol concentration and antimicrobial properties of 10 distinct ajwain samples were compared, poor correlation for all samples were observed in Figure 4.8 against tested microorganisms. However, no such direct correlation was exhibited against all tested pathogenic microbes. Ajwain sample 5 and 6 showed higher microbial inhibition whereas thymol was not detected in the sample.

2. Correlation between antimicrobial properties (ZoI) and total non-polar bioactive compounds

Total bioactive compounds identified in ajwain samples by GCMS analysis were added and combined effect on antimicrobial properties were studied. The results obtained in correlation analysis of total non-polar bioactive concentration and antimicrobial properties are presented in Table 4.12.

Table 4.12 - Correlation between antimicrobial properties (ZoI) and total non-polar bioactive compounds

Ajwain samples	mg total bioactive compound/100 g ajwain sample	Zone of Inhibition (mm)					
		Bacillus	***Listeria***	***Kocuria***	***E.coli***	***Salmonella***	***Pseudomonas***
1	536.92	18	14	14	14	12	9
2	418.13	9	14	16	9	9	9
3	497.19	9	12	14	9	9	14
4	476.76	9	6	6	9	9	9
5	62.58	12	12	15	12	9	9
6	5.39	9	6	6	10	9	4
7	556.49	12	6	6	14	14	9
8	473.41	9	6	6	12	12	9
9	670.16	15	14	14	14	14	14
10	665.67	12	15	14	12	14	12

Zone of Inhibition measured in mm. Disc diameter 6mm excluded from the readings. Mean of n=3 replicates ± S.D.

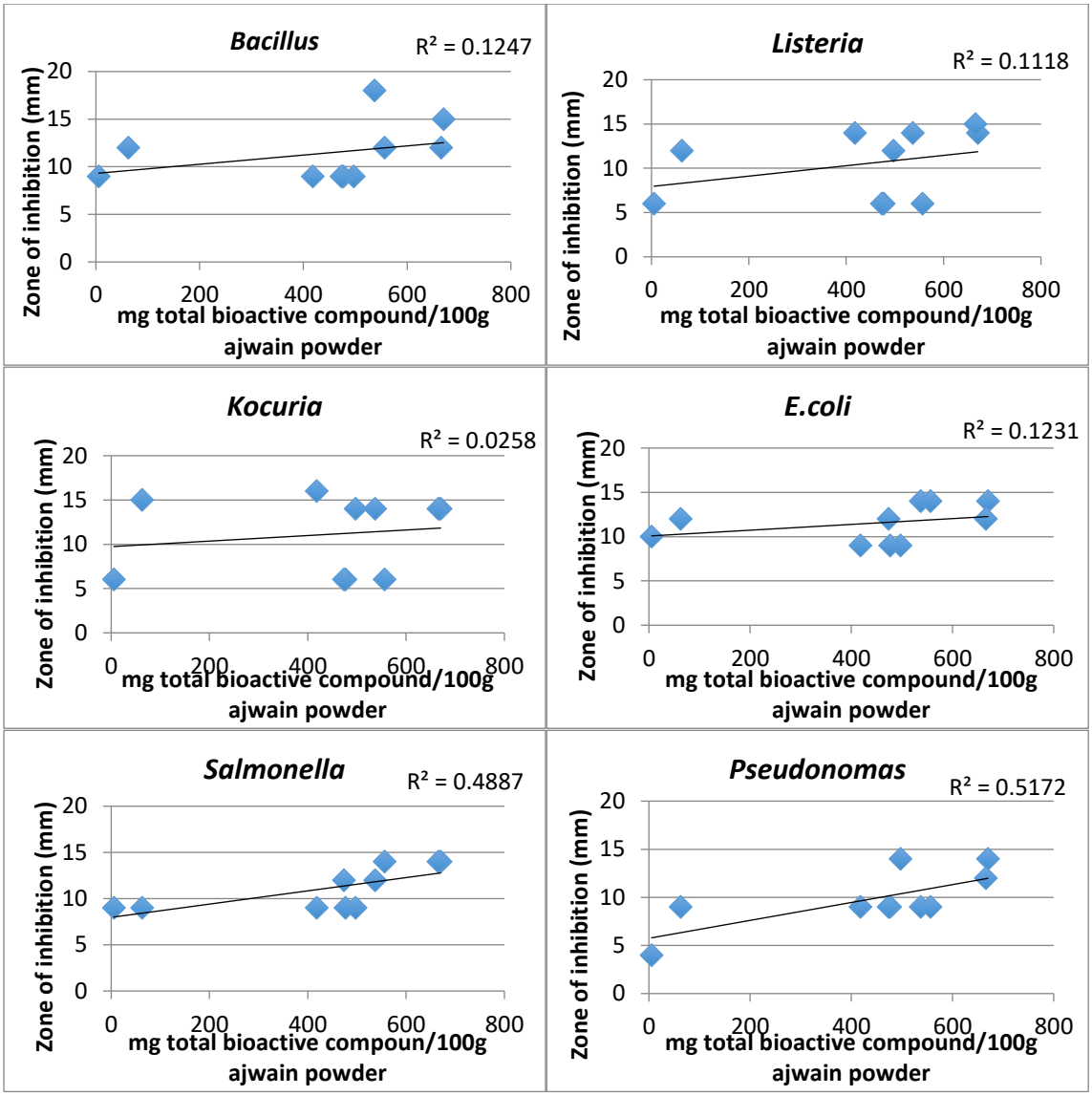


Figure 4.9 – Effect of total non-polar bioactive compounds of ajwain ethanolic extracts on antimicrobial properties of ajwain extracts against different microorganisms

The largest correlations identified for *Salmonella* and *Pseudomonas* as compared to other microorganisms. When total bioactive concentration and antimicrobial properties of 10 distinct ajwain samples were compared, poor correlation against tested pathogenic microbes were observed in Figure 4.9. Ajwain sample number 5 and 6 showed very little amount of non-polar bioactive compound concentration and effective inhibition against pathogenic microorganisms. Therefore, there is need to identify polar compounds in ajwain extracts, which might be responsible for antimicrobial inhibition and possible justification of research question. It was assumed that some other bioactive compound might be responsible for antimicrobial action or these compounds act synergistically with other compounds.

Comparative data showing concentration of non-polar bioactive compounds in 10 different varities of ajwain samples (mg compound/100g ajwain powder) in Table 4.13. Effect of non-polar phytochemical compounds of ajwain ethanolic extracts on antimicrobial properties of ajwain extracts against different microorganisms (values represent R^2 cofficient) in Table 4.14.

Table 4.13 – Concentration of non-polar bioactive compounds in 10 different varities of ajwain samples (mg compound/100g ajwain powder)

Ajwain sample	ρ-cymene	ϒ-terpinene	Thymol	Total non-polar compounds
1	67	164	302	537
2	38	87	278	418
3	56	159	274	497
4	75	269	112	476
5	44	0	0	62
6	0	0	5	5
7	7	232	302	556
8	52	128	274	473
9	79	255	315	670
10	75	226	337	665

Table 4.14 - Effect of non-polar phytochemical compounds of ajwain ethanolic extracts on antimicrobial properties of ajwain extracts against different microorganisms (values represent R^2 cofficient)

	Bacillus	***Listeria***	***Kocuria***	***E.coli***	***Salmonella***	***Pseudomonas***
ρ-cymene	0.2169	0.2858	0.1887	0.2001	0.3806	**0.5132**
ϒ-terpinene	0.0709	0.0031	0.013	0.0752	0.3642	0.3466
Thymol	0.1184	0.1742	0.0717	0.1421	0.4763	0.4005
Total non-polar compound	0.1247	0.1118	0.0258	0.1231	0.4887	**0.5172**

The largest correlations identified for *Pseudomonas* followed by *Salmonella* as compared to other microorganisms. Interestingly, ρ-cymene showed good correlations as compared to thymol or total non-polar compound against selected microorganisms.

4.4.8 Analysis of aglycones after enzyme hydrolysis of glycosides

To overcome complexity of polyphenol analysis, the ajwain extracts were incubated with hesperinidase enzyme and cellulase. Hydrolysis conditions and enzyme incubation time was optimised by using combination of enzymes were performed. First, different combination of enzymes were incubated with ajwain extracts and production of aglycones was compared. A combination of enzyme incubated for 24 hours, proved to be more efficient to obtain a higher degree of aglycone than any other enzyme combination of ajwain extracts. HPLC-DAD chromatograms of ajwain samples were far less comlex after hydrolysis (enzyme treatment on the samples). Aglycone of hydrolysed ajwain sample were identified and confirmed with reference standard compounds.

These hydrolysed ajwain samples showed number of polar phenolic compounds in the samples. Some of the compounds were in high concentration as compared to others. Polar phenolic compounds such as caffeic acid, trans-cinnamic acid, and flavonoids such as, epicatechin, apigenin, luteolin, quercetin and kampferol was found in abundant compound in all ajwain samples. It was assumed that these compounds might be responsible for antimicrobial effect in ajwain samples. The correlation between these compounds and antimicrobial properties were studied. However, compounds such as epicatechin, total phenolic acid and total flavonoids correlations were presented, and all the rest of the correlations were presented in tables. The correlation between caffeic acid, trans-cinnamic acid and quercitin are presented in appendix C. When caffeic acid concentration and antimicrobial properties of 10 distinct ajwain samples were compared, no direct correlation against all tested pathogenic microbes were observed. Similarly, the correlation between trans-cinnamic acid and quercitin shows no direct correlation against tested microoganisms. It was assumed that some other polar phenolic compound might be responsible for antimicrobial action or these compounds act synergistically with other compounds. The correlation with other bioactive compounds presented in comparative tables (Table 4.16,4.17 and 4.21, 4.22).

4.4.9 Correlation between antimicrobial properties and polar phenolic compounds

1. Total polar phenolic acid

In ajwain samples, compounds identified by HPLC analysis were combined and total polar phenolic compound were studied. The results obtained in correlation analysis of total phenolic acid (mg/100g ajwain powder) and antimicrobial properties are presented in Table 4.15.

Table 4.15 - Correlation between antimicrobial properties (ZoI) and total phenolic acid of ajwain samples

Ajwain sample	mg total phenolic acid/100g ajwain powder	Antimicrobial inhibition (Zone of inhibition in mm)					
		Bacillus	***Listeria***	***Kocuria***	***E.coli***	***Salmonella***	***Pseudomonas***
1	1527	18	14	14	14	12	9
2	1870	9	14	16	9	9	9
3	1081	9	12	14	9	9	14
4	1010	9	6	6	9	9	9
5	1535	12	12	15	12	9	9
6	641	9	6	6	10	9	4
7	1864	12	6	6	14	14	9
8	2067	9	6	6	12	12	9
9	2010	15	14	14	14	14	14
10	1479	12	15	14	12	14	12

Zone of Inhibition measured in mm. Disc diameter 6mm excluded from the readings. Mean of n=3 replicates ± S.D.

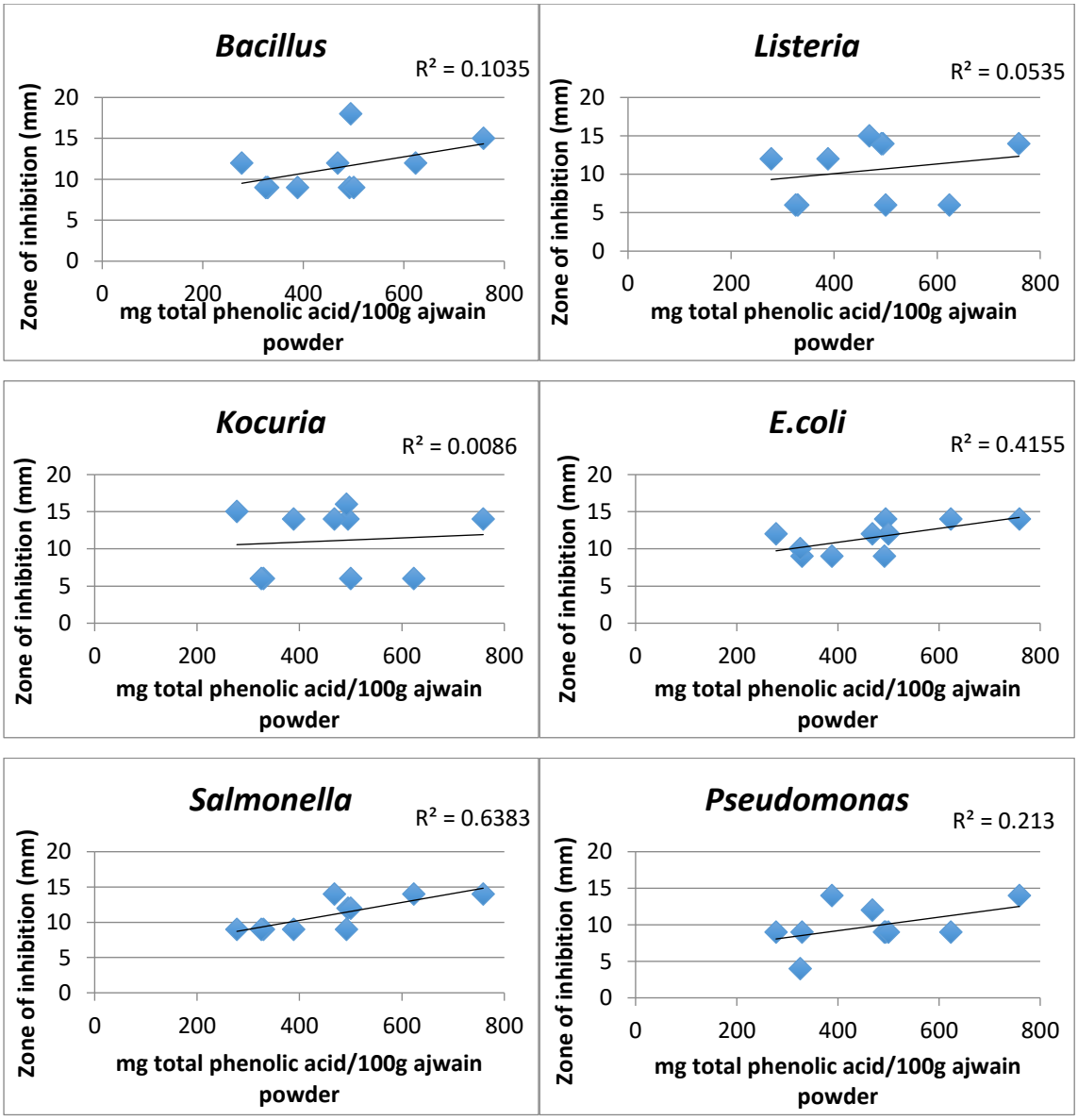


Figure 4.10- Effect of total phenolic acid of ajwain ethanolic extracts on antimicrobial properties of ajwain extracts against different microorganisms

When total phenolic acid concentration and antimicrobial properties of 10 distinct ajwain samples were compared, some good correlation against tested gram-negative pathogenic microbes were observed in Figure 4.10. Ajwain sample 5 showed less concentration of total phenolic acid as compared to other ajwain samples. It was assumed that some other polar phenolic compound might be responsible for antimicrobial action or these compounds act synergistically with other compounds.

Table 4.16 – Concentration of polar phenolic acid compounds in 10 different varities of ajwain samples (mg phenolic acid compound/100g ajwain powder)

Ajwain sample	4-Hydroxy benzoic acid	Procatechinic acid	Gallic Acid	Vanillic acid	Syringic acid	Trans-cinnamic acid	Chlorogenic acid	Caffeic acid	Ferulic acid	Rosmarinic acid	p-Coumaric acid	Total phenolic acids
1	24.9	23.6	23.4	24.7	21.7	111.2	15.2	1208	25.4	21	28.4	**1527**
2	23.6	22.6	23.4	22.6	21.5	105.4	15.2	1560	25.8	20.9	29	**1870**
3	23.3	22	23.4	22.7	20.8	73.9	15.2	810	23.3	21.1	25.4	**1081**
4	21.7	22.5	23.4	20.5	23.1	78.6	15.2	732.4	24.5	23.2	25.1	**1010**
5	31.2	29.1	23.4	23.8	20.6	22.7	19.1	1153	151.3	33.4	27.8	**1535**
6	20.7	20.2	23.4	20.5	19.7	108.4	15.2	347.3	21.6	21.1	22.9	**641**
7	33.1	28.2	23.4	29.3	28.8	116.9	15.2	1513	26.8	20.9	28.5	**1864**
8	26.6	24.4	23.4	23.9	22.5	111.3	15.2	1743.3	27	20.9	29	**2067**
9	30.8	27.3	23.7	30.4	28.8	123	15.2	1650.3	28.3	21.2	31.6	**2010**
10	25.3	22.7	23.4	24.4	22.1	85	15.2	1180.8	28.9	20.9	30.2	**1479**

Table 4.17 - Effect of polar phenolic acid compounds of ajwain ethanolic extracts on antimicrobial properties of ajwain extracts against different microorganisms (values represent R^2 cofficient)

	Bacillus	*Listeria*	*Kocuria*	*E.coli*	*Salmonella*	*Pseudomonas*
4-Hydroxy benzoic acid	0.1845	0.0109	0.0135	**0.567**	0.341	0.1379
Procatechinic acid	0.17	0.0089	0.0172	0.4493	0.1699	0.1177
Gallic Acid	0.1667	0.0869	0.0528	0.1715	0.1911	0.1699
Vanillic acid	0.3537	0.0471	0.0222	**0.6822**	**0.6703**	0.2424
Syringic acid	0.1229	0.0132	0.0329	0.3763	**0.5224**	0.1572
Trans-cinnamic acid	0.039	0.0201	0.0968	0.0986	0.2675	0.0042
Chlorogenic acid	0.0046	0.038	0.0955	0.0069	0.1002	0.0012
Caffeic acid	0.0896	0.0682	0.0518	0.2976	0.35	0.1487
Ferulic acid	0.0082	0.0461	0.105	0.0136	0.074	0.0006
Rosmarinic acid	0.0006	0.0164	0.0586	0.0005	0.1427	0.0016
p-Coumaric acid	0.2703	0.2752	0.211	0.4055	**0.5394**	0.4032
Total phenolic acids	0.1035	0.0673	0.0536	0.3301	0.3513	0.1371

4.4.10 Correlation between antimicrobial properties and polar flavonoids compounds

1. Epicatechin

After hydrolysis of ajwain samples, highest concentration of epicatechin was found as compared to others phenolic compounds. It was assumed that, Epicatechin might be responsible for antimicrobial effect in ajwain samples. Hence, it was selected for correlation with antimicrobial properties. The results obtained in correlation analysis of epicatechin (mg/100g ajwain powder) and antimicrobial properties are presented in Table 4.18.

Table 4.18: Correlation between antimicrobial properties (ZoI) and epicatechin of ajwain samples

Ajwain sample	mg Epicatechin/100g ajwain powder	Antimicrobial inhibition (Zone of Inhibition in mm)					
		Bacillus	***Listeria***	***Kocuria***	***E.coli***	***Salmonella***	***Pseudomonas***
1	155.7	18	14	14	14	12	9
2	162.2	9	14	16	9	9	9
3	102.8	9	12	14	9	9	14
4	43.9	9	6	6	9	9	9
5	22.3	12	12	15	12	9	9
6	21.7	9	6	6	10	9	4
7	233.4	12	6	6	14	14	9
8	151.7	9	6	6	12	12	9
9	348.4	15	14	14	14	14	14
10	152.3	12	15	14	12	14	12

Zone of Inhibition measured in mm. Disc diameter 6mm excluded from the readings. Mean of n=3 replicates ± S.D.

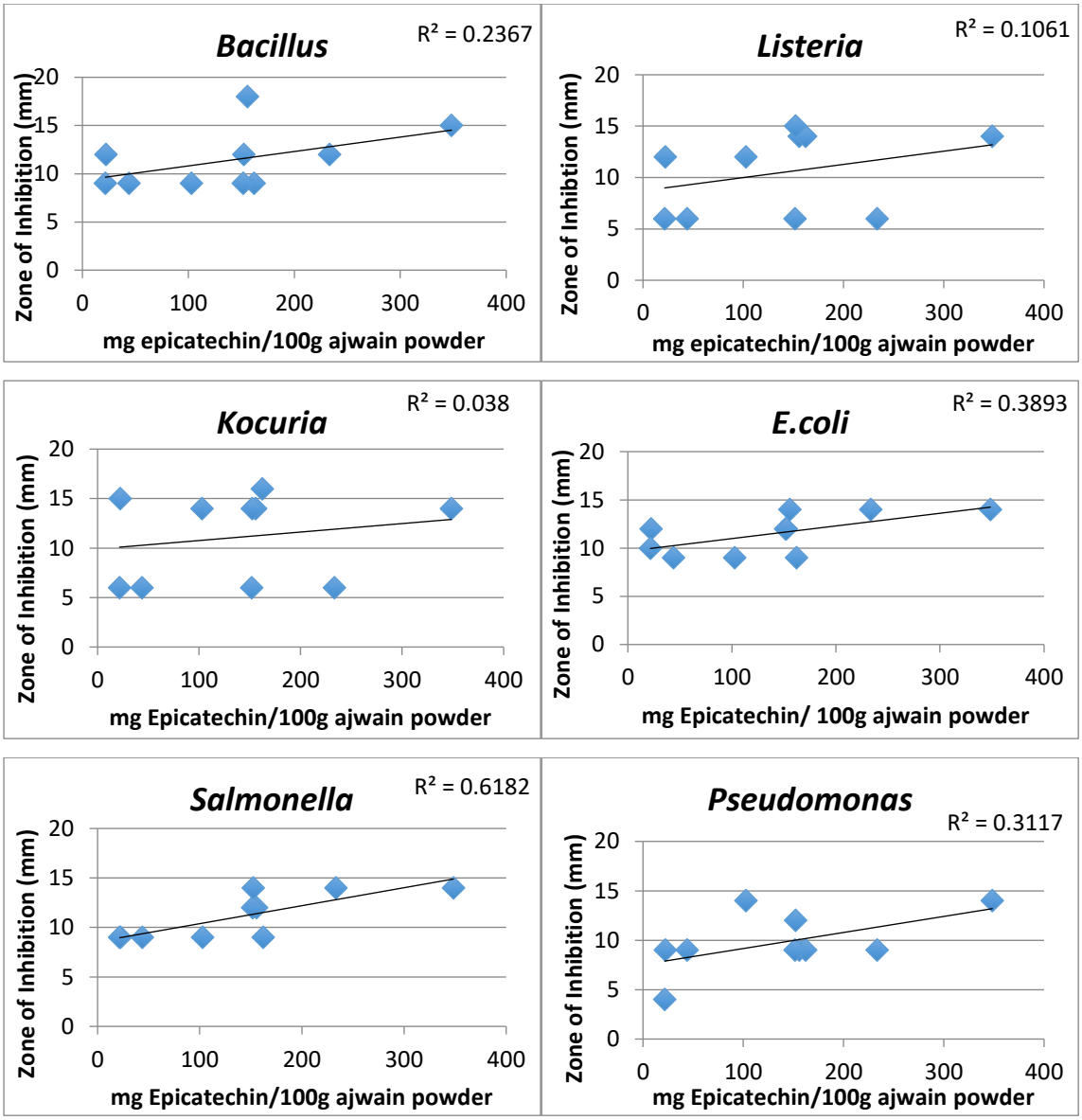


Figure 4.11 - Effect of epicatechin of ajwain ethanolic extracts on antimicrobial properties of ajwain extracts against different microorganisms

The highest correlation identified for *Salmonella* followed by *E.coli* and *Pseudomonas* as compared to other microorganisms. When epicatechin concentration and antimicrobial properties of 10 distinct ajwain samples were compared, good correlation against all tested pathogenic microbes were observed in Figure 4.10 except for ajwain sample 4, 5 and 6. It was assumed that individual polar phenolic compound might not be responsible for total antimicrobial action or these compounds act synergistically with other compounds.

2. Total polar flavonoids

In ajwain samples, compounds identified by HPLC analysis were combined and total flavonoid compounds were studied. The results obtained in correlation analysis of total flavonoids (mg/100g ajwain powder) and antimicrobial properties are presented in Table 4.19.

Table 4.19: Correlation between antimicrobial properties (ZoI) and flavonoids of ajwain samples

Ajwain sample	mg total flavonoids/100g ajwain powder	Antimicrobial inhibition (Zone of inhibition in mm)					
		Bacillus	***Listeria***	***Kocuria***	***E.coli***	***Salmonella***	***Pseudomonas***
1	391	18	14	14	14	12	9
2	454	9	14	16	9	9	9
3	309	9	12	14	9	9	14
4	233	9	6	6	9	9	9
5	601	12	12	15	12	9	9
6	198	9	6	6	10	9	4
7	579	12	6	6	14	14	9
8	475	9	6	6	12	12	9
9	732	15	14	14	14	14	14
10	420	12	15	14	12	14	12

Zone of Inhibition measured in mm. Disc diameter 6mm excluded from the readings. Mean of n=3 replicates ± S.D.

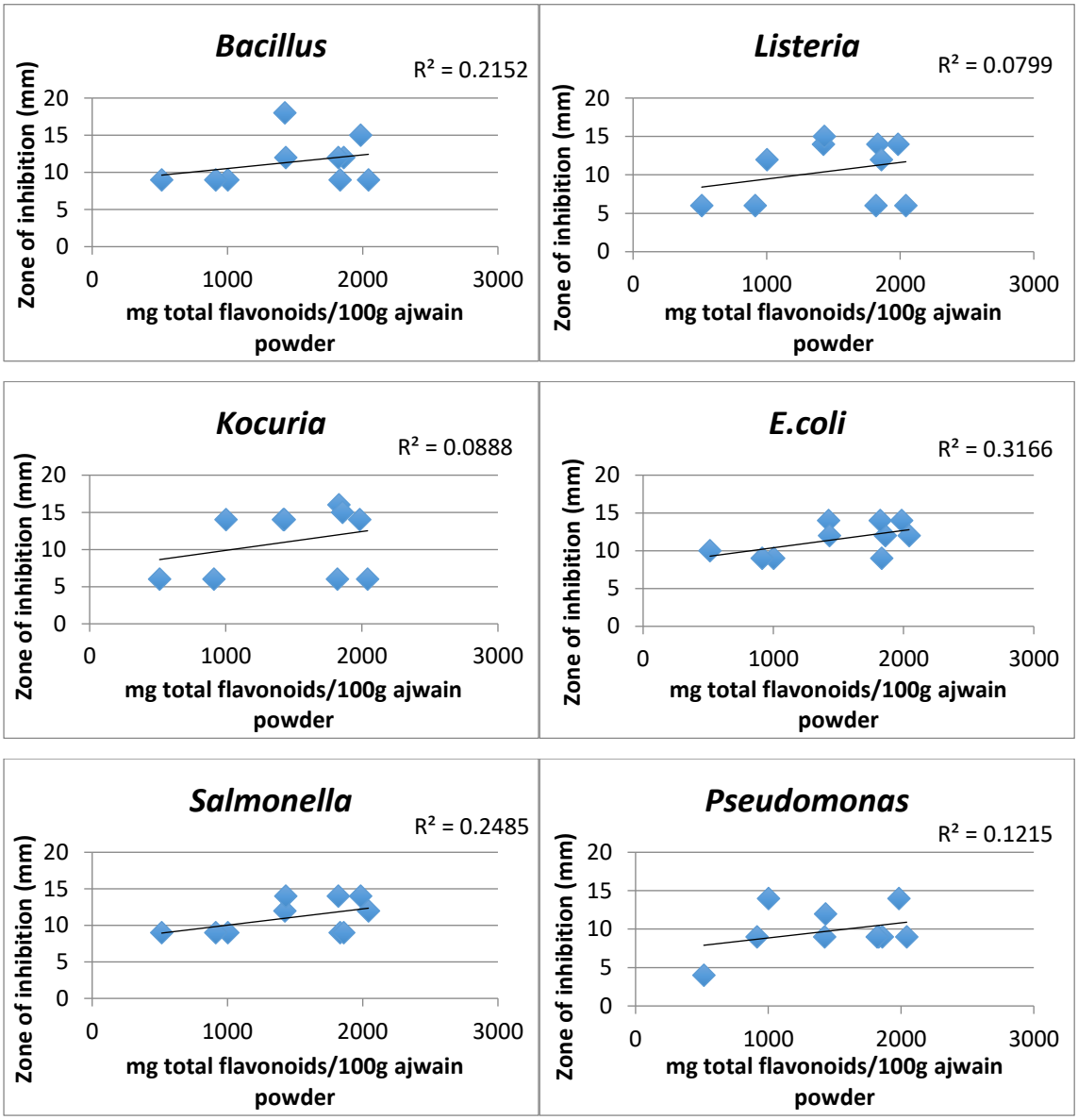


Figure 4.12 - Effect of total flavonoids of ajwain ethanolic extracts on antimicrobial properties of ajwain extracts against different microorganisms

When total flavonoid concentration and antimicrobial properties of 10 distinct ajwain samples were compared, some correlation against tested gram-negative pathogenic microbes were observed in Figure 6. Ajwain sample 6 showed less concentration of total flavonoid as compared to other ajwain samples. It was assumed that some other polar phenolic compound might be responsible for antimicrobial action or these compounds act synergistically with other compounds.

3. Total polar phytochemical compounds (phenolic acids and flavonoids)

In ajwain samples, the concentration of all phenolic acids and flavonoid compounds identified by HPLC analysis were combined and total polar phytochemical compounds were studied. The results obtained in correlation analysis of total polar phytochemical compounds (mg/100g ajwain powder) and antimicrobial properties are presented in Table 4.20.

Table 4.20: Correlation between antimicrobial properties (ZoI) and total polar phytochemical compounds of ajwain samples

Ajwain sample	mg total phenolic compound/100g ajwain powder	Antimicrobial inhibition (Zone of inhibition in mm)					
		Bacillus	***Listeria***	***Kocuria***	***E.coli***	***Salmonella***	***Pseudomonas***
1	1918.9	18	14	14	14	12	9
2	2324.4	9	14	16	9	9	9
3	1390.3	9	12	14	9	9	14
4	1243.4	9	6	6	9	9	9
5	2136.7	12	12	15	12	9	9
6	839.5	9	6	6	10	9	4
7	2443	12	6	6	14	14	9
8	2542.4	9	6	6	12	12	9
9	2743.1	15	14	14	14	14	14
10	1899.52	12	15	14	12	14	12

Zone of Inhibition measured in mm. Disc diameter 6mm excluded from the readings. Mean of n=3 replicates ± S.D.

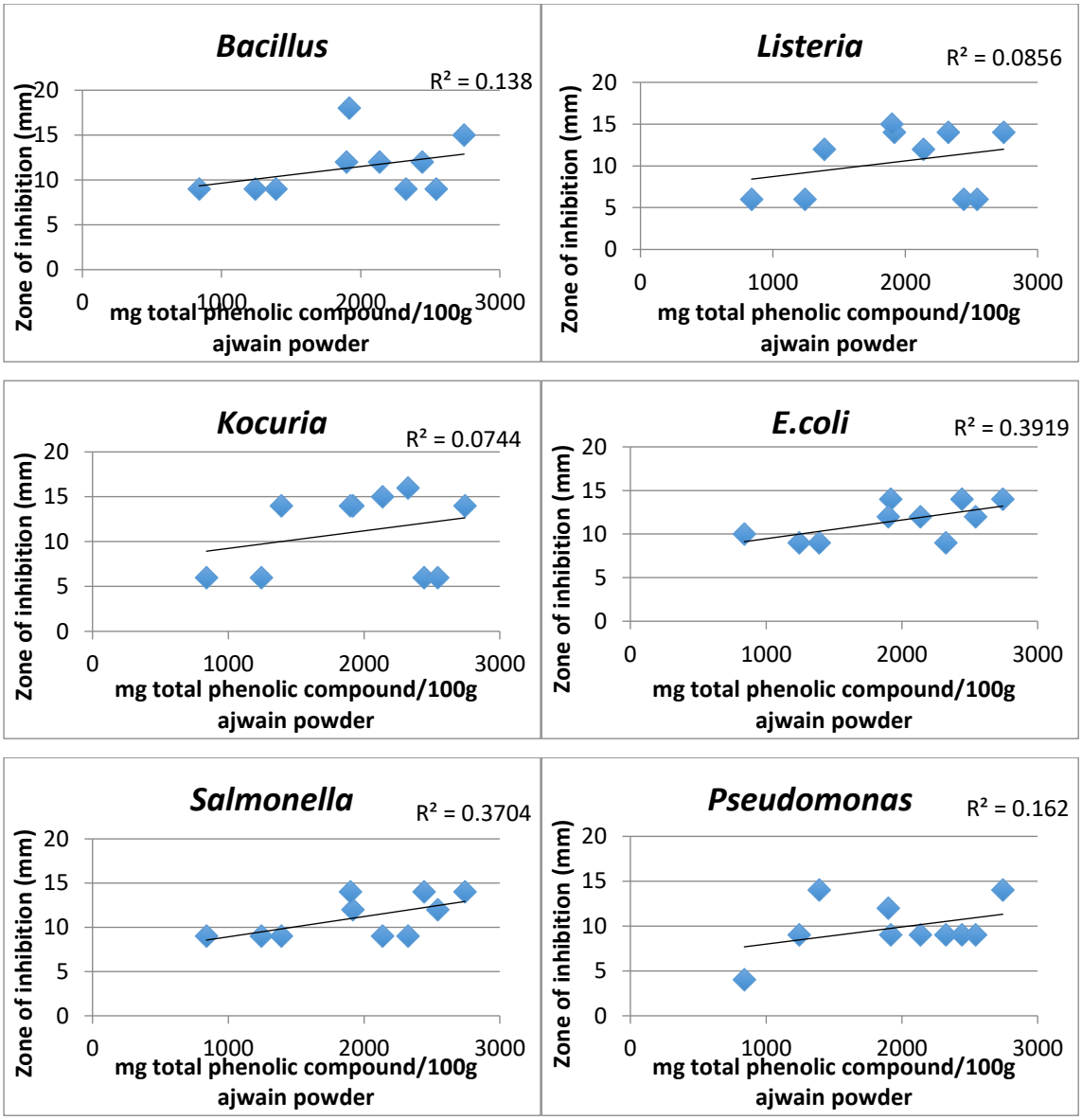


Figure 4.13- Effect of total polar phenolic compounds of ajwain ethanolic extracts on antimicrobial properties of ajwain extracts against different microorganisms

The data points appeared closer to linearity and R^2 value of *E.coli* and *Salmonella* was found to be 0.39 and 0.37 (closer than for any other microorganisms). When total phytochemical compounds concentration and antimicrobial properties of 10 distinct ajwain samples were compared, good correlation compared to individual compound correlation was observed against all tested Gram-negative pathogenic microbes than Gram-positive pathogens in Figure 4.13. Ajwain sample 6 showed less concentration of total phenolic compounds and less antimicrobial inhibition properties as compared to other ajwain samples.

Table 4.21 – Concentration of polar bioactive flavonoid compounds in 10 different varities of ajwain samples (mg compound/100g ajwain powder)

	Epigallocatechin Gallate	Catechin	Epicatechin	Naringenin	Apigenin	Luteolin	Rutin	Quercetin	Kaempferol	Total flavonoids	Total polar compd
1	29.5	30.5	155.7	21	43.3	28.3	22.3	29	31.8	391	**1918**
2	30.2	30.1	162.2	21.4	71.4	35.4	22.4	39.8	41.5	454	**2324**
3	26.2	27.6	102.8	20.3	34.3	26.3	22.5	26	23.2	309	**1390**
4	25.9	23.8	43.9	20.8	26.1	26.6	24	25.6	16.5	233	**1243**
5	23.8	30.8	22.3	22.2	273.3	76.3	25.5	101.4	25.7	601	**2137**
6	23.5	23.8	21.7	20.8	23.6	23.9	22	22.8	16.4	198	**839**
7	29.7	50.9	233.4	21.2	96.8	35.5	22.3	39.7	49.4	579	**2443**
8	30.1	35.9	151.7	21.3	90.7	36.1	22.2	40.7	46.2	475	**2542**
9	32.9	60.2	348.4	21.6	115.5	35.4	22.2	39.6	56.7	732	**2743**
10	31.5	30.3	152.3	21.2	70.12	31.4	22.2	22.9	38.7	420	**1899**

Table 4.22 - Effect of polar flavonoid compounds of ajwain ethanolic extracts on antimicrobial properties of ajwain extracts against different microorganisms (values represent R^2 cofficient)

	Bacillus	*Listeria*	*Kocuria*	*E.coli*	*Salmonella*	*Pseudomonas*
Epigallocatechin Gallate	0.1756	0.13	0.0615	0.265	**0.6357**	0.3228
Catechin	0.1852	0.0066	0.0001	0.5117	**0.5358**	0.1725
Epicatechin	0.2367	0.1061	0.038	0.3893	**0.6182**	0.3117
Naringenin	0.0811	0.0671	0.0981	0.2012	0.0387	0.0042
Apigenin	0.0336	0.0384	0.1095	0.1228	0.0007	0.0073
Luteolin	0.0111	0.0499	0.1117	0.0487	0.0171	0.0074
Rutin	0.0057	0.0008	0.0203	0.024	0.2196	0.0007
Quercetin	0.0087	0.0127	0.0874	0.0416	0.0368	0.002
Kaempferol	0.1222	0.0426	0.0229	0.412	**0.6133**	0.2136
Total flavonoids	0.2152	0.1212	0.1249	0.4787	0.3326	0.1957
Total polar compd	0.138	0.0856	0.0744	0.3919	0.3704	0.162

4.4.11 Correlation between antimicrobial properties ans total phytochemical compound in ajwain extract (non-polar and polar)

Total non-polar bioactive compound identified using GCMS and total polar phenolic compound identified using HPLC were combined and studied for correlation with antimicrobial effect. The results obtained in correlation analysis of total phytochemical compounds (mg/100g ajwain powder) and antimicrobial properties are presented in Table 4.23.

Table 4.23: Correlation between antimicrobial properties (ZoI) and total non-polar and polar phytochemical compounds of ajwain samples

Ajwain sample	mg total phytochemical compound/100g ajwain powder	Antimicrobial inhibition (Zone of inhibition in mm)					
		Bacillus	***Listeria***	***Kocuria***	***E.coli***	***Salmonella***	***Pseudomonas***
1	2455.82	18	14	14	14	12	9
2	2742.53	9	14	16	9	9	9
3	1887.49	9	12	14	9	9	14
4	1720.16	9	6	6	9	9	9
5	2199.28	12	12	15	12	9	9
6	844.89	9	6	6	10	9	4
7	2999.49	12	6	6	14	14	9
8	3015.81	9	6	6	12	12	9
9	3413.26	15	14	14	14	14	14
10	2565.19	12	15	14	12	14	12

Zone of Inhibition measured in mm. Disc diameter 6mm excluded from the readings. Mean of n=3 replicates ± S.D.

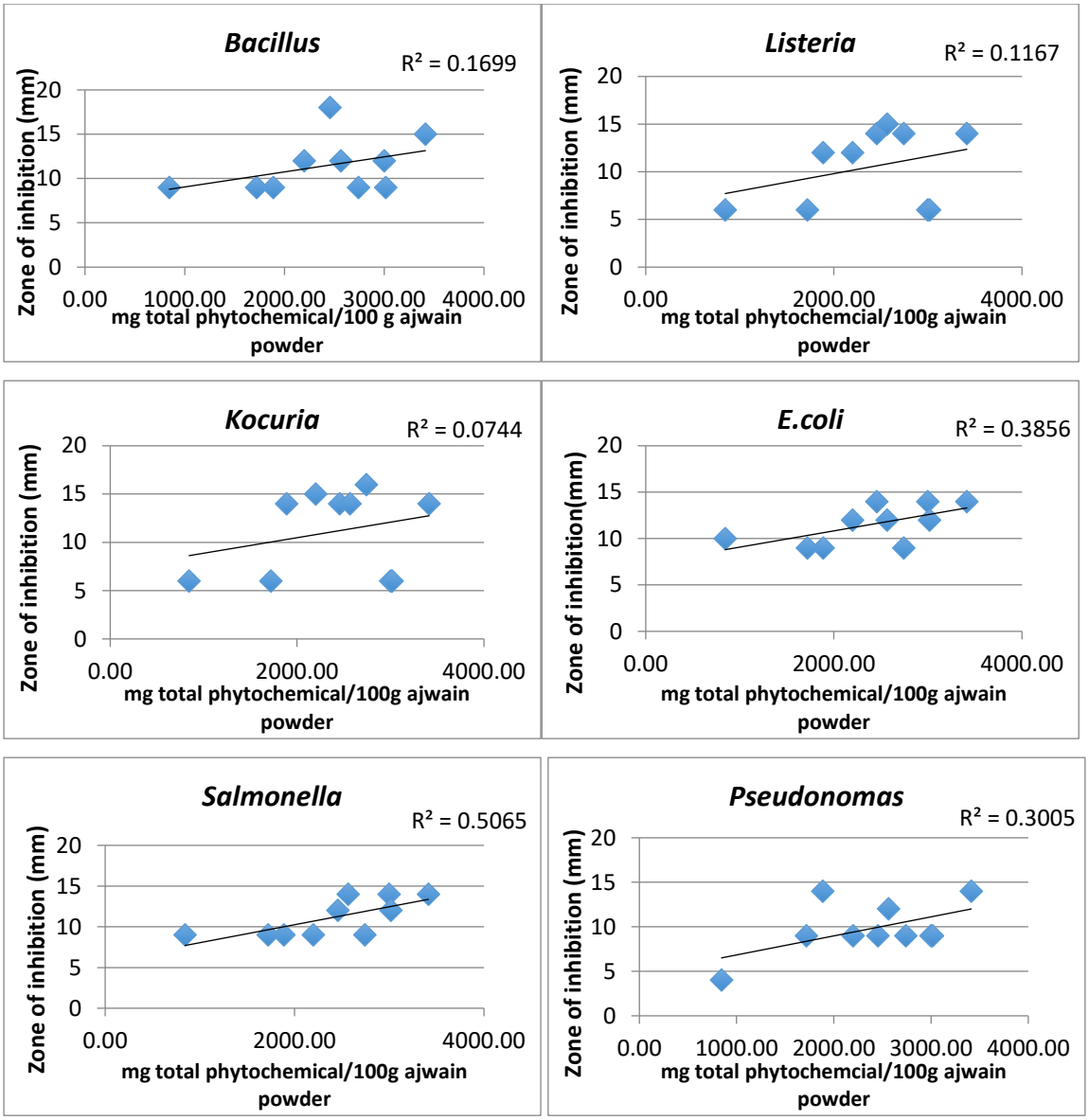


Figure 4.14- Effect of total (non-polar and polar) phytochemical compounds of ajwain ethanolic extracts on antimicrobial properties of ajwain extracts against different microorganisms

The highest correlations identified for *Salmonella* followed by *E.coli* and *Pseudomonas* was found to be 0.5, 0.38 and 0.30 (closer than for any other microorganisms). When total non-polar and polar phytochemical compounds concentration and antimicrobial properties of 10 distinct ajwain samples were compared, good correlation compared to individual compound correlation was observed against all tested Gram-negative pathogenic microbes than Gram-positive pathogens in Figure 4.13. Ajwain sample 6 showed less concentration of total phytochemical compound and less antimicrobial inhibition properties as compared to other ajwain samples.

4.5 Discussion

In the present study, it was observed that ultrasound-assisted ethanol extract of ajwain contained thymol as a major compound and p-cymene and γ-terpinene as major non-phenolic compounds were quantified. Similar to these results, Chahal *et al* (2017) reported chemical composition of ajwain seeds essential oil is influenced by various factors such as method of extraction of spice extract. This result shown in Table 4.18, the extraction of essential oil from which plant part such as seeds or leaf plays an important role. In all the compared studies, major compound identified were similar to present study such as thymol, p-cymene and r-terpinene along with trace compounds. However, the method of extraction of ajwain sample is different in all comparative study.

In a contrasting study by Moein *et al*. (2015), reported γ-terpinene (48.07%), p-cymene (33.73%), and thymol (17.41%) as the major compounds of the oil from Iran. Whereas in present study, thymol was identified as major compound followed by r-terpinene and p-cymene. It is assumed that the difference in results is due to climatic conditions of two different countries, in our study samples are collected from India and compared study is from Iran. In the addition, another study reported, the major components of Indian origin ajwain oil was determined as p-cymene (76.27%), thymol (13.30%), dl-limonene (3.23%), 1,8-cineole (2.58%) and γ-terpinene (1.68%) in Kedia *et al*. (2015) report. In this study the difference in result with our study is due to time of harvesting and collection of samples is different. Alternatively, a study by Nagalakshmi *et al* (2000), reported similar results with present study, thymol (39.36%), γ-terpinene (30.97%), p-cymene (19.47%), b-pinene (5.45%) and a-pinene (1.48%) were the major components of the oil of another Indian sample. Comparative discussion of phenolic compounds identified in ajwain seeds in past 20 years research (Table 4.18).

In present study, qualitative analysis by GCMS identified thymol (57.67%), p-cymene (15.1%) and r-terpinene (24.26%) as a major compounds whereas α-pinene, camphene, β-pinene, β-mycrene, α-phellandrene, caryophyllene were found in trace amounts. These analyses were performed in triplicates and mean results were presented. The same procedure was performed on 10 distinct varieties (collected from different states of India, having different soil and climatic conditions) of ajwain spices. The analysis were performed in triplicates and mean results were presented. The chemical compound concentration of bioactive

compounds of these samples of ajwain extracts were compared. The variation in 10 distinct ajwain sample bioactive compound composition was observed which is due to number of factors such as samples were collected from different regions of India, different climatic conditions, soil conditions, harvesting time.

Table 4.24 – Comparative discussion of phenolic compounds identified in ajwain seeds in past 20 years research

Ajwain oil extraction method	**Compounds identified**	**Reference**
Supercritical fluid (CO_2) extraction of ajwain oil	γ-terpinene (14.2%), p-cymene (23.1%) and thymol (62.0%).	Chahal *et al* (2017)
hydro-distillated extraction of oil	thymol (49.0%), γ-terpinene (30.8 %), p-cymene (15.7%), β-pinene (2.1%), myrcene (0.8%) and limonene (0.7%).	Chahal *et al* (2017)
Ajwain seed essential oil Ajwain plant leaves	thymol (40%), p-cymene (15.6%) and γ-terpinene (11.9%), β-pinene (4%), limonene (4%), carvacrol (5%), camphene and myrcene present in trace amounts. thymol (42.7- 46.2%), γ-terpinene (38.5 -38.9%) and p-cymene (14.1-13.9%)	Abdolali *et al* (2007)
Ajwain essential oil	thymol (87.75%) and carvacrol (11.17%), p-cymene (60.78%) and γ-terpinene (22.26%).	Nagalakshmi *et al.* (2000)
Ajwain essential oil (Iran sample)	γ-terpinene (48.07%), p-cymene (33.73%), and thymol (17.41%)	Moein *et al*. (2015)
Ajwain essential oil (Indian origin sample)	p-cymene (76.27%), thymol (13.30%), dl-limonene (3.23%), 1,8-cineole (2.58%) and γ-terpinene (1.68%)	Kedia *et al*. (2015)
Ajwain essential oil (Indian origin sample)	thymol (39.36%), γ-terpinene (30.97%), p-cymene (19.47%), b-pinene (5.45%) and a-pinene (1.48%)	Nagalakshmi *et al* (2000)

1. Effect of non-polar compounds concentration on antimicrobial properties

Thymol was identified by GCMS in ajwain extracts, major constitute in the non-polar bioactive compound concentration. Thymol is a monoterpenoid similar to carvacrol, having one hydroxyl (-OH) group at phenolic ring. Studies showed that thymol interact with bacterial cell membrane and cause structural and functional damage to cytoplasmic membrane (Hyldgaard et al., 2012; Pisoschi *et al,* 2018). Studies showed that thymol which is found in thyme essential oil transmit antioxidant properties (Lee *et al,* 2004). According to another study by Lira Mota *et al,* (2012), the major compounds of thyme include p-cymene and thymol and antifungal activity against *Rhizopus oryzae* was observed mainly due to thymol. In the study by Paul *et al,* (2011), reported thymol, as major component in *T.ammi* (ajwain oil) possess antibacterial effect against food spoilage bacteria and supported possibility that some other minor compound involve in synergetic effect. In a similar study by Singh *et al,* (2004) reported identification of 26 compound in ajwain essential oil, thymol is major compound identified and showed fungi-toxic effect.

p-cymene is another important compound in non-polar constitute of bioactive components in ajwain extracts. In the present study, correaltion graph shows ρ-cymene has potential antimicrobial inhibition against Gram-negative microorganisms (Correlation graph provided in appendix C). Study showed that it is a precursor of carvacrol and has been reported to showed weak antibacterial activity and works synergistically with carvacrol (Burt, 2004). Trace compounds such as a-pinene, b-pinene and b-caryphyllene has been reported to have antimicrobial activities (Dorman *et al,* 2000). It has been assumed and has been suggested by other studies that antimicrobial properties of non-polar bioactive compounds could be responsible by major and minor components, it is possible that major compounds is regulated by minor compounds as well as these compounds exhibit synergetic effect with other compounds to exert antimicrobial activity (Gupta *et al,* 2013).

In a study by Rahimmalek *et al* (2017), found essential oil composition variation of ajwain due to various environmental conditions such as soil and climatic factors. According to GC–MS analysis, thymol (48.84–61.44%), γ-terpinene (26.96–19.46%) and p-cymene (15.09–20.32%) were the major non-polar aromatic components. He suggested that high essential oil and thymol content (phenolic chemotype) can be obtained in poor, undeveloped, relatively stony and shallow soil orders in arid and semi-arid conditions, while non-phenolic cyclic chemotype

(p-cymene and r-terpinene) needs higher humidity and soil depth. Moreover, the texture of the soil influenced the ajwain essential oil composition. In the current study, ajwain samples were collected from 10 distinct source from India (Table 4.1 on page 100). All the samples were different in size, shape, aroma and flavour. It is assumed that difference in bioactive composition is due to soil and climate conditions of the region were these samples grown, processed and collected. Studies showed that aromatic profile of plants could be affected by genetic, climatic factors and soil conditions.

2. Effect of polar phenolic compounds concentration on antimicrobial properties

In the present study, quantitative analysis of 10 distinct ajwain sample was studied using HPLC (Table 4.10 on page 128). The ajwain samples were enzyme treated with Hesperinidase and cellulase enzyme for 24 hr incubation time (Table 4.8 on page 122). This enzyme treatment method helps to hydrolyse the samples resulting in better separation of the polar compounds, which were compared with non-enzyme treated samples (Table 4.9 on page 125). Some polar phenolic compounds such as epicatechin, caffeic acid, trans-cinnamic acid, and quercetin were found in higher concentration. It was assumed that these compounds might be responsible for antimicrobial activity. However, no literature on these polar compounds shown highest concentration in ajwain sample are responsible for antimicrobial effect was found. Therefore, correlation graphs between polar phenolic compounds and antimicrobial effect was studied (Chapter 4.4.9).

In literature, it was found that some polar phenolic compounds are responsible for antimicrobial activity. The polar phenolic compound such as cinnamic acid, caffeic acid, catechol, quinones have potential antimicrobial effects (Cowan, 1999). Flavonoids reported to have antimicrobial activity, form complexes with cell wall proteins (Cowan, 1999). In a study by Tajkarimi *et al,* (2010) reported that chemicals such as saponin, flavonoids, thiosulfinates and glucosinolates showed antimicrobial effect. Thiosulfinates extracted from garlic showed extreme antimicrobial activity against Gram-negative microorganisms. Glucosinolates show broad range of antimicrobial effect with direct or synergistic effect when used with other substances (Tajkarimi *et al,* 2010). Studies showed that phenolic compounds such as caffeic acid, chlorogenic acid and protocatechuic acid were responsible for the antibacterial activity of coffee (Dogasaki *et al,* 2002). In another study by Kim and Fung (2004), reported antimicrobial activity of tea in both Gram-positive and Gram-negative

microorganisms due to phenolic compounds such as epicatechin, catechin, chlorogenic acid, gallic acid, epigallocatechin gallate.

3. Correlation between antimicrobial activity and phytochemical compounds

Correlation between non-polar bioactive compound and antimicrobial properties were studied. The highest correlation with thymol concentration was identified for *Salmonella* and *Pseudomonas* (R^2 <0.4) and poor correlation for other microorganisms was observed. According to many studies described earlier, reported thymol is responsible for antimicrobial activity. However, no such direct correlation for all ajwain sample was observed against tested foodborne pathogens in this study. No direct correlation with other bioactive compounds such as p-cymene, r-terpinene was observed (correlation graphs for p-cymene and r-terpinene were presented in appendix). In the present study, correaltion graph shows ρ-cymene has potential antimicrobial inhibition against Gram-negative microorganisms such as *Salmonella* (R^2 <0.6). Total non-polar bioactive compounds show good correlation for *Salmonella* and *Pseudomonas* (R^2 <0.4) and weak correlation for few ajwain samples (except ajwain sample 5 and 6). This result suggested that some other compounds might be responsible for antimicrobial action or there might be some synergetic effect with other compound.

When correlation between polar phenolic compound and antimicrobial properties were studied, good correlation with epicatechin was observed for *Salmonella* followed by *E.coli, Pseudomonas, Bacillus, Listeria* and *Kocuria* (R^2 <0.6, 0.38, 0.3, 0.2, 0.1 and 0.02 respectively). The polar phenolic acid such as vanillic acid, syringnic acid and p-coumaric acid showed higher correlations for gram-negative microorganisms such as *Salmonella* (Table 4.16, 4.17). The polar flavonoids such as epigallocatechin gallate, catechin and Kaempferol shows higher correlation for Gram-negative microbes such as *Salmonella* (Table 4.21 and 4.22). However, trans-cinnamic acid and quercetin individual compound shows very poor correlations against mricrooganisms. However, some good correlation was observed with total polar phenolic compounds. This might show that these polar phenolic compounds were also responsible for antimicrobial action. These results might show that these are the compounds which shows synergetic effect with non-polar compounds. There are very few compounds that have a clear individual correlation between concentration and antimicrobial effect, but this correlation is

indeed stronger for multiple weaker compounds contributing to overall effect in total non-polar and polar phytochemical compound. However, this study needs further research to find out which compound shows synergetic effect. Possible mechanism of antimicrobial action could be studied by using standard reference phenolic compound and studying individual compound effect on antimicrobial activity and correlations against microorganisms.

4.6 Conclusion

In the present study, major non-polar bioactive compounds and a wide range of phenolic compounds were detected in ajwain samples. The results showed that the 10 distinct ajwain samples differ in phenolic profile as well as the amount of polyphenols. A simple HPLC method has been developed for the determination of phenolic compounds with high commercial interest. A simple sample preparation is required prior to HPLC analysis. The technique developed is sensitive, reproducible, and accurate.

Correlation between non-polar bioactive compounds and polar phenolic compound with antimicrobial properties against foodborne pathogens were studied. Overall, weak correlation was observed between individual compound and antimicrobial activity except for *Salmonella* and *Pseudomonas* with thymol, epicatechin and some other polar bioactive compounds. A possible explanation might be different mechanism of action of the antimicrobial activity exhibited by each phenolic compound, presence of other non-identified compounds in the extract, as well as potential synergetic effects between phenolic compounds. Total non-polar and polar phytochemical compounds showed stronger correlation as compared to individual compounds on antimicrobial activity, suggesting multiple weaker compounds are contributing to overall effect rather than single individual compound except epicatechin for gram-negative miroorganisms.

However, further study is required to understand the mechanism of pure standard bioactive compounds with respect to antimicrobial properties, different concentration and acceptable in food products is needed. So that quality parameters could be set for spice extracts and could be use as natural alternative to synthetic preservatives in food preservation.

Chapter 5

Anti-glycation and antioxidant properties of ajwain and nutmeg

5.1 Introduction

Diabetes mellitus is a group of metabolic diseases characterised by hyperglycaemia and risk of long-term complications affecting the eyes, blood vessels, nerves, skin and kidneys (Ahmed, 2005). According to WHO, (2013), around 344 million people worldwide have diabetes. Diabetes can affect various organs and systems such as causing improper function of gastrointestinal tract and making the affected individual more susceptible to infection. In diabetes, delayed wound healing is a major problem. Glucose is a main source of energy in maintaining health. The high concentration of glucose that is due to inadequate secretion of insulin is defined as hyperglycaemia. Hyperglycaemia causes increased protein glycation and the gradual build-up of advanced glycation end-products (AGEs) in body tissues. Protein glycation is a complex addition reaction between the free amino proteins and the carbonyl groups of sugars. AGEs are complex, heterogeneous molecules that cause protein cross-linking, exhibit browning and generate fluorescence. Formation of AGEs generate auto-oxidation reactions and yield oxygen free radicals (Ahmed, 2005) which can be controlled with antioxidants. It is well known that plants possess anti-oxidative properties and are related to the presence of phenolic compounds (Ramkissoon *et al.,* 2013).

Phenolic compounds are compounds having polyphenolic structure i.e. one or more aromatic ring with at least one hydroxyl group attached such as terpenes, flavonoids (Ramkissoon *et al.*, 2013; Bi *et al.,* 2017). Polyphenolic compounds widely found in plants such as fruits, vegetables, spices and herbs (Bi *et al*., 2017). In the previous chapter, polyphenolic compounds were identified in ajwain, nutmeg extracts, which possess potential antimicrobial effect. Many researchers suggested that polyphenols might play an important role in preventing oxidative stress related diseases and reducing the risk of diabetes (Ramkissoon *et al.,* 2013; Bi *et al.,* 2017). In this regard, number of plant-based materials such as spices and herbs contain polyphenolic compounds known to possess anti-oxidative properties. Therefore, the antioxidant agents may be responsible for the retardation of AGE formation by preventing further oxidation. Most studies have focussed on developing natural plant-

based products that could effectively inhibit AGE formation and have antioxidant properties. There could be a synergistic effect of polyphenolic compounds present in natural plant-based materials responsible for anti-glycation and antioxidant properties in treatment of diabetes (Ramkissoon *et al.,* 2013). In the present study, anti-glycation and antioxidant properties of spice extracts were determined in relation to their phytochemical characteristics. Glucose is a major precursor of AGE formation. Although studies indicate that methylglyoxal is considered as more effective and reactive reducing sugar than glucose (Ramkissoon et al, 2013; Ahmad et al, 2007).

The natural plant-based material such as common household spices and herbs can make an important contribution to prevent AGEs formation. The use of spices and herbs in food has been suggested to not only increase the antioxidant content but also provide anti-diabetic effect (Bi *et al*., 2017). Spices such as cinnamon, cloves, and allspice have been suggested to possess anti-glycation effect by inhibiting the formation of AGEs and antioxidant effect (Bi *et al.,* 2017) because of their high phenolic compound content (Kaefer and Milner, 2008). Studies showed that major bioactive compounds of spices and herbs possess potential beneficial effects for diabetes management such as cinnamaldehyde in cinnamon spice (Babu *et al*, 2007), curcumin in turmeric spice (Bi *et al*, 2017), tannins (flavonoids) in green tea extract (Babu *et al*, 2007). A number of natural polyphenolic compounds such as caffeic acid, chlorogenic acid, epigallocatechin, and quercetin has been reported to inhibit AGEs (Popova *et al*, 2010; Bi *et al*, 2017).

In the present study, antioxidant properties of spice extracts were determined. The total phenolic content in ajwain and nutmeg extracts was determined using the Folin-Ciocalteu's colorimetric method. This method is based on the oxidation of phenolic compound by Folin Ciocalteau reagent which is a mixture of phosphotungstic acid ($H_3PW_{12}O_{40}$) and phosphomolybdic ($H_3PMo_{12}O_{40}$). After the oxidation reaction the reagent is reduced producing a blue colour under alkaline conditions which is supplied by adding sodium carbonate. The maximum absorption of the blue chromophore at 760nm is measured and is proportional to the total quantity of the phenolic compounds originally present.

The ferric reducing antioxidant power (FRAP assay) i.e Fe^{3+} to Fe^{2+} was determined. This assay relies on reduction ability of antioxidants, ferric ion and tripyridyltriazine (TPTZ) acts as reagents. Ferric ion (111)-TPTZ complex is reduced to Ferric ion (11)- TPTZ complex producing an intensive blue colour and the absorption is monitored in a spectrophotometer at 593nm. The absorption provides information about the antioxidative capacity (Papadopoulos., 2008). The molecule in the antioxidant acts as reductants which donates electrons which stabilises free radicals which reduces the damage they have on cells and organs (Biovision., 2018).

Radical scavenging activity of the spice extracts against 2,2-diphenyl-2-picrylhydrazyl hydrate (DPPH) was determined spectrophotometrically. The DPPH method is rapid, simple, accurate and inexpensive assay for measuring the ability of different compounds to act as free radical scavengers or hydrogen donors, and to evaluate the antioxidant activity of foods and beverages (Marxen et al., 2007; Perez-Jimenez et al., 2008). This assay is based on the scavenging and reducing ability of antioxidant towards 2,2-Diphenyl-1-picrylhydrazyl radical Antioxidants acts as hydrogen donor, DPPH• accepts hydrogen from the antioxidant. DPPH• is one of the few stable and commercially available organic nitrogen radicals. Antioxidants react with the free radical DPPH• to produce DPPH upon absorption of hydrogen atom from the antioxidant, purple colour is changed to yellow hence causing reduction in UV absorption at 517nm. The absorption is measured with a spectrophotometer (Chandra., 2016). Lower absorbance of the reaction mixture indicated higher radical scavenging activity.

The comparison between the total phenol content, antioxidant properties and anti-glycation potential of spice extracts was also demonstrated. The main purpose of this study is to compare the anti-glycation potential to antioxidant properties of ajwain and nutmeg spice extracts. This work is an attempt to find link between common household spices and home-based recommendations for diabetes treatment.

5.2 Aim and Objectives

The aim of this study was to investigate and compare the effect of ajwain and nutmeg spice extracts on the production of AGEs *in vitro* and on their antioxidant properties.

The objectives were: -

- To assess and compare the effects of different reducing sugars (methylglyoxal and glucose) on cross-linked AGEs *in vitro.*
    - To examine and compare the ability of ajwain spice extract to inhibit the production of cross-linked AGEs *in vitro.*
    - To examine and compare the ability of nutmeg spice extract to inhibit the production of cross-linked AGEs *in vitro.*
- To measure and compare the effect of ultrasound-assisted extraction of ajwain and nutmeg spice extracts on antioxidant properties *in vitro* using total phenolic content (TPC), ferric reducing antioxidant power (FRAP) assay and 2,2-diphenyl-2-picrylhydrazyl hydrate (DPPH) assay.

5.3 Methodology

5.3.1 Preparation of spice extract for anti-glycation activities

The spices (ajwain and nutmeg) were ground in mill and powdered. Aqueous spice extracts were prepared by mixing in water in a 1:10 ratio. Ethanol extracts were also prepared using the same 1:10 ratio of solute to solvent. The mixtures were allowed to stir in a magnetic stirrer for 24 hr for stirring method and for ultrasound-assisted extraction method, 20 KHz for 5 min was used. The total content was collected and centrifuged at 9000 rpm for 10 mins and the supernatants were filtered through Whatman filter paper no. 4 and stored for later use.

Anti-glycation tests were based mainly on glycation of proteins, measurement of crosslinked AGEs by SDS-PAGE and image analysis of SDS PAGE gels. Spices extracts were tested as inhibitors to stop or delay AGEs formation. Water based and ethanol based 1:10 ratio of spice extract that showed good results for antimicrobial activity was selected to be tested for anti-glycation and antioxidant activities.

5.3.2 Anti-glycation effects of spice extracts

1. Chemicals required and final concentrations used for stock solution

Lysozyme was purchased from Sigma, UK, Methyglyoxal from Sigma, UK and D-Glucose – BDH, UK. **Lysozyme (10mg/ml):** Stock solution of Lysozyme was prepared by dissolving 0.09g in 3ml phosphate buffer. Final concentration of lysozyme 10mg/ml. **0.1 M sodium phosphate buffer:** Phosphate buffer solution (0.1 M), pH=7 which was adjusted to pH=7.4 with sodium hydroxide. 3mM sodium azide was added to prevent any bacterial growth. **0.1 M Methylglyoxal or 0.5M Glucose:** Stock solution of methylglyoxal was prepared by dissolving 2.161g of 40% aqueous solution of methylglyoxal in 100 ml distilled water.

2. Glycation of proteins *in vitro*

The detection of glycated proteins was based on a previously described method (Ahmad *et al.*, 2007). 10mg/ml lysozyme was incubated with 0.1M methylglyoxal or 0.5M glucose in 0.1 M sodium phosphate buffer containing 3mM sodium azide (which inhibit the growth of microorganisms) at pH 7.4. Different concentrations of spice extracts as inhibitor (15 to 100mg/ml) were included in the incubation mixture as shown in Tables 5.1 and 5.2. Control

sample was prepared without addition of sugar or inhibitor. All 0.1M methylglyoxal samples were then incubated at 37°C for 5 -7 days and 0.5M glucose samples for 15 -18 days, after which the samples were further analysed using SDS PAGE gels.

Table 5.1: Incubation mixture for protein glycation using different concentrations of spice extract with lysozyme and 0.1M methylglyoxal in 0.1M sodium phosphate buffer, pH 7.4 at 37^0C

	A Control	B Positive control	Samples			
			C 15mg/ml	D 30mg/ml	E 45mg/ml	F 100mg/ml
Lysozyme (10mg/ml)	250µl	250µl	250µl	250µl	250µl	250µl
Methylglyoxal (0.1M)	-	250µl	250µl	250µl	250µl	250µl
Spice extract (inhibitor)	-	-	250µl	250µl	250µl	250µl
Buffer	750µl	500µl	250µl	250µl	250µl	250µl

Table 5.2: Incubation mixture for protein glycation using different concentrations of spice extract with lysozyme and 0.5M glucose in 0.1M sodium phosphate buffer, pH 7.4 at 37^0C

	A Control	B Positive control	Samples			
			C 15mg/ml	D 30mg/ml	E 45mg/ml	F 100mg/ml
Lysozyme (10mg/ml)	250µl	250µl	250µl	250µl	250µl	250µl
Glucose (0.5M)	-	250µl	250µl	250µl	250µl	250µl
Spice extract (inhibitor)	-	-	250µl	250µl	250µl	250µl
Buffer	750µl	500µl	250µl	250µl	250µl	250µl

5.3.3 Measurement of cross-linked AGEs by Sodium Dodecyl Sulphate-Polyacrylamide Gel (SDS PAGE) electrophoresis

1. Materials/instruments required

Xcell surelock mini-cell electrophoresis tank and lid (Invitrogen), Zoom Dual power unit (Invitrogen), Water bath set at 70°C, Pipettes (P10-1000), 1.5 ml Eppendorf tubes, 500 µl Eppendorf tubes, 1.0 ml pipette tips, 200 µl pipette tips, NuPAGE round gel loading tips (Invitrogen catalog no. LC1001), NuPAGE Precast Novex gels, NuPAGE Anti-oxidant (Invitrogen catalog no. NP0005), NuPAGE LDS sample buffer (4x) (Invitrogen catalog no. NP0007), NuPAGE MES buffer (20x) (Invitrogen catalog no. Np0002-02), Mark unstained standard (1x), Simply blue safe stain (Invitrogen catalog no. LC6065), DL- dithiothreitol (Sigma), Methanol (Analytical grade), Glacial acetic acid (Analytical grade), and Square petri dished (Sterilin).

2. Reagent preparation for SDS PAGE

DL-dithiothreitol (DTT) solution was prepared using 77.5 mg/ml in a 1.5 ml Eppendorf tube. Aliquot of the sample prepared into a 500 µl Eppendorf tube by adding 25µl NuPAGE LDS sample buffer (4x), 10 µl 77.5mg/ml DTT and 65 µl spice sample. Mixture was vortex for 10-20s., heated in a 70°C water bath for 10 min and then cool to room temperature.

Running buffer was prepared by measuring 50ml of NuPAGE MES (20x) and added 1L water. MES buffer is used to separate low-high molecular weight proteins (2.5-200 kDa). Transferred 200ml to a 500 ml Duran bottle, added 0.4ml of NuPAGE Antioxidant solution, and mixed.

Pre cast gel was removed from the packaging and gently the comb was removed, taking care not to disturb the wells. The comb and wrapper was disposed in the normal waste bin. The gel plates and wells were washed with deionised water to remove any gel pieces and residual storage buffer. The wells of the gel were rinsed (3x) with diluted MES buffer, using a P1000 automatic pipette. The white tape from the front gel plate was removed.

3. Loading of samples and gel electrophoresis

The electrophoresis tank was set up, by positioning an empty tank + magnetic stirring bar, onto a magnetic stirrer. The electrode was secured into the tank. The gel plates were fixed on SDS-PAGE electrophoresis apparatus. The gels were inserted either side of the buffer core, ensuring that the back-gel plates of each gel are facing inwards. The gel plates were locked in

place using the gel tension wedge. The upper buffer chamber was filled with NuPAGE MES containing antioxidant, until the buffer is covering the tops of the gels but not overflowing into the lower buffer chamber. The lower buffer chamber was checked for any leaks. In case of any leaks, the buffer was poured out, re-secure the buffer core and the gels. The upper chamber was re-filled and check for leaks. The lower buffer chamber was poured with SDS running buffer solution (without antioxidant) until the level reaches halfway up the gel.

The samples were loaded to gel, 10µl per sample depending on protein concentration. 10ul of ColorBurst® electrophoresis marker was loaded in the first well. 10ul of both control samples were loaded to 2nd and 3rd wells. In rest of wells SDS page inhibitor solution was loaded. The lid was secure and attached to the power at 250V power supply. Voltage 200 V, Current 350 mA, Wattage 100W, Time 35 min was used. Electrophoresis was carried out until the blue bands reached the separating gel approximately in 35 minutes. When the dye front was reached to about 1cm from the bottom, the apparatus was shut automatically as electrophoresis completed. The gels were taken out carefully and were removed from the sealed gel plated by opening with the flat end of a spatula. The gel were transferred to a sandwich box or square petri-dish.

4. Staining Procedure

The gels were transferred into fixing solution (50% methanol and 10% acetic acid) for 30 min. 50ml of simply blue safe stain was added to the gel and was placed on shaker for about 30 min and left in blue stain for overnight. The stain was decanted and rinsed with deionised water (on shaker) for 2-3 times, Until the background of the gels were cleared.

5. Image analyser

The gel images were analysed using Odyssey Fc Imager (Li-COR, UK). The gels were placed in Odyssey Fc imaging tray into imaging drawer. In the Image studio software on desktop, under work area file was created to save gel images. In Acquiring tab, drop down menu, desired channel(s) – 600, 700, 800 and/or Chemi selected (channel 600 selected for this study), integration time was selected (longer integration time lead to improved signal-to-noise ratio). The Image was then acquired using Acquire button on the software. From Image studio, gel image were exported to image studio files and were saved. After integration of gels, bands were compared within the same gel. Integrated Density (I.D) of bands was measured and

analysed and the percentage inhibition of crosslinked AGEs was calculated using the following formula:

% inhibition = (I.D without inhibitor – I.D with inhibitor) / I.D without inhibitor x 100 (1)

5.3.4 Methods for antioxidant activity of ajwain and nutmeg extracts

1. Total phenolic content

The total phenolic content in ajwain and nutmeg extracts was determined using the Folin-Ciocalteu's colorimetric method according to Skerget et al (2005). Gallic acid is used as standard. Samples of spice extracts (0.5ml) and 2.5ml of Folin-Ciocalteu's phenol reagent (diluted 10 times) were mixed with 2 ml sodium carbonate (Na_2CO_3), incubated at 50^0C for 5 minutes. Mixture were cooled to room temperature before measuring the absorbance at 760nm using a spectrophotometer (Thermo, electron Coorparation, Genesis 10-S, at 760nm). Each assay was performed in triplicate.

Standard curve

The standard curve was prepared using gallic acid solutions, the absorbance measured at 760nm and results expressed as mg/L of gallic acid equivalents. A phenolic content in spice extracts in gallic acid equivalents was calculated as described below:

C = c*v/m (2)

Where: C is total phenolic compounds in spice extract in GAE

c: the concentration of gallic acid determined from the calibration curve (mg/L)

v: the volume of spice extracts (ml)

m: the weight of spice extract (g)

2. Ferric reducing antioxidant power (FRAP) assay

The ferric reducing antioxidant power (FRAP assay) i.e Fe^{3+} to Fe^{2+} was determined according to the procedure of Benzie and Strain (1999). Spice extracts (0.1ml) was added to 3ml of freshly prepared FRAP reagent consisted of 300 mM acetate buffer (pH 3.6), 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) solution in 40 mM HCL and 20 mM $FeCl_3 \cdot 6H_2O$ (10:1:1). The samples were incubated in water bath at 37^0C for 4min and the absorbance was recorded before and after incubation at 593nm using spectrophotometer (Thermo, electron Coorparation, Genesis 10-S, 593nm). Aqueous solutions of known Fe^{2+} ($FeSO_4.7H_2O$) concentrations in the range of 0.01-1.00mM were used for calibration. The total antioxidant capacity, the FRAP value of the sample was calculated and expressed as mmol Fe^{2+} equivalents (Fe) per Kg [mmol Fe/Kg]. All samples were analysed in three replicates.

3. Determination of the radical scavenging ability using the 2,2-diphenyl-2-picrylhydrazyl hydrate (DPPH) assay

Radical scavenging activity of the spice extracts against 2,2-diphenyl-2-picrylhydrazyl hydrate (DPPH) was determined spectrophotometrically using the method of Brand-Williams *et al* (1995). DPPH solution was prepared in methanol and 0.06mM was used. Aqueous and ethanolic ajwain and nutmeg spice extracts (0.1ml) were added to the 3.9ml of DPPH solution. The decrease in absorbance measured using spectrophototmeter (Thermo, electron Coorparation, Genesis 10-S) at 517nm was determined after placing samples test tubes in dark for 30min. The absorbance of the DPPH radical without antioxidant (negative control), at t=0min was measured. The standard value for DPPH used was 1.544 and the ability to scavenge the DPPH radical was calculated using the following equation:

$$\textbf{DPPH (radical scavenging activity) \%} = [(A_{Control} - A_{Sample} / A_{Control}) \times 100] \quad \textbf{(3)}$$

Where $A_{Control}$ is the absorbance of the control (initial DPPH), A_{sample} is the absorbance of the sample at t=30min. The results were expressed as the free radical scavenging activity (%). The higher the radical scavenging activity, the higher the antioxidant effect of the product. The experiments were carried out in triplicates.

5.4 Results

5.4.1 Effect of spices (ajwain and nutmeg) extracts on 0.1M methylglyoxal-derived AGEs

1. The effect of ajwain spice stirring ethanol extract on formation of MG-derived cross-linked AGEs

Methylglyoxal is known as the most reactive reducing sugar. The monomers, dimers, and trimers of the cross-linked methylglyoxal-lysozyme AGEs were inhibited substantially depending on the concentration of ethanol extract of ajwain spice as shown in Figure 5.1. The reduced electrophoretic mobility of cross-linked AGEs with higher molecular weights were demonstrated in lane c-f compared to control sample (Lane b). The effect of different concentrations of ethanolic extract of spice (ajwain) clearly showed a reduction in the intensity of the dimer bands, which was used to measure the percentage of inhibition. Lysozyme incubated alone in lane a and lysozyme with methylglyoxal in lane b (control sample).

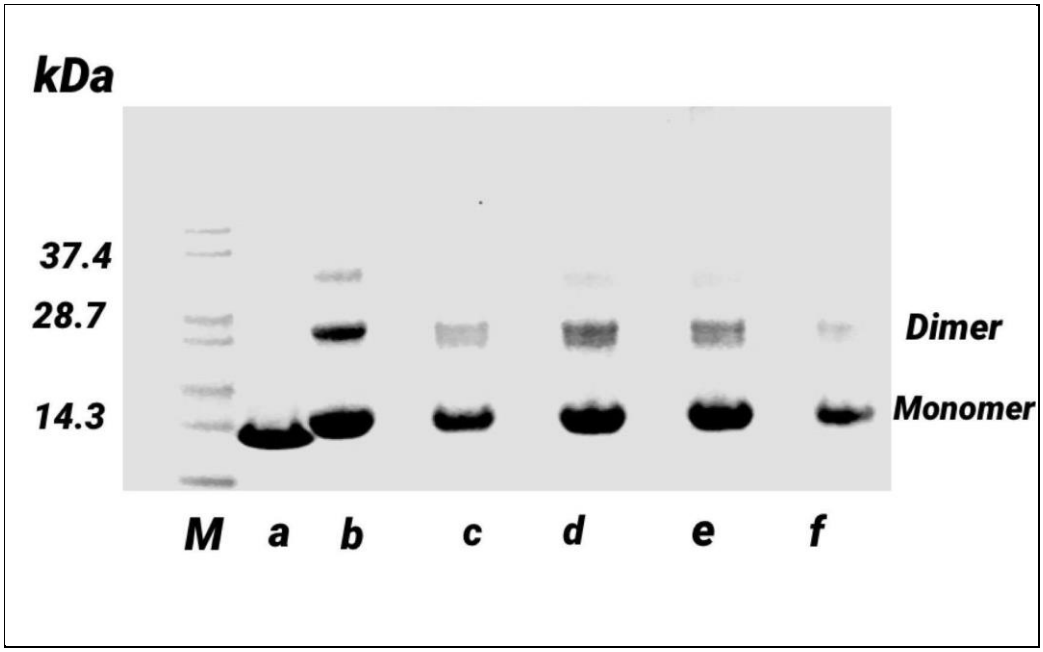


Figure 5.1: Gel shows the effect of ajwain extract on methylglyoxal derived AGEs. Lysozyme (10 mg/ml) incubated alone (lane a) or in the presence of 0.1 M methylglyoxal (lane b) and the effect of different concentrations (c) 15 mg/ml, (d) 30 mg/ml, (e) 45 mg/ml, (f) 100 mg/ml, of ethanol extract of ajwain in 0.1 M sodium phosphate buffer pH 7.4 at 37 °C for 5days. The result is a representative figure of at least three independent experiments.

2. **The effect of ultrasound-assisted ethanolic extract of ajwain on formation of methylglyoxal-derived crosslinked AGEs**

The monomers, dimers, and trimers of the cross-linked methylglyoxal-lysozyme AGEs were inhibited substantially depending on the concentration of ultrasound-assisted ethanol extract of ajwain as shown in Figure 5.2. The reduced electrophoretic mobility of cross-linked AGEs with higher molecular weights were demonstrated in lane c-f compared to control sample (Lane b). The effect of different concentrations of ethanolic extract of spice (ajwain) clearly showed a reduction in the intensity of the dimer bands, which was used to measure the percentage of inhibition.

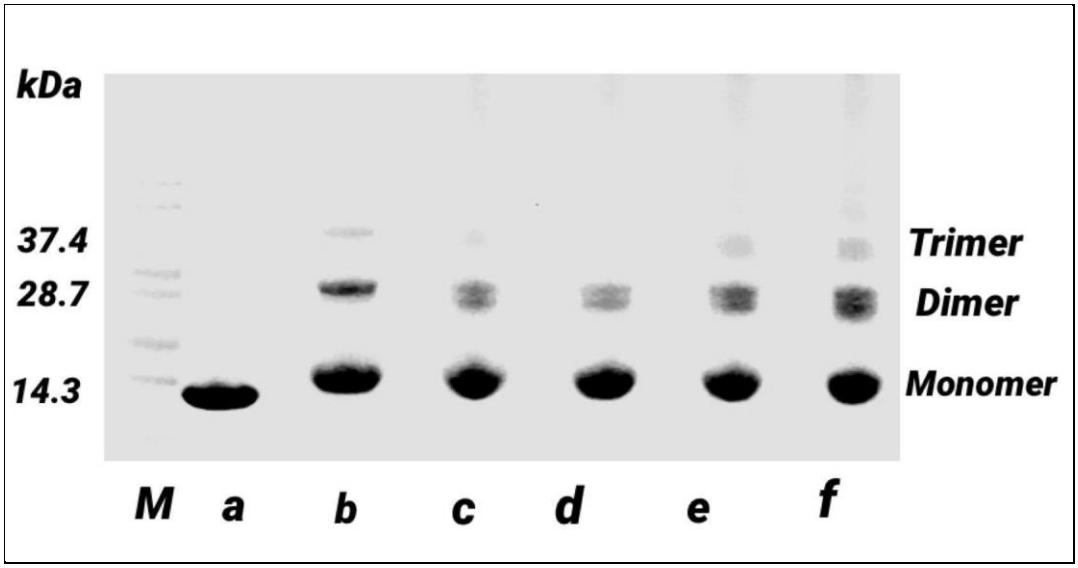


Figure 5.2: Gel shows the effect of ultrasound-assisted ethanolic extract of ajwain on methylglyoxal-derived AGEs. Lysozyme (10 mg/ml) incubated alone (lane a) or in the presence of 0.1 M methylglyoxal (lane b) and the effect of different concentrations (c) 15 mg/ml, (d) 30 mg/ml, (e) 45 mg/ml, (f) 100 mg/ml, of ethanolic extract of ajwain in 0.1 M sodium phosphate buffer pH 7.4 at 37 °C for 5days. The result is a representative figure of at least three independent experiments.

3. The effect of nutmeg spice stirring ethanol extract on formation of methylglyoxal-derived cross-linked AGEs

The monomers, dimers, trimers and tetramers of the cross-linked methylglyoxal-lysozyme AGEs were inhibited substantially depending on the concentration of ethanolic extract of nutmeg spice as shown in Figure 5.3. The reduced electrophoretic mobility of cross-linked AGEs with higher molecular weights were demonstrated in lane c-f compared to control sample (Lane b). The effect of different concentrations of ethanolic extract of nutmeg spice clearly showed a reduction in the intensity of the dimer bands, which was used to measure the percentage of inhibition.

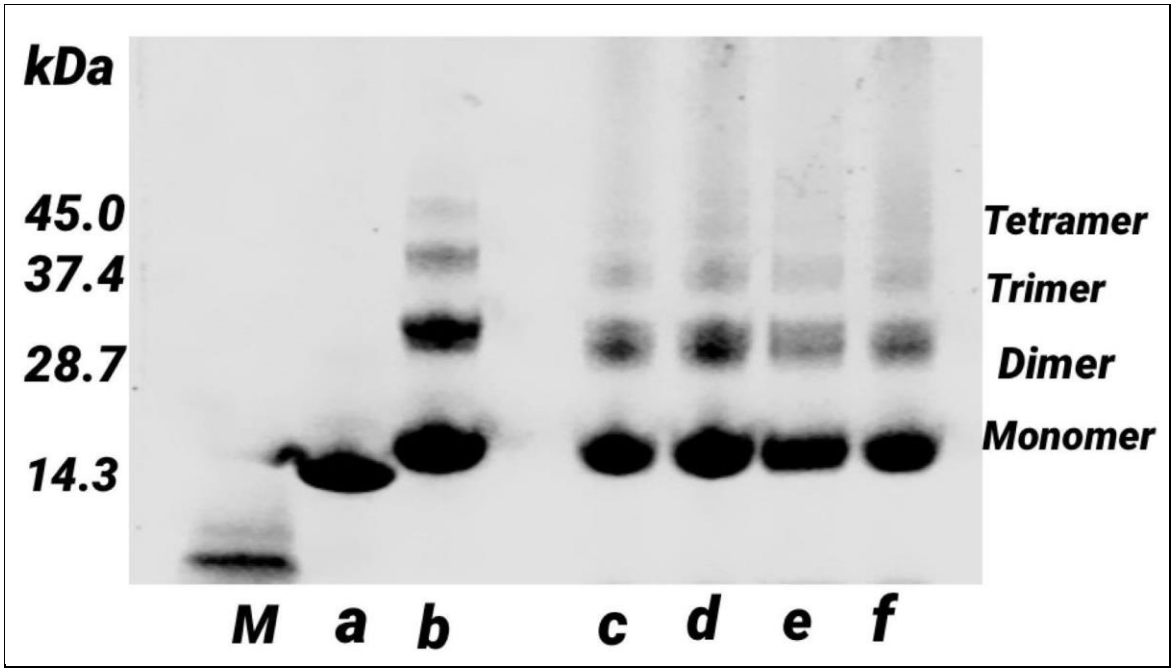


Figure 5.3: Gel shows the effect of nutmeg extract on methylglyoxal-derived AGEs. Lysozyme (10 mg/ml) incubated alone (lane a) or in the presence of 0.1 M methylglyoxal (lane b) and the effect of different concentrations (c) 15 mg/ml, (d) 30 mg/ml, (e) 45 mg/ml, (f) 100 mg/ml, of ethanolic extract of nutmeg in 0.1 M sodium phosphate buffer pH 7.4 at 37 °C for 5 days. The result is a representative figure of at least three independent experiments.

4. **The effect of ultrasound-assisted ethanolic extract of nutmeg on formation of methylglyoxal-derived cross-linked AGEs**

The monomers, dimers, trimers and tetramers of the cross-linked methylglyoxal-lysozyme AGEs were inhibited substantially depending on the concentration of ethanolic extract of nutmeg spice as shown in Figure 5.4. The reduced electrophoretic mobility of cross-linked AGEs with higher molecular weights were demonstrated in lane c-f compared to control sample (Lane b). The effect of different concentrations of ethanolic extract of nutmeg spice clearly showed a reduction in the intensity of the dimer bands, which was used to measure the percentage of inhibition.

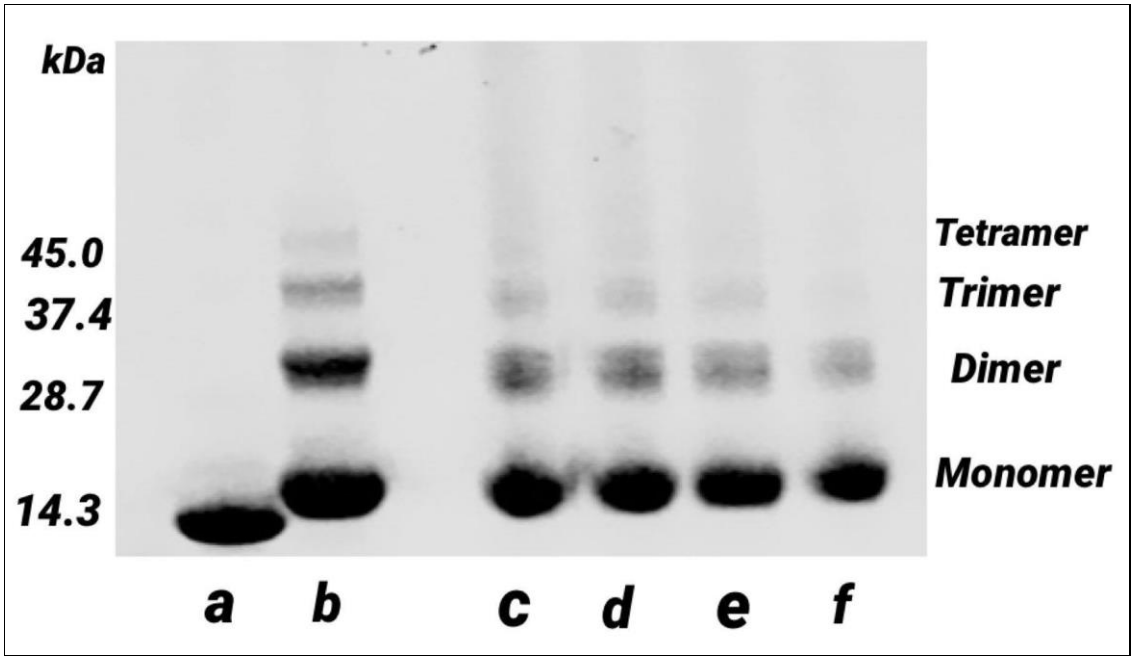


Figure 5.4: Gel shows the effect of ultrasound-assisted ethanolic extract of nutmeg on methylglyoxal-derived AGEs. Lysozyme (10 mg/ml) incubated alone (lane a) or in the presence of 0.1 M methylglyoxal (lane b) and the effect of different concentrations (c) 15 mg/ml, (d) 30 mg/ml, (e) 45 mg/ml, (f) 100 mg/ml, of ethanolic extract of nutmeg in 0.1 M sodium phosphate buffer pH 7.4 at 37 °C for 5days. The result is a representative figure of at least three independent experiments.

5. **The effect of aqueous ajwain extract on formation of methylglyoxal-derived cross-linked AGEs**

The effect of different concentrations of aqueous extract of spice (ajwain) does not show a reduction in the intensity of the dimer bands, which were used to measure, the percentage of inhibition. Hence, results were not represented.

5.4.2 Effect of spices (ajwain and nutmeg) extracts on 0.5M glucose derived AGEs

6. **The effect of ajwain ethanolic extract on formation of glucose-derived cross-linked AGEs**

Lysozyme incubated in the presence of glucose produces sufficient cross-linked AGEs.

The monomers and dimers of the cross-linked glucose-lysozyme AGEs were inhibited substantially depending on the concentration of ethanolic extract of ajwain spice as shown in Figure 5.5. The reduced electrophoretic mobility of cross-linked AGEs with higher molecular weights were demonstrated in lane c-f compared to control sample (Lane b). The effect of different concentrations of ethanolic extract of ajwain was clearly showed a reduction in the intensity of the dimer bands, which was used to measure the percentage of inhibition.

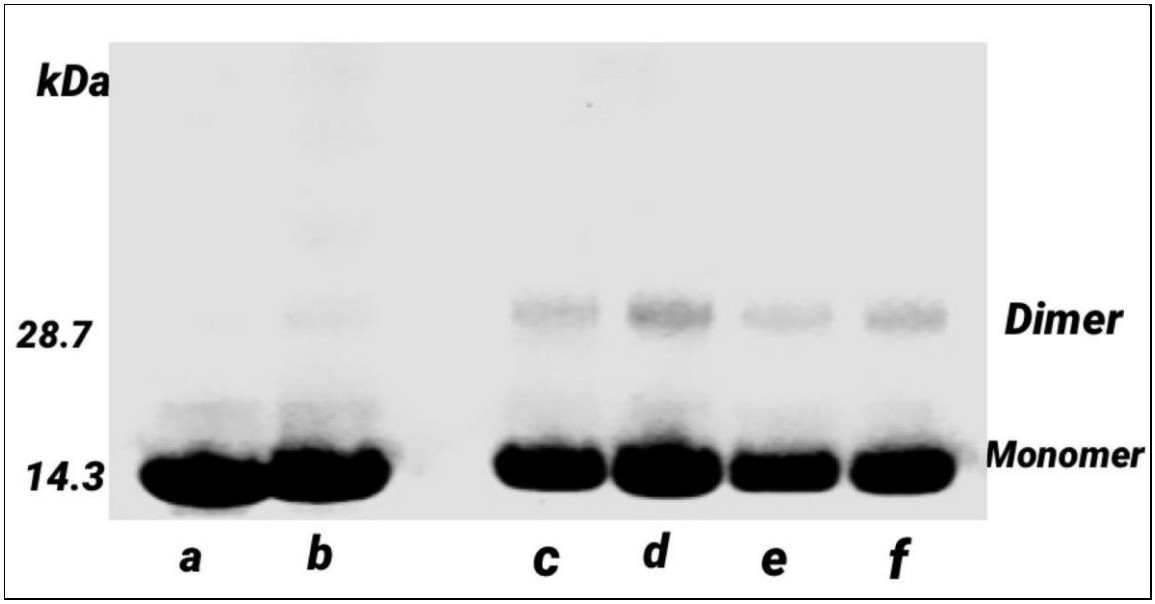


Figure 5.5: Gel shows the effect of ethanolic extract of ajwain on glucose-derived AGEs. Lysozyme (10 mg/ml) incubated alone (lane a) or in the presence of 0.5M Glucose (lane b) and the effect of different concentrations (c) 15 mg/ml, (d) 30 mg/ml, (e) 45 mg/ml, (f) 100 mg/ml, of stirring ethanol extract of ajwain in 0.1 M sodium phosphate buffer pH 7.4 at 37 °C for 18 days. The result is a representative figure of at least three independent experiments.

7. The effect of ultrasound-assisted ethanolic extract of ajwain on formation of glucose-derived cross-linked AGEs

The monomers and dimers of the cross-linked glucose-lysozyme AGEs were inhibited substantially depending on the concentration of ethanolic extract of ajwain spice as shown in Figure 5.6. The reduced electrophoretic mobility of cross-linked AGEs with higher molecular weights were demonstrated in lane c-f compared to control sample (Lane b). The effect of different concentrations of ethanolic extract of ajwain clearly showed a reduction in the intensity of the dimer bands, which was used to measure the percentage of inhibition.

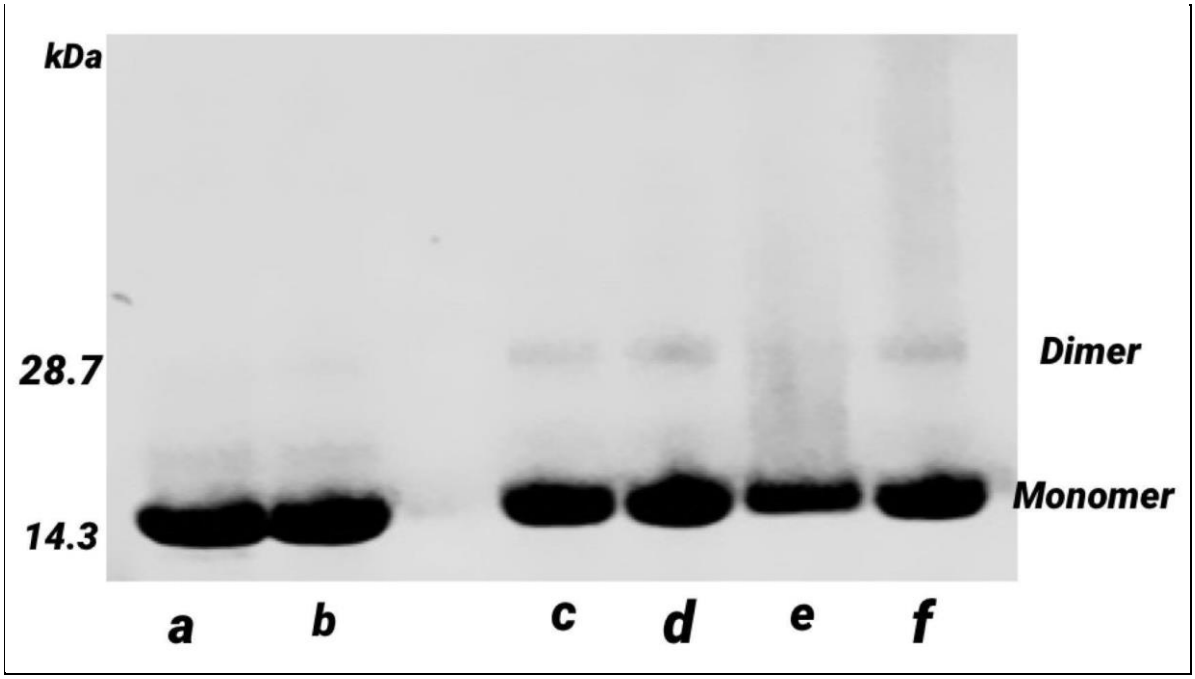


Figure 5.6: Gel shows the effect of ultrasound assisted ethanolic extract of ajwain on glucose-derived AGEs. Lysozyme (10 mg/ml) incubated alone (lane a) or in the presence of 0.5M Glucose (lane b) and the effect of different concentrations (c) 15 mg/ml, (d) 30 mg/ml, (e) 45 mg/ml, (f) 100 mg/ml, of ethanol extract of ajwain in 0.1 M sodium phosphate buffer pH 7.4 at 37 °C for 18 days. The result is a representative figure of at least three independent experiments.

8. The effect of nutmeg ethanol extract on formation of glucose-derived cross-linked AGEs

The monomers and dimers of the cross-linked glucose-lysozyme AGEs were inhibited substantially depending on the concentration of ethanolic extract of nutmeg as shown in Figure 5.7. The reduced electrophoretic mobility of cross-linked AGEs with higher molecular weights were demonstrated in lane c-f compared to control sample (Lane b). The effect of different concentrations of ethanolic extract of nutmeg was clearly showed a reduction in the intensity of the dimer bands, which was used to measure the percentage of inhibition.

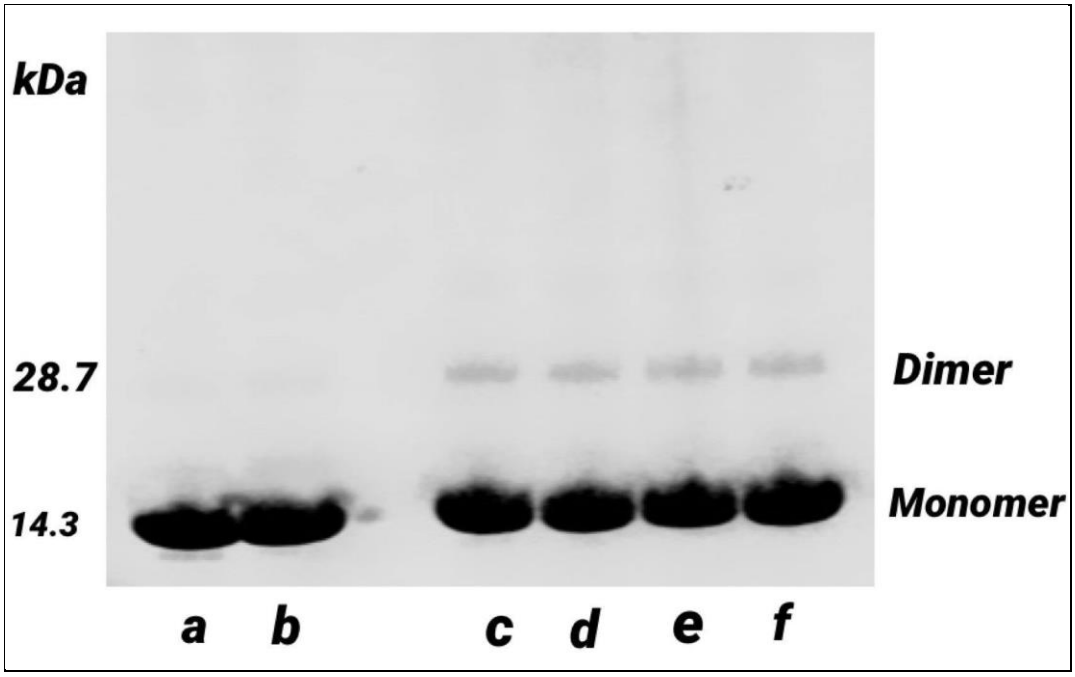


Figure 5.7: Gel shows the effect of ethanolic extract of nutmeg on glucose-derived AGEs. Lysozyme (10 mg/ml) incubated alone (lane a) or in the presence of 0.5M Glucose (lane b) and the effect of different concentrations (c) 15 mg/ml, (d) 30 mg/ml, (e) 45 mg/ml, (f) 100 mg/ml, of ethanol extract of nutmeg in 0.1 M sodium phosphate buffer pH 7.4 at 37 °C for 18 days. The result is a representative figure of at least three independent experiments.

9. The effect of ultrasound-assisted ethanolic extract of nutmeg on formation of glucose-derived cross-linked AGEs

The monomers and dimers of the cross-linked glucose-lysozyme AGEs were inhibited substantially depending on the concentration of ethanolic extract of nutmeg spice as shown in Figure 5.8. The reduced electrophoretic mobility of cross-linked AGEs with higher molecular weights were demonstrated in lane c-f compared to control sample (Lane b). The effect of different concentrations of ethanolic extract of nutmeg spice clearly showed a reduction in the intensity of the dimer bands, which was used to measure the percentage of inhibition.

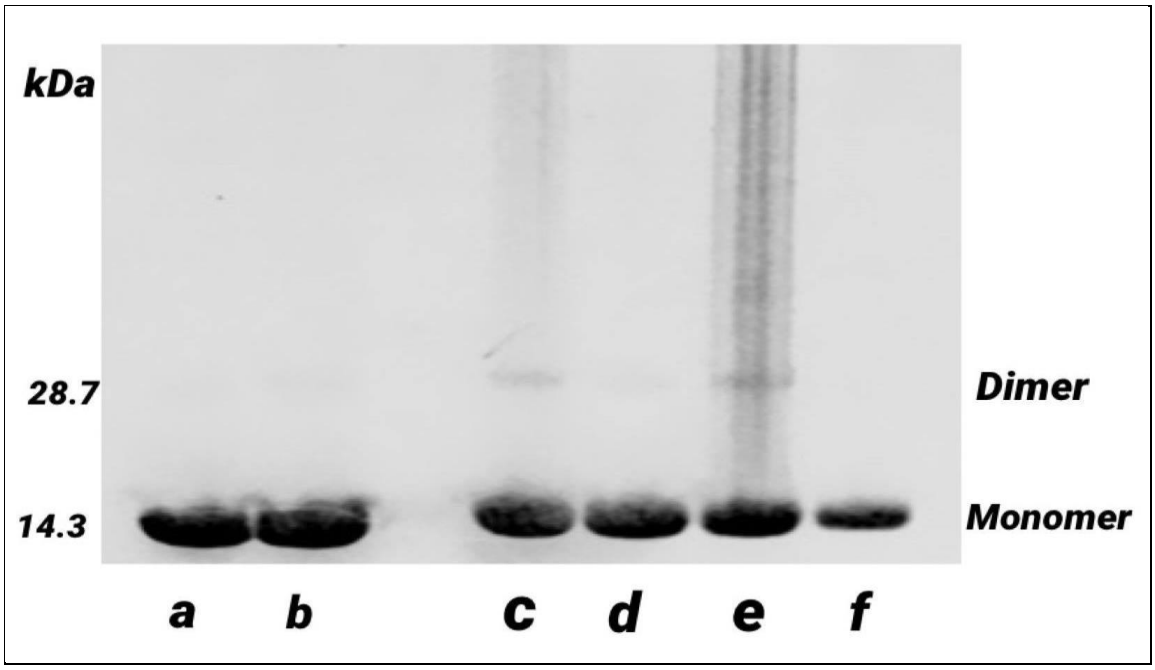


Figure 5.8: Gel shows the effect of ultrasound-assisted ethanol extract of nutmeg on glucose-derived AGEs. Lysozyme (10 mg/ml) incubated alone (lane a) or in the presence of 0.5M Glucose (lane b) and the effect of different concentrations (c) 15 mg/ml, (d) 30 mg/ml, (e) 45 mg/ml, (f) 100 mg/ml, of ethanol extract of nutmeg in 0.1 M sodium phosphate buffer pH 7.4 at 37 °C for 18 days. The result is a representative figure of at least three independent experiments.

10. The effect of aqueous ajwain extract on formation of glucose-derived cross-linked AGEs

The effect of different concentrations of aqueous extract of ajwain spice does not showed reduction in the intensity of the dimer bands, which was used to measure the percentage of inhibition. Hence, results were not represented.

Table 5.3 shows comparative mean result presentation of percentage inhibition of all ajwain and nutmeg samples. Highest inhibition was observed in ajwain samples at 100mg/ml concentration.

Table 5.3– Mean results representation of % inhibition of AGEs by spice extracts

	% Inhibition							
	0.1M Methylglyoxal		0.5M Glucose		0.1M Methylglyoxal		0.5M Glucose	
Concentration (mg/ml)	Ajwain Stirring Ethanol	Ajwain Ultrasound Ethanol	Ajwain Stirring Ethanol	Ajwain Ultrasound Ethanol	Nutmeg Stirring Ethanol	Nutmeg Ultrasound Ethanol	Nutmeg Stirring Ethanol	Nutmeg Ultrasound Ethanol
15	45	43.3	29	18.5	43	43.2	11.5	22.3
30	55.2	47	30.8	20	45.7	47.6	21.7	35
45	66.6	53.8	31.6	35.6	51.4	52	28	46.2
100	**77.9**	**66**	36.7	**38.2**	57	**60.7**	35	**48**

Comparison of effect of stirring and ultrasound-assisted ethanol extract of ajwain and nutmeg on methylglyoxal and glucose-induced AGE formation. Each value represented the mean ± S.D (n=3). Values in bold text shows the highest value in the column.

Production of crosslinked AGE *in vitro* was inhibited by ethanol extracts of ajwain and nutmeg. This inhibition was dependent on ajwain or nutmeg ethanol extract concentrations as maximum inhibition was observed in the sample with 100mg/ml of ajwain or nutmeg extracts as shown in Table 5.3.

5.4.3 Antioxidant properties of spice extracts

1. **Total phenolic content using Folin Ciocalteau method**

Table 5.4 - Values of absorbance at 760nm and standard concentration

Total Phenol	
mg/L Gallic acid	Average values of standard measured at Absorbance 760nm
50	0.593
100	1.082
150	1.563
200	2.045
250	2.523

The values from table was used to plot a standard curve (figure 5.9) which was used to find the corresponding concentration of the spices and herbs extracts.

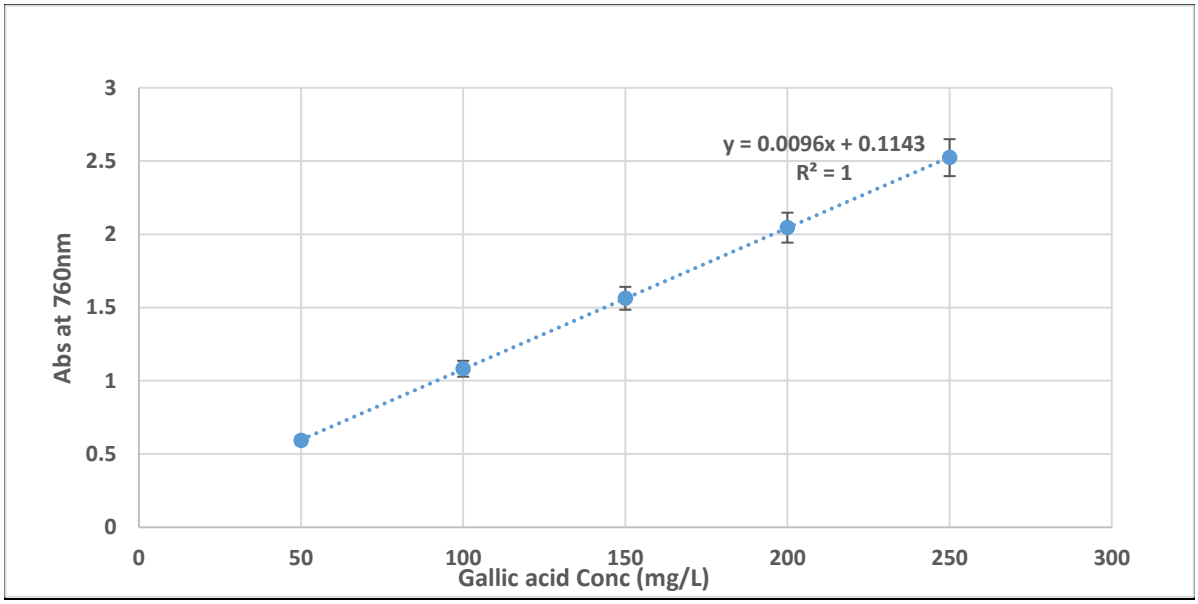


Figure 5.9– Standard curve for total phenolic content of Gallic acid. Gallic acid was used as a standard solution for total phenolic contents. Each value represent the mean ± SD (n=3).

Table 5.5- Total phenolic content in ajwain and nutmeg extracts

Spice extract	1	2	3	Average	Concentration mg/L diluted extract	Concentration in mg/L undiluted extract (multiply with dilution factor 10)
Ajwain aqueous extracts	1.349	1.368	1.382	1.366	130.42 ± 1.72	1304.20 ± 17.2
Ajwain Ethanol extracts	1.612	1.683	1.618	1.63	158.68 ± 4.10	**1586.84 ± 41.01**
Nutmeg aqueous extracts	0.371	0.376	0.385	0.377	27.39 ± 0.73	273.99 ± 7.39
Nutmeg Ethanol extracts	1.345	1.455	1.503	1.43	137.50 ± 8.43	1375.03 ± 84.37

The total phenolic content of ajwain and nutmeg was calculated using standard curve in figure 5.9. The aqueous extract of ajwain is 130mg GAE/L and nutmeg is 27.3mg GAE/L, whereas ethanol extract of ajwain is the highest 158.6mg GAE/L and nutmeg is 137.5mg GAE/L.

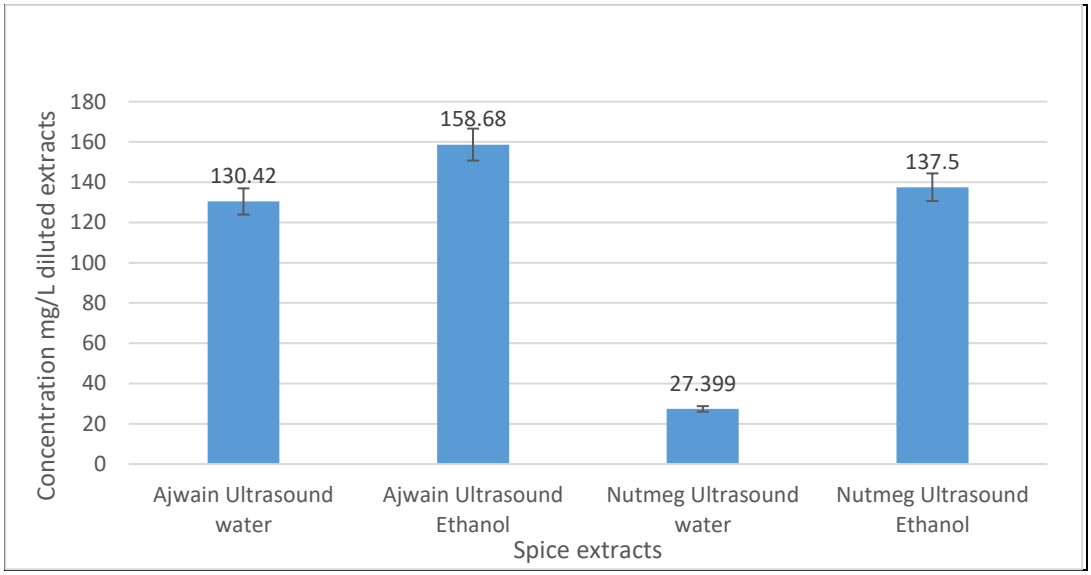


Figure 5.10– Total phenolic content of ajwain and nutmeg extracts in 1g sample. Each value represents the mean ± SD (n=3).

2. FRAP assay

The ferric ion reducing antioxidant power of spice extracts was measured at 593nm. All the analysis were carried out in triplicates and mean values were taken. The standard calibration curve was prepared and shown in Table 5.5 and figure 5.19.

Table 5.6 – Absorbance values for standard calibration curve

Fe2+ conc mM		Absorbance at 593nm			Average Abs
		1	2	3	
0.01	T0	0.009	0.009	0.007	0.009
	T4	0.01	0.01	0.009	
0.025	T0	0.016	0.018	0.018	0.018833
	T4	0.02	0.02	0.021	
0.05	T0	0.03	0.032	0.033	0.035167
	T4	0.037	0.039	0.04	
0.1	T0	0.067	0.068	0.069	0.069167
	T4	0.069	0.071	0.071	
0.25	T0	0.169	0.177	0.177	0.174667
	T4	0.171	0.177	0.177	
0.5	T0	0.36	0.358	0.362	0.360167
	T4	0.362	0.358	0.361	

The values from table was used to plot a standard curve (figure19) which was used to find the corresponding concentration of the spices extracts

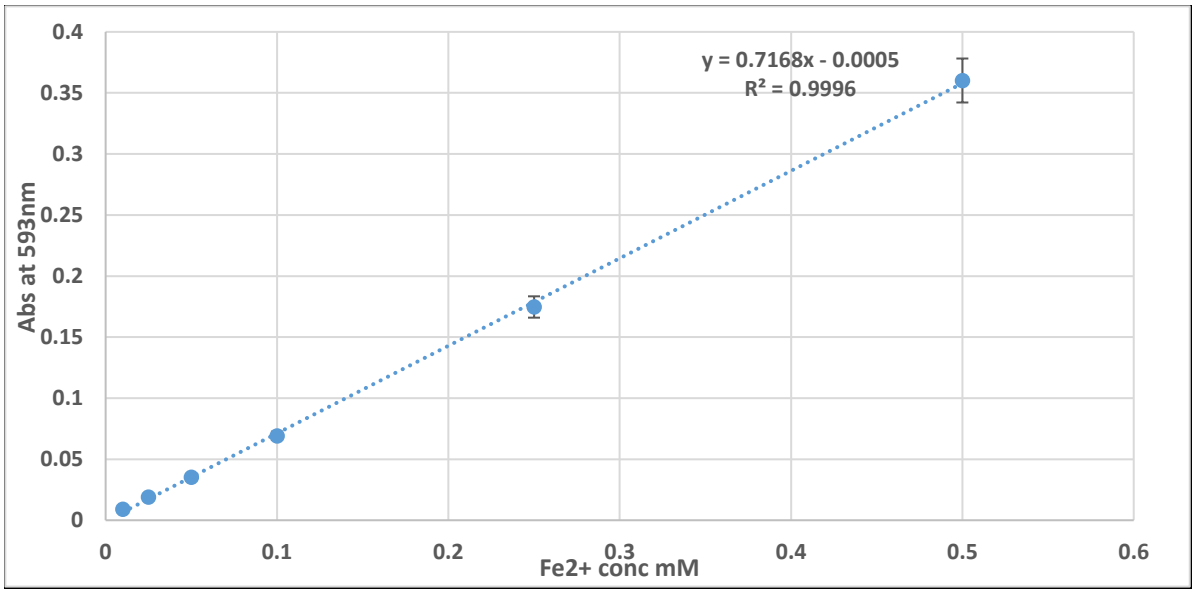


Figure 5.11– Standard FRAP curve. Each value represent the mean ± SD (n=3).

Table 5.7 - Calculated concentration values using the x value of the standard curve

Spice extract	time in min	1	2	3	Average Absorbance at 593nm	Concentration mg/L diluted extract	Concentration in mg/L undiluted extract (multiply with dilution factor 10)
Ajwain aqueous extract	Before t=0	0.708	0.736	0.729	0.745	1.04 ± 0.02	10.4 ± 0.20
	After t= 4	0.983	0.976	0.936	0.918	1.28 ± 0.03	**12.81 ± 0.35**
Ajwain ethanol extract	Before t=0	0.239	0.212	0.275	0.278	0.388 ± 0.04	3.8 ± 0.44
	After t=4	0.483	0.38	0.506	0.479	0.66 ± 0.09	6.69 ± 0.93
Nutmeg aqueous extract	Before t=0	0.201	0.327	0.25	0.308	0.43 ± 0.08	4.3 ± 0.88
	After t=4	0.304	0.552	0.355	0.454	0.63 ± 0.18	6.34 ± 1.82
Nutmeg ethanol extract	Before t=0	0.463	0.734	0.78	0.976	1.36 ± 0.23	13.62 ± 2.38
	After t=4	0.694	1.072	1.059	1.3	1.82 ± 0.29	**18.2 ± 2.99**

Values represent mean of n=3, ± S.D readings.

The FRAP values for ajwain and nutmeg samples were calculated using standard FRAP curve in Figure 5.11. The ajwain aqueous extract contain higher concentration than ethanol extracts in 1g sample. However, nutmeg aqueous extract contains lower concentration than ethanol extracts in 1 g sample.

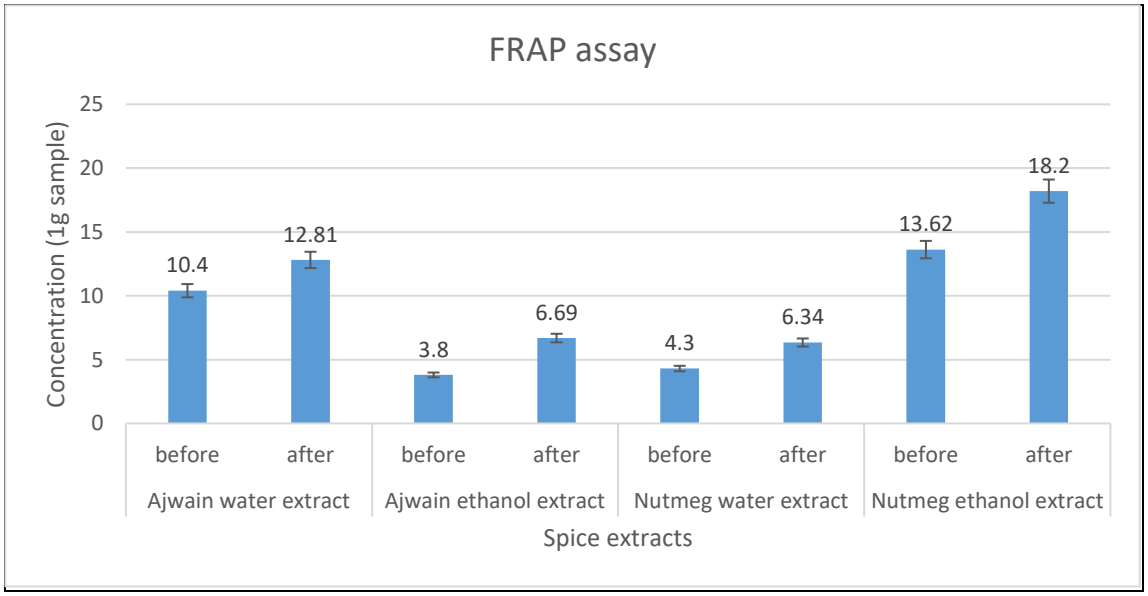


Figure 5.12 – Ferric Reducing Antioxidant capacity of ajwain and nutmeg extracts in 1g sample. Each value represent the mean ± SD (n=3).

3. **DPPH assay -** 2,2-diphenyl-2-picrylhydrazyl hydrate assay

Table 5.8 – Radical scavenging activity of spice extracts

Spice extracts	After			Average	%RSA
	1	2	3		
Ajwain water extract (mg/10ml)	0.161	0.153	0.154	0.149 ± 0.00	90.34 ± 0.28
Ajwain water extract (mg/L diluted extract)	0.242	0.156	0.419	0.449 ± 0.13	70.89 ± 8.68
Ajwain ethanol extract (mg10ml)	0.335	0.23	0.205	0.126 ± 0.06	**91.79 ± 4.46**
Ajwain ethanol extract (mg/L diluted extract)	0.674	0.651	0.648	0.631 ± 0.01	59.08 ± 0.92
Nutmeg water extract (mg/10ml)	0.353	0.347	0.354	0.352 ± 0.00	77.18 ± 0.03
Nutmeg water extract (mg/L diluted extract)	0.679	0.682	0.673	0.672 ± 0.00	56.47 ± 0.45
Nutmeg ethanol extract (mg/10ml)	0.146	0.28	0.238	0.313 ± 0.06	79.70 ± 6.85
Nutmeg ethanol extract (mg/L diluted)	0.562	0.543	0.522	0.502 ± 0.02	67.46 ± 0.20

Values represent mean ± SD of n=3 readings. % RSA in the samples was calculated using the formula.

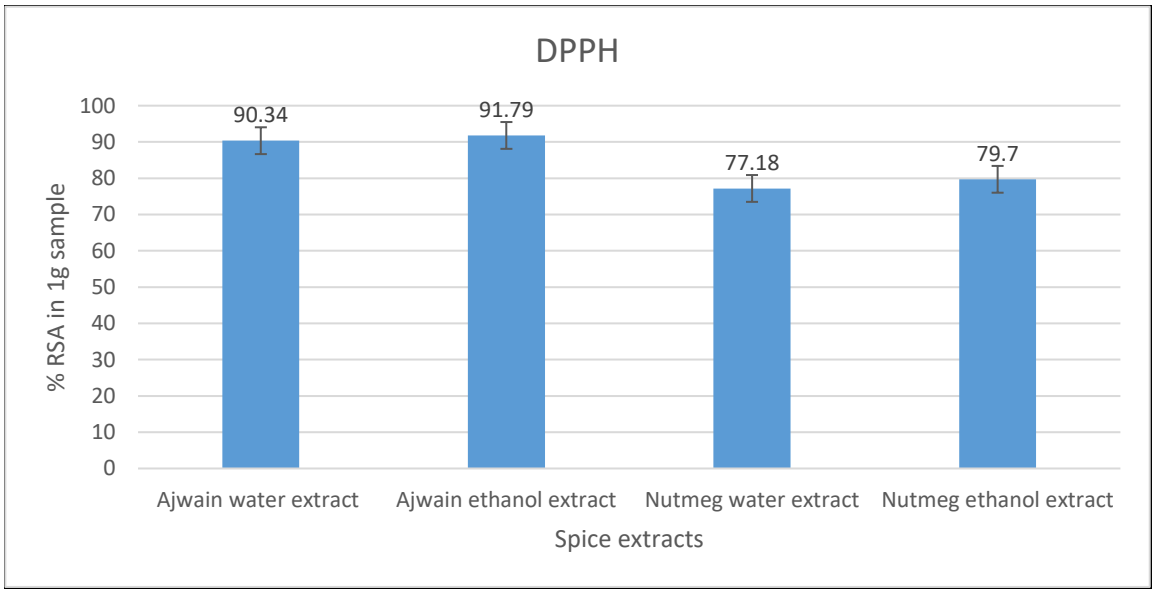


Figure 5.13– Radical scavenging activity of ajwain and nutmeg extracts in 1g sample. Each value represent the mean ± SD (n=3)

Radical scavenging activity of spice extracts was calculated using the formula. The percentage RSA of ajwain ethanol extracts was found to be highest 91.7% and for nutmeg ethanol extract is 79.7%.

5.4.4 Comparison between TPC, FRAP, DPPH and anti-glycation ability of ajwain and nutmeg extracts

In Table 5.9 the properties of TPC, FRAP, DPPH and anti-glycation of ajwain and nutmeg extracts were compared with each other.

Table 5.9– Comparison between TPC, FRAP, DPPH and anti-glycation properties of ajwain and nutmeg extracts

Spice extracts (1g sample)	TPC (mg GAE/L)	FRAP (mg/L)	DPPH (% RSA)	Anti-glycation (% inhibition) Methylglyoxal	Anti-glycation (% inhibition) Glucose
Ajwain aqueous extract	130.42 ± 1.72	12.81 ± 0.35	90.34 ± 0.28	ND	ND
Ajwain ethanol extract	158.68 ± 4.10	6.69 ± 0.93	**91.79** ± 4.46	**66**	**38.2**
Nutmeg aqueous extract	27.39 ± 0.73	6.34 ± 1.82	77.18 ± 0.03	ND	ND
Nutmeg ethanol extract	137.50 ± 8.43	**18.2** ± 2.99	79.7 ± 6.85	60.7	48

ND – not detected in the samples

When TPC and percentage inhibition of glycation (both methylglyoxal and glucose) were compared, ajwain and nutmeg ethanol extracts exhibited a high value as compared to aqueous method. On the other hand, aqueous extracts of ajwain and nutmeg showed low TPC with no percentage inhibition of anti-glycation activity. In contrast, ultrasound-assisted ethanol extracts of ajwain and nutmeg extracts demonstrated high TPC with relatively high anti-glycation property.

Comparison of FRAP to anti-glycation capacity revealed a high value for nutmeg ethanol extracts, while ajwain ethanol extracts displayed low anti-glycation capacity with FRAP. On the other hand, aqueous extracts of ajwain and nutmeg showed low FRAP with no percentage inhibition of anti-glycation activity. In contrast, ultrasound-assisted ethanol extracts of ajwain and nutmeg extracts demonstrated high FRAP with relatively high anti-glycation property.

The link between DPPH and anti-glycation expressed a high value of ajwain and nutmeg ethanol extracts as compared to aqueous extracts which showed high DPPH scavenging

activity with no anti-glycation activity. In contrast, ultrasound-assisted ethanol extracts of ajwain and nutmeg demonstrated high DPPH with relatively high anti-glycation property.

5.5 Discussion

In the present study, glycation of proteins was examined *in vitro* by incubation of proteins with different reducing sugars (methylglyoxal and glucose) for different time periods. Lysozyme protein was used to detect cross-linked AGEs production and measured by SDS PAGE technique. Many researchers have studied glycation of lysozyme *in vitro*, Lysozyme contain 6 lysine and 11 arginine per molecules. Glycation of lysozyme can be achieved by methylglyoxal and glucose (Mashilipa *et al*, 2011; Ramkissoon *et al*, 2013). In the present study, ajwain and nutmeg ultrasound-assisted ethanol extracts showed significant inhibitory potential against protein glycation. Ajwain and nutmeg inhibited *in vitro* AGE formation to 66% and 60% at 100mg/ml concentration respectively. A study by Ramkissoon *et al*. (2013) showed that the positive control aminoguanidine inhibited AGEs formation by 75.9%. However, this method cannot be directly compared to the present study as fluorescent method was used to determine AGEs formation whereas the present study examined crosslinked AGEs by SDS PAGE. The mechanism of action of anti-glycation properties of spice extracts was not explored. However, correlation between total phenol content, anti-oxidative properties and inhibition of glycation was explored. The present study suggest that the anti-glycation properties of spice extracts was correlated with antioxidant activity. Ramkissoon *et al*, (2013) suggested similar results in their study.

The determination of chemical composition of ajwain spice in previous chapter showed that the presence of bioactive polyphenolic compounds such as terpenoids, flavonoids in the spice extract. Ramkissoon *et al,* (2016) reported that the antioxidant and protein glycation inhibition properties of plant material (ten different spice extracts such as thyme, garlic, ginger) are related to the free radical scavenging property of phenolic compounds and flavonoids. In the present study, spice extracts (ajwain and nutmeg) have polyphenolic compounds that may contribute to the anti-glycation and antioxidant properties. The spice extract has shown potential antioxidant properties thus might be helpful in preventing the oxidative stress related diseases. However, the antioxidant properties of the spice extract

might not be the only reason for anti-glycation activities. Some proposed mechanism of anti-glycation action includes – breaking the cross-linked structures in the formed of AGEs, blocking the carbonyl groups and inhibiting the formation of late-stage Amadori products (Adisakwattana *et al.*, 2012; Ramkissoon *et al.,* 2016).

In the present study, different concentrations (mg/ml) of ajwain and nutmeg extracts using stirring and ultrasound-assisted extraction have been studied to investigate inhibitory effect on cross-linked AGEs *in vitro.* The results shown that ajwain and nutmeg extracts have potential in lysozyme-reducing sugars system. The formation of cross-linked AGEs induced by incubation of lysozyme (Elosta *et al,* 2012). This study illustrates for the first time that high to low concentrations of ajwain and nutmeg extracts have significant inhibitory effects on cross-linked AGEs *in vitro.* However, a study by Aljohi *et al* (2016) compared anti-glycation and antioxidant properties of *Momordica charantia* and reported potential effect of different concentration of *M. charantia* on cross-linked AGEs.

The antioxidant results obtained from this study indicate that ajwain extracts more effective than nutmeg extract. This result matches with the similar study, by Choudhary *et al* (2017), reported methanolic extract of ajwain contain higher amount of total phenolic content and showed >90% DPPH radical scavenging activity. The ethanol extracts were found to have higher phenolic content than water (aqueous) extracts. These differences might be due to bioactive compound extraction with solvents. Similar study by Embuscado (2015), explained that antioxidant activity of spices can be determined by total phenol content, FRAP and DPPH and antioxidant efficacy of spices such as thyme, nutmeg and other spice extracts depend upon phenolic profile. The strong antioxidant properties of the spice extract are correlated with the high level of phenolic compounds (Hwang *et al,* 2013).

In the present study, spice extracts (ajwain and nutmeg) indicate potential anti-glycation and antioxidant properties that may offer potential in glycation pathways and against oxidative stress related diseases either as dietary supplements or as glycation inhibitors. However, further investigation are required from crude extract for proper drug development.

5.6 Conclusion

The spice extracts contain polyphenols that possess anti-glycation and antioxidant properties. Daily intake of spice in food may increase antioxidants and decrease the risk of diabetic complications. However, there is insufficient evidence and needs further studies in humans with spices intake to confirm novelty in diabetes management. There is insufficient evidence from current study to support the mechanism of action of the individual phenolic compounds in relation to anti-diabetic effect and further studies are required.

Chapter 6

Overall conclusion

6.1 Introduction

Natural antimicrobial extracts from spices and herbs have shown to have some antimicrobial effect on the major spoilage foodborne microorganisms was studied. The antimicrobial effect was somehow potentiate by the addition of spice and herbs extracts in food industry to prevent growth of foodborne pathogens and food spoilage microorganisms and to enhance the shelf life. Natural spices and herbs extract possess antimicrobial properties, could be good alternative to synthetic chemical preservatives and food additives in food. Spice extracts have shown to inhibit the growth of foodborne pathogenic microorganisms. (Tajkarimi *et al.,* 2010; Burt 2004). Therefore, the use of plant-based antimicrobials has potential to improve the safety and quality of food products. Natural plant-based ingredients can be used in combination with other traditional food preservation methods to improve antimicrobial efficacy and ensure safety of food products.

6.2 Optimization and screening of spices and herbs based on antimicrobial activity

Isolation and purification of antimicrobial extracts from natural plants is a critical step. Conventional techniques for extraction are time-consuming and require a large volume of solvent (Chemat *et al.,* 2017). Ultrasound-assisted extraction method was studied for the extraction of spices and herbs, due to high extraction efficiency, reduced extraction time with high reproducibility (Tiwari, 2015; Asbahani *et al*., 2015)**.** Ethanol extracts of spices and herbs resulted in greater antimicrobial effect than extraction with water. When ethanol was combined with ultrasound-assisted extraction process of spices and herbs showed effective inhibitory activity against foodborne pathogenic microorganisms. From antimicrobial properties analysed using agar disc diffusion assay, it could be seen that ultrasound-assisted ethanol extracts of ajwain, nutmeg, cumin, clove, bay leaves and Indian gooseberry showed effective inhibitory effect against the tested pathogens such as *Bacillus cereus, Listeria monocytogenes, Kocuria rhizophilia, E.coli, Salmonella typhimurium* and *Pseudomonas aeruginosa*. Further antimicrobial properties of six selected spices were anlysed for MIC and

MBC, to determine lowest effective concentration of spice extracts against foodborne pathogens.

6.3 Effect of spice extract on morphology and cellular structure of selected microorganisms

Effect of spice extract and pure bioactive compound on morphological changes and cellular structure in selected bacterial cells was studied using scanned electron microscopy (SEM) images and before and after treated, bacterial images were compared. SEM images reflect the morphological alterations of bacteria membrane when treated with ajwain extracts was reported. Disrupted cell membrane structure and changes in bacteria cells may be due to cell membrane lysis and transformation on permeability of membrane. Therefore, the changes can lead to loss of inner cell content. As indicated in SEM images of pure bioactive compounds, showed morphological alterations of bacterial cell. These bioactive (phenolic) compounds such as thymol, p-cymene and carvacrol responsible for antimicrobial action including cellular disruption, leakage of cell components, pore formation and cell lysis. It is assumed that bioactive compounds in spice extracts is responsible agent for antimicrobial inhibition of bacterial cells. However, the exact mode of antimicrobial action of specific natural components in plant extracts against target microorganisms are still unknown (Gyawali *et al*., 2015). Therefore, these results suggest further investigation of possible mode of action of plant extracts on pathogenic microorganisms. Further research for the justification of this statement was require.

6.4 Bioactive compounds in ajwain extract and potential antimicrobial inhibition

Bioactive chemical compounds composition of ajwain extracts was studied using gas chromatography mass spectrometry (GCMS). The major non-polar chemical compound was found to be thymol, p-cymene and r-terpinene along with a-pinene and b-pinene in traces. However, to understand link between antimicrobial properties and non-polar bioactive compound, correlation graphs were studied. There was no/weak direct correlation was found between either of expected and reported bioactive compound published in literature. Studies shows that chemical structure of bioactive compound such as hydroxyl group of thymol plays

an important role in antimicrobial efficacy (Pisoschi *et al,* 2018). Another compound p-cymene is a precursor for carvacrol compound and work synergistically with compounds responsible for antimicrobial action (Burt, 2004). It was assumed that there might be synergetic effect between major and minor bioactive compounds that are responsible for antimicrobial properties of spice extracts. Therefore, to overcome this problem, the extraction of polar phytochemical compounds of ajwain extracts were studied with and without enzyme treatment using HPLC.

The ajwain polar phytochemical compounds were compared before and after enzyme treatment. The combine use of hesperinidase and cellulase enzyme as an efficient method for releasing phenolic acid and flavonoids were reported. To understand link between antimicrobial properties and polar phytochemicals, correlated graphs were studied. It was found that there is some correlation between phytochemical content of spice extract with antimicrobial properties. However, weak direct correlations with individual polar bioactive compounds were found except for thymol and epicatechin against *Salmonella* and *Pseudomonas*. Stronger correlation was found with total non-polar and polar phytochemical compounds. It was assumed that these non-polar and polar bioactive compounds act synergistically to show increase antimicrobial action against foodborne pathogenic microorganisms.

6.5 Antiglycation and antioxidant properties of spice extracts

In the present study, anti-glycation and antioxidant properties of spice (ajwain and nutmeg) extract was also studied. The spice extracts, ajwain and nutmeg ultrasound-assisted ethanol extracts showed significant inhibitory potential against protein glycation. However, to understand link between potential bioactive compounds present in spice extract and responsible for anti-glycation, antioxidant properties, comparative graphs were studied. The ethanol extracts were found to have higher phenolic content than water (aqueous) extracts. These differences might be due to bioactive compound extraction with solvents. The strong antioxidant properties of the spice extract are related with the high level of phenolic compounds. Further investigation to identify individual phenolic compounds in relation to anti-diabetic effect is required to understand mechanism of action on diabetes and its

complications, *in-vivo* study is required or some clinical trials to understand the potential use of spices in daily basis and any further recommendations. The purpose to investigate the anti-glycation and antioxidant properties of common spices and herbs is an attempt to find the link between common spices and diabetes. Research on spices were carried out to find a link that could lead to practical home-based recommendations for dietary modifications as the treatment for diabetes.

6.6 Is antimicrobial activity of spices and herbs effective in food system?

The antimicrobial effect of spice and herbs extracts has been demonstrated mainly *in vitro*. In food system, the amount of spices required are so high than used in laboratory work. Spices have strong aroma and flavour even at low concentration. The use of high levels are not always acceptable organoleptically. Number of *in vitro* studies have been done on antimicrobial potential of spices, which did not produce marked inhibition as many pure standard phenolic compounds. This is because of phytochemicals in these spice extracts that act synergistically with other compounds may influence by presence of other nutrients in foodstuffs and with food heat processing techniques. Moreover, amount of phytochemicals in spices and herbs depend on number of factors such as geographical, ecological, seasonal conditions, age of plant, harvesting time, and method of extraction. These are the major issues for their application as natural food preservatives. Spices and herbs are natural ingredient must themselves be free from growth of pathogenic microorganisms. Besides all these issues when consumer demand for natural preservative, spices and herbs could be good alternative for natural antimicrobial agent. The problems with the use of spices could be resolved by using combine range of processes, using individual or combination of spices may be with other plant extracts in low concentration to give natural, safe and quality food product.

6.7 Gap in the knowledge for setting quality standards for spices

One of the study aims was to establish key quality markers for producers wishing to use natural plant extracts as alternatives to synthetic chemical preservatives in food safety and

preservation. Ultrasound-assisted ethanol extracts of Indian origin spices and herbs showed potential antimicrobial properties in inhibition of foodborne pathogenic microorganisms. There is gap in knowledge of setting quality standards for spices trading. The quantification of bioactive components present in ajwain spices extract has showed potential antimicrobial properties, could be useful to those who need to perform analysis on ajwain spice. Therefore, quality standards could be set for ajwain spice trading. The bioactive phytochemical component in spice (ajwain) extract showed potential could be relevant to quality maker.

6.8 Further research recommendation

In the present study, correlation between individual phenolic compound and antimicrobial properties showed weak correlation whereas stronger for multiple (total) phenolic compounds contributing to overall antimicrobial effect. However, further research is warranted to determine the mechanisms by which phenolic compounds exert their antimicrobial properties, and how this might then translate into optimising the phenolic profile and thus potency, of natural, plant-derived food preservatives. The results highlight strong antimicrobial, anti-glycation and antioxidant properties of ajwain due to good source of phenolic compounds. Therefore, ajwain may be formulated as plant-based food preservative a good alternative to synthetic chemical preservative for enhancement of shelf life.

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Appendix A - Optimization of spice and herbs based on different ratios

Table 1 – Optimization of spice and herb water extracts based on different ratios

		Water extracts - stirring method					
		Bacillus	***Listeria***	***Kocuria***	***E.coli***	***Salmonella***	***Pseudomonas***
	01:10	0	0	0	3	4	4
	01:20	2	0	0	0	0	0
Coriander	01:30	2	0	0	0	0	0
	01:10	4	3	6	0	0	4
	01:20	2	0	4	0	0	3
Cumin	01:30	1	0	2	0	0	0
	01:10	4	3	4	0	0	3
	01:20	2	0	2	0	0	0
Thyme	01:30	1.5	0	1	0	0	0
	01:10	0	0	0	0	0	0
	01:20	0	0	0	0	0	0
	01:30	1	0	0	0	0	0
	01:40	0.5	0	1	0	0	0
Fenugreek	01:50	1	0	2	0	0	0
	01:10	0	0	0	0	0	0
Mustard	01:20	0	4	0	0	0	0

Disc diameter 6mm excluded from all the readings. ZoI measuresd in mm.

Table 2 – Optimization of spice and herbs ethanol extracts based on different ratios

		Ethanol extracts - stirring method					
		Bacillus	***Listeria***	***Kocuria***	***E.coli***	***Salmonella***	***Pseudomonas***
	01:10	12	9	9	12	12	9
	01:20	9	6	6	6	8	6
Coriander	01:30	9	4	4	4	4	4
	01:10	12	12	9	12	9	6
	01:20	9	9	6	9	9	6
Cumin	01:30	9	9	6	9	9	4
	01:10	14	9	9	12	9	10
	01:20	12	6	6	9	9	9
Thyme	01:30	10	6	6	9	9	9
	01:10	9	6	6	6	9	6
	01:20	9	8	6	6	6	6
	01:30	9	8	8	6	6	6
	01:40	9	6	8	6	6	4
Fenugreek	01:50	9	6	8	8	6	4
	01:10	9	6	9	9	9	8
Mustard	01:20	9	6	7	9	9	7
Ethanol		9	6	6	9	9	4

Disc diameter 6mm excluded from all the readings. ZoI measuresd in mm. If the value against selected microorganism is same as Ethanol value that shows no inhibition against pathogen.

2. Optimization of spice and herb extracts using different ethanol concentration

Table 3 – Optimization of spice and herb ethanol extracts using different ethanol concentration on coriander sample

	Ethanol concentration – Coriander Stirring method		
	50%	**70%**	**Absolute**
Bacillus	0	0	6
Listeria	0	4	6
Kocuria	4	6	6
E.coli	0	4	6
Salmonella	0	4	6
Pseudomonas	4	6	6

Disc diameter 6mm excluded from all the readings. ZoI measuresd in mm.

3. Optimization of spice and herb extraction using different methods

Table 4– Optimization of spice and herb extraction using different methods on fenugreek ethanol extract

	Fenugreek - 1:10 ratio used, ethanol extracts			
	Separating funnel	**Stirring**	**Ultrasound**	**Ethanol**
Bacillus	9	9	9	9
Listeria	6	8	8	6
Kocuria	6	9	9	6
E.coli	9	9	9	9
Salmonella	9	12	12	9
Pseudomonas	6	8	6	4

Disc diameter 6mm excluded from all the readings. ZoI measuresd in mm. If the value against selected microorganism is same as Ethanol value that shows no inhibition against pathogen.

Table 5– Optimization of extraction methods using coriander water or ethanol extracts

	Coriander 1:10 ratio – Different extraction methods				
		Ethanol			
	Water	**Ultrasound**	**Separating funnel**	**Stirring**	**Ethanol**
Bacillus	0	12	12	12	9
Listeria	4	14	9	9	6
Kocuria	0	6	6	6	6
E.coli	6	12	12	9	9
Salmonella	4	12	12	12	9
Pseudomonas	4	9	9	9	4

Disc diameter 6mm excluded from all the readings. ZoI measuresd in mm.

4. Optimization of spice and herb ultrasound extraction based on different settings

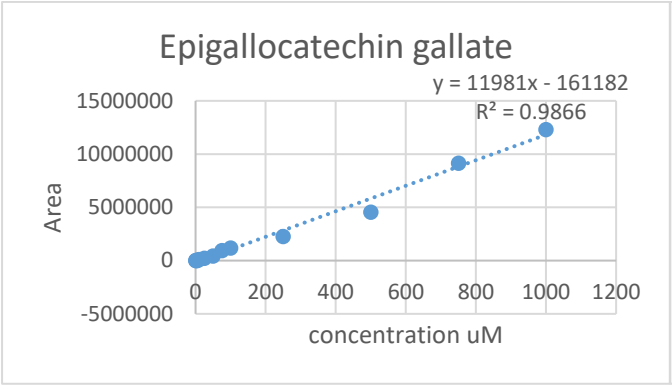
Table 6– Optimization of spice and herb ultrasound-assisted extraction based on different ultrasound settings on fenugreek sample

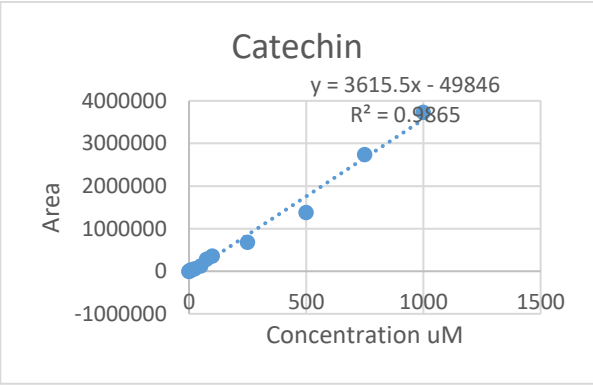
	Ultrasound setting 20% and 100% amplitude - Ethanol extracts of Fenugreek						
	01:10		01:20		01:30		
	20%	100%	20%	100%	20%	100%	Ethanol
Bacillus	9	9	9	9	9	9	9
Listeria	14	6	14	9	14	9	6
Kocuria	12	6	15	9	12	12	6
E.coli	12	10	14	9	14	9	9
Salmonella	12	9	12	12	12	12	9
Pseudomonas	9	9	9	9	12	9	4

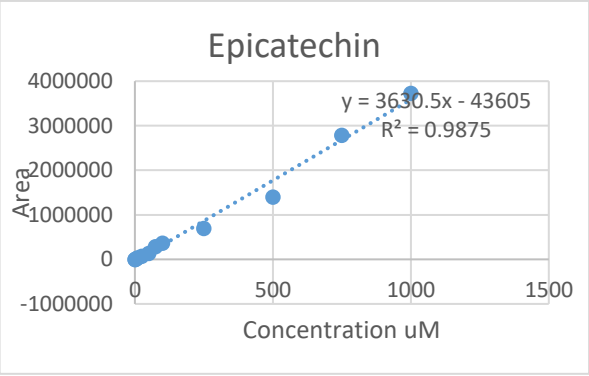
Disc diameter 6mm excluded from all the readings. ZoI measuresd in mm.

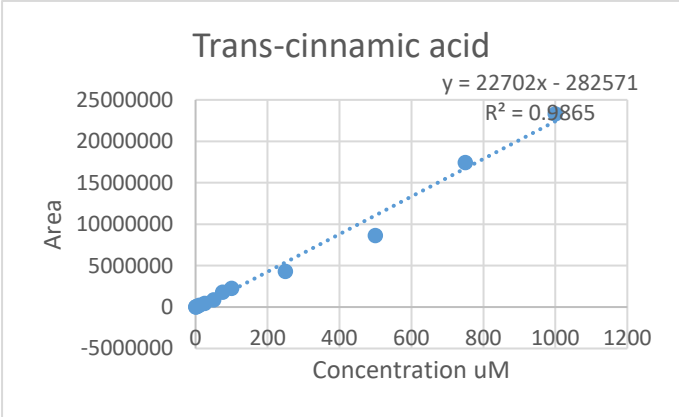
Appendix

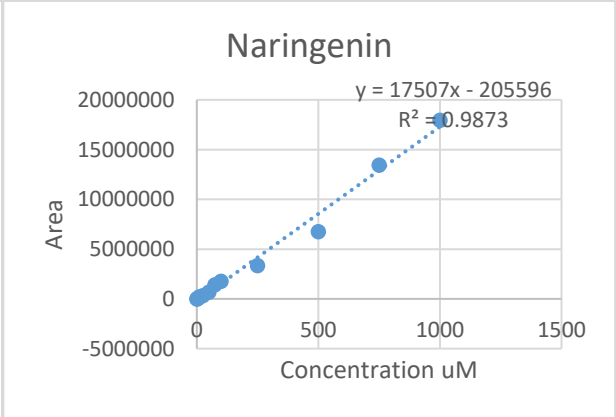
B - Standard curves for polar phenolic compounds

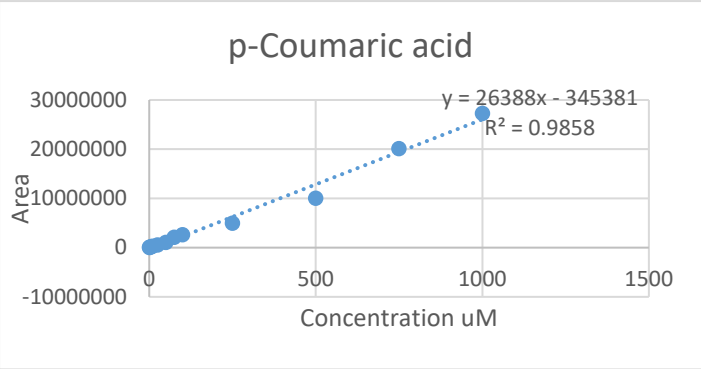











p-Coumaric acid
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R² = 0.9858
Area
Concentration uM
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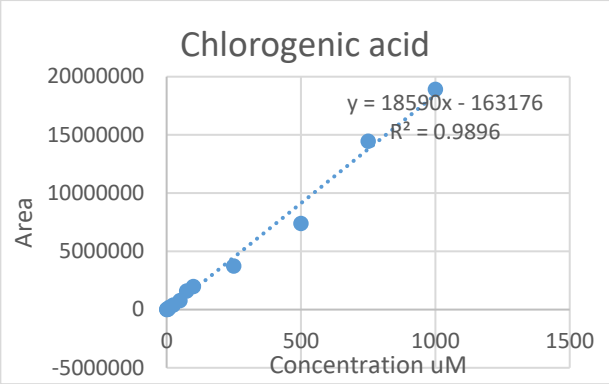
20000000
10000000
0
-10000000
0
500
1000
1500


Chlorogenic acid
y = 18590x - 163176
R² = 0.9896
Area
Concentration uM
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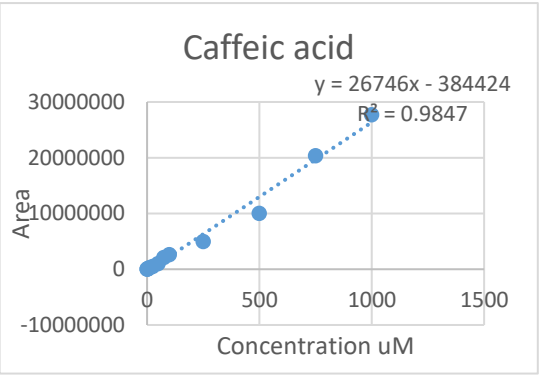
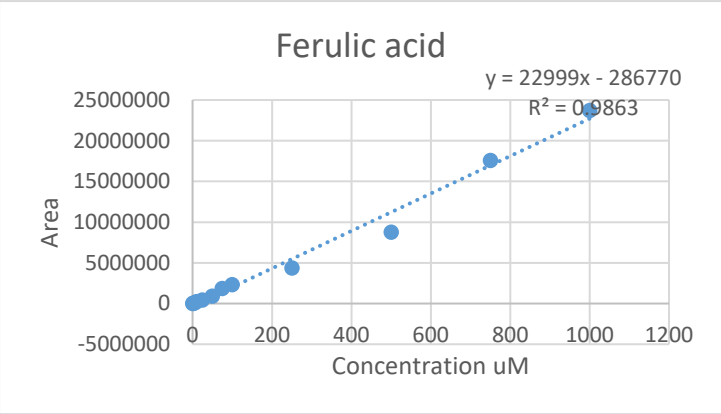
15000000
10000000
5000000
0
-5000000
0
500
1000
1500


Caffeic acid
y = 26746x - 384424
R² = 0.9847
Area
Concentration uM
30000000
20000000
10000000
0
-10000000
0
500
1000
1500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y = 22999x - 286770
R² = 0.9863
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Concentration uM
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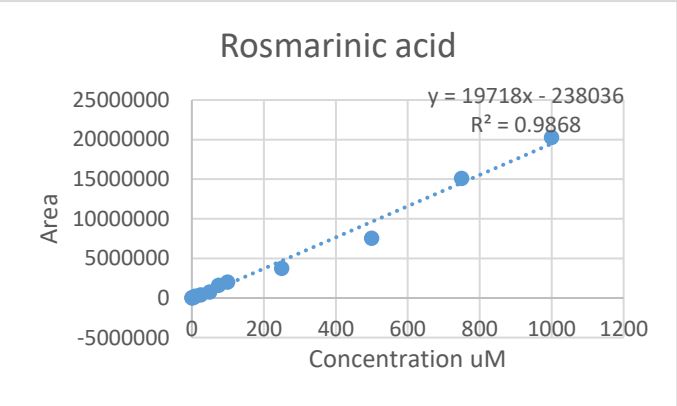
20000000
15000000
10000000
5000000
0
-5000000
0
200
400
600
800
1000
1200


Rosmarinic acid
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R² = 0.9868
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Concentration uM
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20000000
15000000
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5000000
0
-5000000
0
200
400
600
800
1000
1200

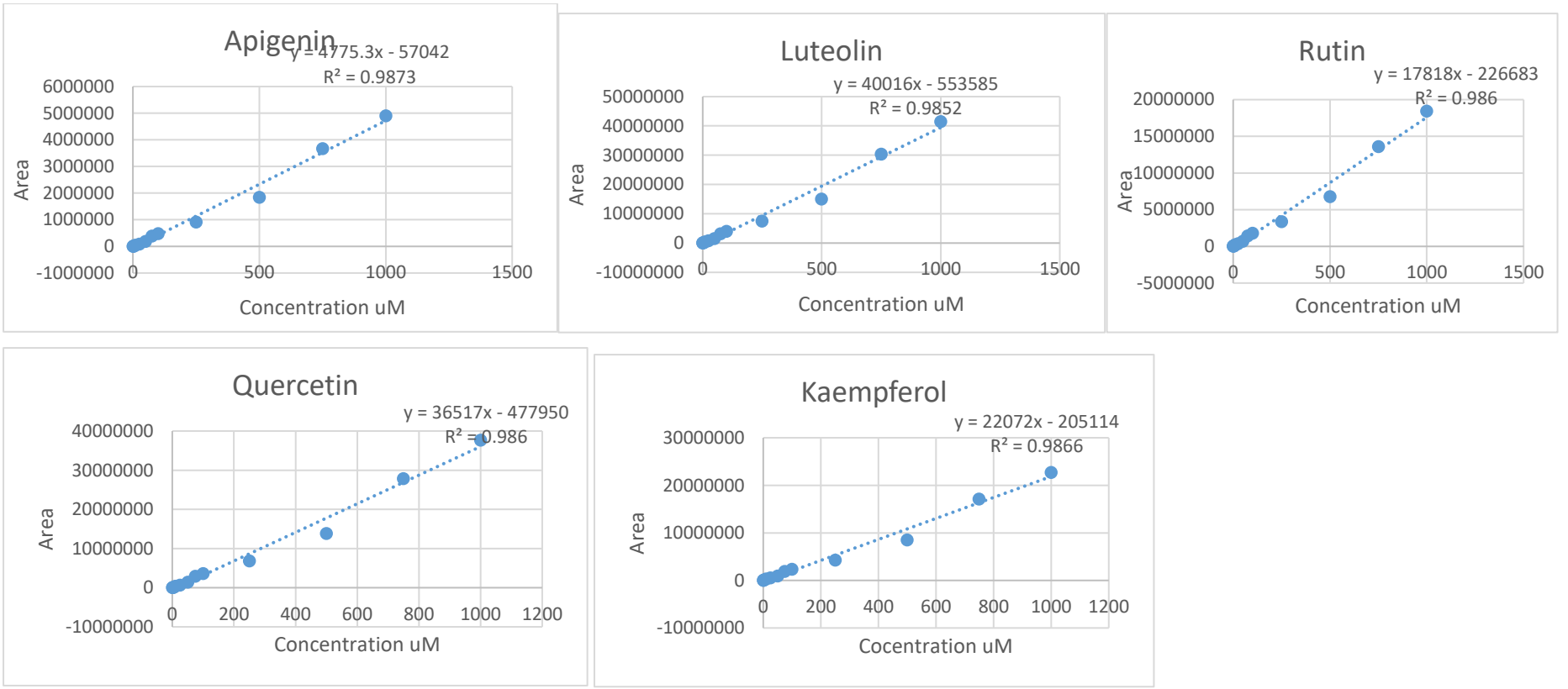

Figure 1- Standard curves for polar phenolic compounds used for quantification of ajwain extract

C- Correlation graphs between non-polar bioactive compounds and antimicrobial properties

1. Correlation between antimicrobial properties (ZoI) and ρ-cymene concentration

Another bioactive compound identified in GCMS analysis was p-cymene. Studies showed that p-cymene is responsible agent and act as precursor for other compound, which act as antimicrobial agent. The results obtained in correlation analysis of p-cymene concentration and antimicrobial properties are presented in Table 1.

Table 1- Correlation between antimicrobial properties (ZoI) and p-cymene concentration (mg/100g of ajwain samples)

Ajwain samples	ρ-cymene concentration (mg/100g ajwain)	Zone of Inhibition (mm)					
		Bacillus	***Listeria***	***Kocuria***	***Ecoli***	***Salmonella***	***Pseudomonas***
1	67.5	18	14	14	14	12	9
2	38.0	9	14	16	9	9	9
3	56.4	9	12	14	9	9	14
4	75.2	9	6	6	9	9	9
5	44.5	12	12	15	12	9	9
6	0	9	6	6	10	9	4
7	71	12	6	6	14	14	9
8	51.9	9	6	6	12	12	9
9	79.3	15	14	14	14	14	14
10	75.6	12	15	14	12	14	12

Zone of Inhibition measured in mm. Disc diameter 6mm excluded from the readings. Mean of n=3 replicates ± S.D. (0 – Not detected in the sample).

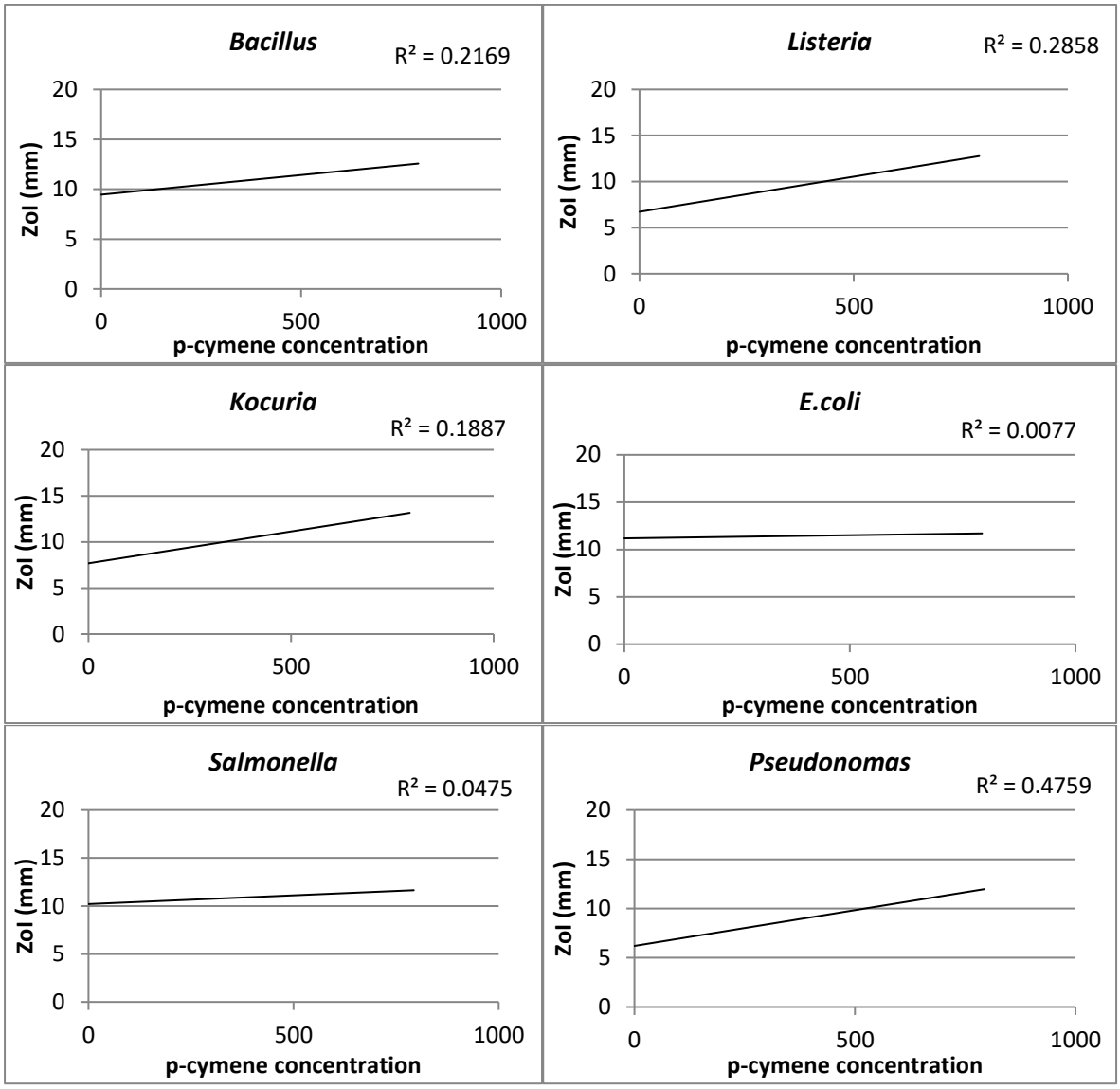

Figure 2– Effect of p-cymene concentration of ajwain ethanolic extracts on antimicrobial properties of ajwain extracts against different microorganisms

When p-cymene concentration and antimicrobial properties of 10 distinct ajwain samples were compared, higher correlation were observed for *Pseudomonas* in Figure 2. Studies showed that p-cymene act as precursor for carvacrol and other bioactive compounds, which are responsible for antimicrobial agents (Burt, 2004; Tajkarimi *et al,* 2010; Pisoschi *et al,* 2018). However, no direct correlation was exhibited against all tested pathogenic microbes. It was assumed that some other bioactive compound might be responsible for antimicrobial action.

2. Correlation between antimicrobial properties (ZoI) and ϒ-terpinene concentration

One more bioactive compound identified in GCMS analysis was r-terpinene. The results obtained in correlation analysis of r-terpinene concentration and antimicrobial properties are presented in Table 2.

Table 2- Correlation between antimicrobial properties (ZoI) and r-terpinene concentration of ajwain samples

Ajwain samples	ϒ-terpinene concentration (mg/100g ajwain)	Zone of Inhibition (mm)					
		Bacillus	***Listeria***	***Kocuria***	***E.coli***	***Salmonella***	***Pseudomonas***
1	164.5	18	14	14	14	12	9
2	87.6	9	14	16	9	9	9
3	159.6	9	12	14	9	9	14
4	269.1	9	6	0	9	9	9
5	0	12	12	15	12	9	9
6	0	9	6	6	10	9	4
7	232.2	12	6	6	14	14	9
8	128.4	9	6	6	12	12	9
9	255.0	15	14	14	14	14	14
10	225.8	12	15	14	12	14	12

Zone of Inhibition measured in mm. Disc diameter 6mm excluded from the readings. Mean of n=3 replicates ± S.D. (0 – Not detected in the sample).

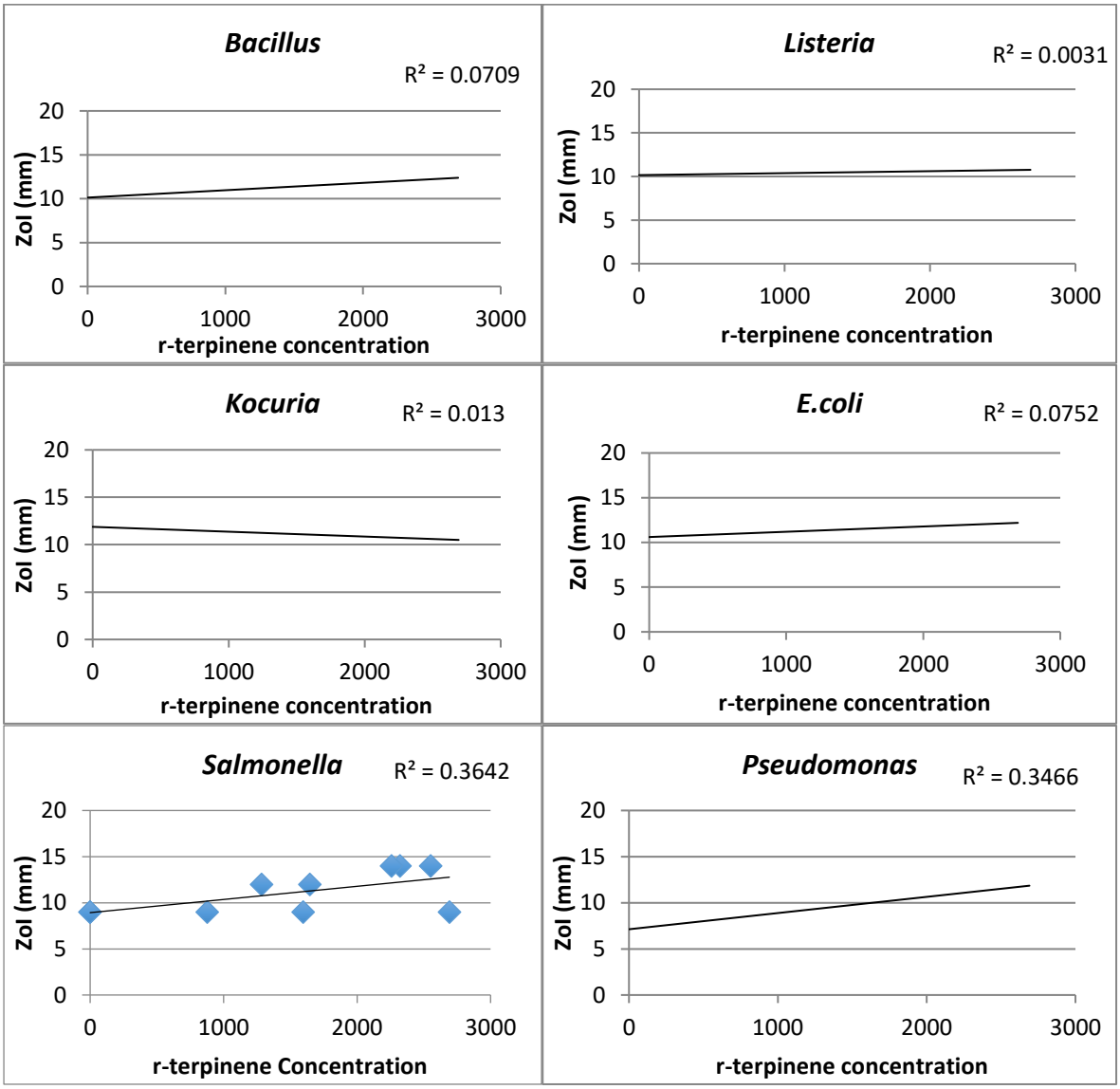

Figure 3– Effect of r-terpinene concentration of ajwain ethanolic extracts on antimicrobial properties of ajwain extracts against different microorganisms

When r-terpinene concentration and antimicrobial properties of 10 distinct ajwain samples were compared, no direct correlation against all tested pathogenic microbes were observed in Figure 3. It was assumed that some other bioactive compound might be responsible for antimicrobial action or these compounds act synergistically with other compounds.

D- Correlation graphs between polar phenolic compounds and antimicrobial properties

1. Caffeic acid

The results obtained in correlation analysis of caffeic acid (mg/100g ajwain powder) and antimicrobial properties are presented in Table 3. The higher concentration of caffeic acid were found in ajwain samples.

Table 3: Correlation between antimicrobial properties (ZoI) and caffeic acid of ajwain samples

Ajwain sample	mg caffeic acid/100g ajwain powder	Antimicrobial inhibition (Zone of inhibition in mm)					
		Bacillus	***Listeria***	***Kocuria***	***E.coli***	***Salmonella***	***Pseudomonas***
1	1208	18	14	14	14	12	9
2	1560	9	14	16	9	9	9
3	810	9	12	14	9	9	14
4	732.4	9	6	6	9	9	9
5	1153	12	12	15	12	9	9
6	347.3	9	6	6	10	9	4
7	1513	12	6	6	14	14	9
8	1743.3	9	6	6	12	12	9
9	1650.3	15	14	14	14	14	14
10	1180.8	12	15	14	12	14	12

Zone of Inhibition measured in mm. Disc diameter 6mm excluded from the readings. Mean of n=3 replicates ± S.D.

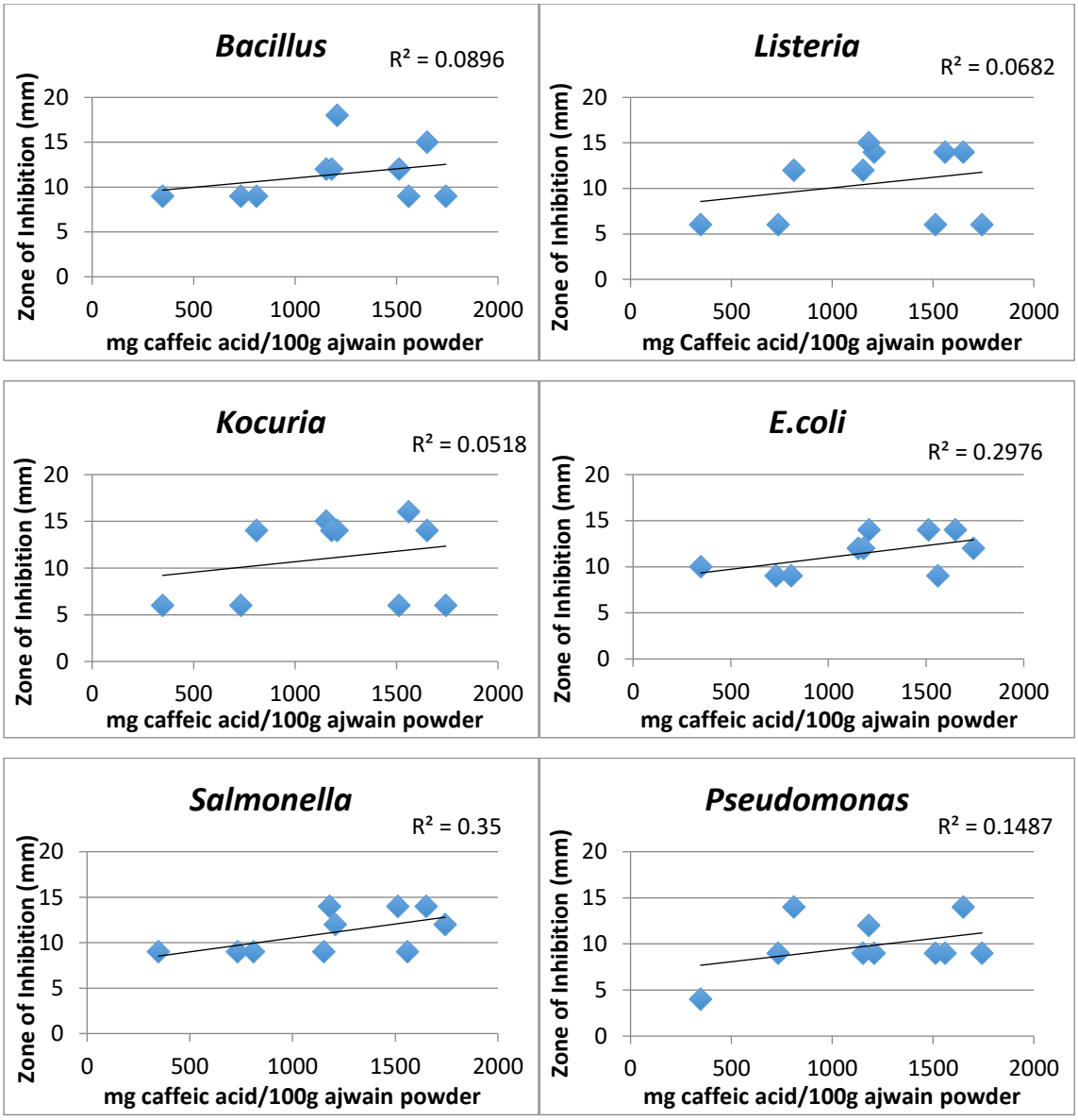

Figure 4- Effect of caffeic acid of ajwain ethanolic extracts on antimicrobial properties of ajwain extracts against different microorganisms

When caffeic acid concentration and antimicrobial properties of 10 distinct ajwain samples were compared, no direct correlation against all tested pathogenic microbes were observed in Figure 4. It was assumed that some other polar phenolic compound might be responsible for antimicrobial action or these compounds act synergistically with other compounds.

2. Trans-cinnamic acid

Trans-cinnamic acid was another abundant compound found after hydrolysis of ajwain samples. Hence, selected for correlation with antimicrobial properties of ajwain samples against foodborne pathogens. The results obtained in correlation analysis of trans-cinnamic acid (mg/100g ajwain powder) and antimicrobial properties are presented in Table 4.

Table 4: Correlation between antimicrobial properties (ZoI) and trans-cinnamic acid of ajwain samples

Ajwain sample	mg trans-cinnamic acid/100g ajwain powder	Antimicrobial inhibition (Zone of Inhibition in mm)					
		Bacillus	***Listeria***	***Kocuria***	***E.coli***	***Salmonella***	***Pseudomonas***
1	111.2	18	14	14	14	12	9
2	105.4	9	14	16	9	9	9
3	73.9	9	12	14	9	9	14
4	78.6	9	6	6	9	9	9
5	22.7	12	12	15	12	9	9
6	108.4	9	6	6	10	9	4
7	116.9	12	6	6	14	14	9
8	111.3	9	6	6	12	12	9
9	123	15	14	14	14	14	14
10	85	12	15	14	12	14	12

Zone of Inhibition measured in mm. Disc diameter 6mm excluded from the readings. Mean of n=3 replicates ± S.D.

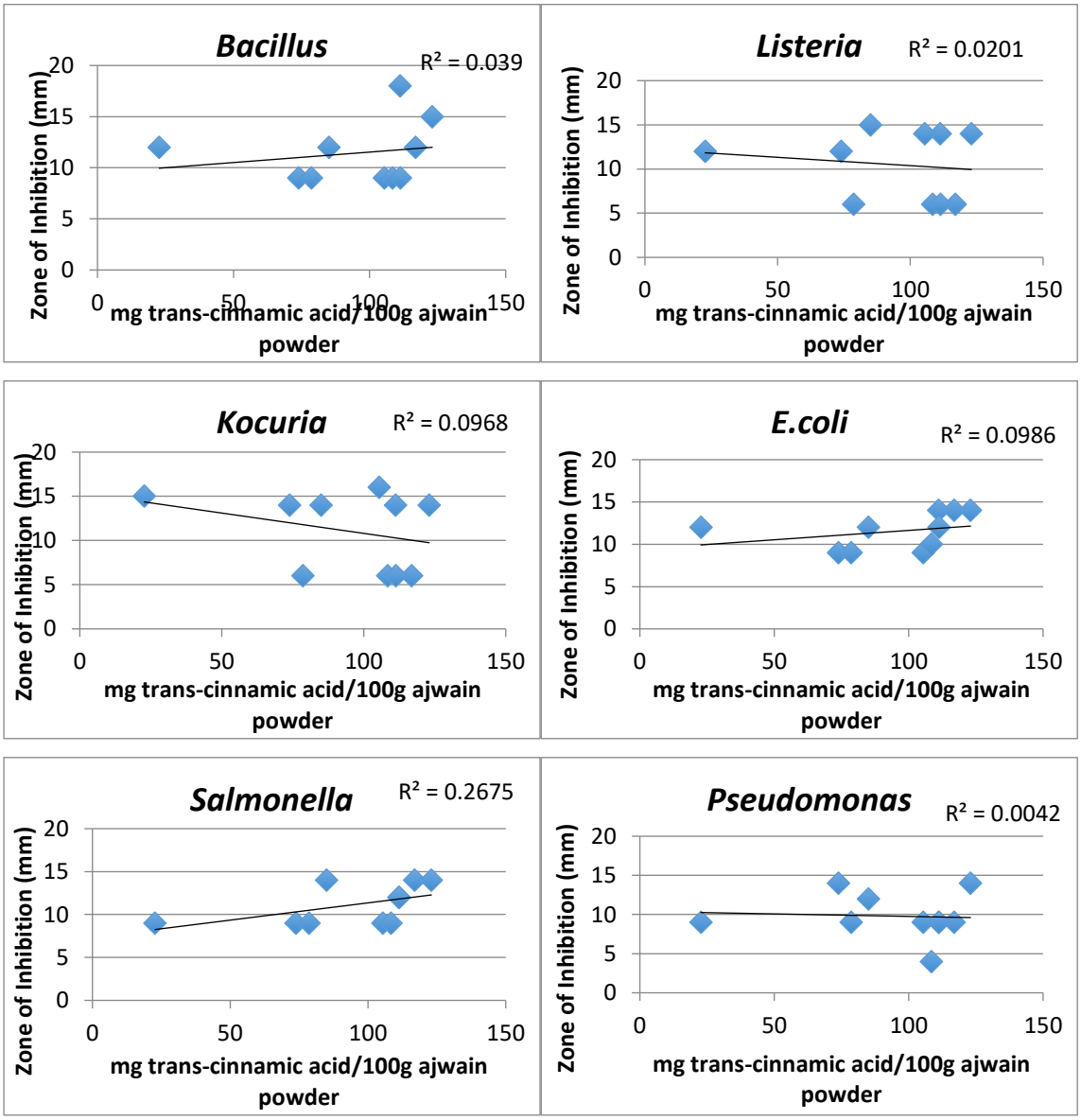

Figure 5 - Effect of trans-cinnamic acid of ajwain ethanolic extracts on antimicrobial properties of ajwain extracts against different microorganisms

The closer data points were found for *Salmonella*, R^2 value was very low as compared to other compounds. When trans-cinnamic acid concentration and antimicrobial properties of 10 distinct ajwain samples were compared, very poor correlation against all tested pathogenic microbes were observed in Figure 5. Ajwian sample 10 showed low concentration of trans-cinnamc acid and high antimicrobial effect against pathogenic microbes. It was assumed that some stronger polar phenolic compound might be responsible for antimicrobial action or these compounds act synergistically with other compounds.

3. Quercetin

After looking at most abundant phenolic compounds such as epicatechin and trans-cinnamic acid, individual effect on antimicrobial properties of ajwain samples, flavonoid such as quercetin found in abundant concentration. Hence, selected for correlation study. The results obtained in correlation analysis of quercetin (mg/100g ajwain powder) and antimicrobial properties are presented in Table 5.

Table 5: Correlation between antimicrobial properties (ZoI) and quercetin of ajwain samples

Ajwain sample	mg Quercetin/100g ajwain powder	Antimicrobial inhibition (Zone of inhibition in mm)					
		Bacillus	***Listeria***	***Kocuria***	***E.coli***	***Salmonella***	***Pseudomonas***
1	29	18	14	14	14	12	9
2	39.8	9	14	16	9	9	9
3	26	9	12	14	9	9	14
4	25.6	9	6	6	9	9	9
5	101.4	12	12	15	12	9	9
6	22.8	9	6	6	10	9	4
7	39.7	12	6	6	14	14	9
8	40.7	9	6	6	12	12	9
9	39.6	15	14	14	14	14	14
10	22.9	12	15	14	12	14	12

Zone of Inhibition measured in mm. Disc diameter 6mm excluded from the readings. Mean of n=3 replicates ± S.D.

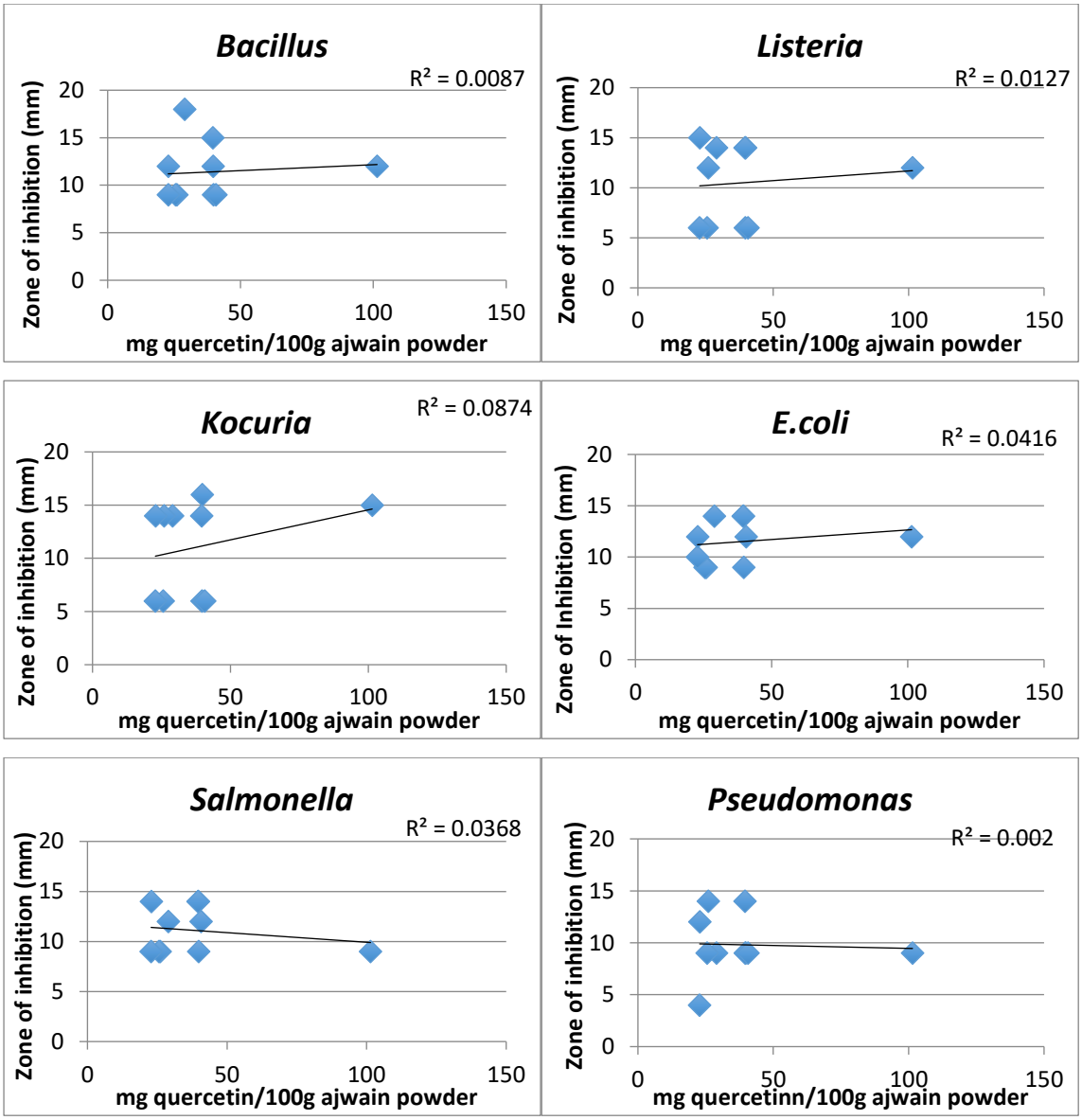

Figure 6 - Effect of quercetin of ajwain ethanolic extracts on antimicrobial properties of ajwain extracts against different microorganisms

The data points were far from linearity for all tested microorganisms was observed for quercetin of all ajwain samples. When quercetin concentration and antimicrobial properties of 10 distinct ajwain samples were compared, very poor correlation against all tested pathogenic microbes were observed in Figure 6. Ajwain sample 5 showed more concentration of quercetin as compared to other ajwain samples.

Publications and Conference presentations

- Article published in IFST Sept 2019 issue 3. Antimicrobial properties of bioactive compounds of Indian spices and herbs in food
- Oral Presentation at 'IFST Young Scientist Competition': Final Round, London Metropolitan University, UK, 18th June 2019. Winner of the competition and awarded Young Postgraduate Scientist of the year 2019.
- Oral Presentation at 'IFST Young Scientist Competition': North of England Regional Round (Winner of the North England regional round), 5th June 2019.
- Image Presentation at Science and Engineering Research Symposium, Manchester Metropolitan University, Sept 2019.
- Participated in 3MT (Minute thesis) competition, Manchester Metropolitan University, UK (May 2019).
- Oral and poster presentation at 11th PGR Conference, Manchester Metropolitan University, March 2019.
- Image of research competition (Thinking creatively about your research and its impact), organised by Manchester Metropolitan University Research and Knowledge Exchange. Abstract and Research Image though not shortlisted, was highly commended by shortlisting team, and was featured in a slideshow as art of the image of research exhibition which took place at the Grosvenor Gallery MMU, UK 26th Feb 2019 till 7th March 2019.
- Participated in Manchester Science Festival (Seven thousand feet), Oct 2018 – Presented Research work and talked about anti-diabetic food such as spices and Karela (bitter melon). Seven thousand feet – Christine Wilcox-Baker received 'making a difference' award and outstanding contribution for patient and public involvement and engagement 2019 by University of Manchester.
- Poster Presentation at Science and Engineering Research Symposium, Manchester Metropolitan University, Sept 2017.
- Oral Presentation at FRIHSC, Manchester Metropolitan University, July 2017.