UNIVERSITY OF PÉCS

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Bioactivities and potential beneficial properties of propolis ethanolic extract, caffeic acid phenethyl ester, and Arabic coffee beans extract

Ph.D. thesis

Ibrahim Ismail Sallam Alfarrayeh

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Signature of the Supervisor

Signature of the Head of Doctoral Shool

PÉCS, 2021.

Dedication

To the soul of my father,

my mother,

my soul mate (Dema),

my lovely daughter (Dana).

Ibrahim Alfarrayeh

Acknowledgments

Praise be to ALLAH, the lord of all creatures who gave me the power to believe in myself and pursue my dreams. I could never have done this without the faith I have in you.

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Ibrahim Alfarrayeh

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List of Abbreviations

Abs	Absorbance
Aif1p	Apoptosis-inducing factor
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
Bir1p	Yeast survivin
CaMca1p	*Candida* metacaspase
CAPE	Caffeic acid phenethyl ester
CAS	Caspofungin
CDR	*Candida* drug resistance
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DMSO	Dimethyl sulfoxide
DSC	Differential scanning calorimetry
DTT	Dithiothreitol
EPS	Extracellular polymeric substances
EGTA	Ethylene glycol-bis(2-aminoethylether)-N N N′N′-tetraacetic acid
GI	Gastrointestinal
Hsp70	Protein chaperone DnaK
IC50	Concentration providing 50% inhibition of DPPH
MBIC	Minimum biofilm inhibitory concentration
MDR	Multi drug resistance
MIC	Minimum inhibitory concentration
MRS	De Man, Rogosa, and Sharpe
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSSA	Methicillin-sensitive *Staphylococcus aureus*
Ndi1p	Yeast apoptosis-inducing factor-homologous mitochondrion-associated inducer of death
Nma111p	Serine protease HtrA2/Omi
Nuc1p	Yeast endonuclease G
PBS	Phosphate-Buffered Saline
PEE	Propolis ethanolic extract

PI	Propidium iodide
ROS	Reactive oxygen species
SD	Standard deviation
SEM	Scanning electron microscopy
SZMC	Szeged Microbiological Collection
TEM	Transmission electron microscope
T_m	Melting temperature
Yca1p	Yeast caspase
YPD	Yeast extract peptone dextrose
Z-VAD-FMK	Carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]- fluoromethylketone

1. Introduction

Contagious diseases caused by various pathogens including bacteria, fungi, parasites, and viruses are a major concern for public health authorities, despite the enormous progress in medicine. Multiple drug resistance in pathogenic microorganisms has been evolved during recent years, due to the evolution and transmission of resistance genes, indiscriminate use of antibiotics in humans, and random use of antimicrobial agents in animals and agriculture. This condition, alongside the undesirable side-effects of some antibiotics and the appearance of previously uncommon infections, is a serious medical problem, so it is necessary to search for new potential active substances against these pathogenic microorganisms, which leads to the production of new effective antibiotics (Alfarrayeh and Tarawneh, 2013; Jamrozik and Selgelid, 2020).

In recent years, the biological activities of natural products have received much attention because of the increasing interest in human health. Many natural products have been studied *in vitro* and *in vivo* by many researchers to investigate their potential antioxidant and antimicrobial properties (Bernardini et al., 2018; Er, 2021; Gyawali and Ibrahim, 2014; Kokoska et al., 2019; Okińczyc et al., 2020). One of the most important natural products that are commonly used for their antimicrobial properties is propolis. It is produced by honeybees from resins and plant exudates. Plants protect themselves from pathogenic microbes primarily by producing secondary metabolites termed phytochemicals, many of which have been isolated and used in medicine for their antioxidant and antimicrobial properties (Berretta et al., 2020). Phytochemical compounds that are promising as antimicrobial agents in humans include all components of propolis (Tiku, 2018). These antimicrobial properties can be recognized by honeybees, which selectively collect these plant products and process them to make propolis. Propolis is then used to protect the beehives against pathogens since honeybees have significantly fewer immune genes than solitary insect species. The consumption of propolis by the bees can support their immune response to different pathogens, which helps them live healthier and longer (Berretta et al., 2020).

Propolis is used in folk and modern medicine for the prevention and treatment of colds, wounds and ulcers, rheumatism, sprains, heart disease, and diabetes (Huang et al., 2014). It has diverse biological properties such as anti-inflammatory (Wang et al., 2013), antimicrobial, antioxidant, antitumor (Bankova et al., 2000; Huang et al., 2014), antiulcer and anti-human immunodeficiency virus (HIV) activities (Ito et al., 2001). Its antioxidant and antimicrobial properties provide scope for

use in food technology as a food preservative. One of the most important advantages is that, unlike the other preservatives, its residues may have a generally favorable effect on health (Bahtiti, 2013).

The chemical composition of propolis is very complex, and more than 340 compounds have been characterized (Ahangari et al., 2018; Ristivojević et al., 2015). Most of its biological properties can be attributed to flavonoids, which are extensively present in propolis (Dalben-Dota et al., 2010; Saddiq and Danial, 2014). However, many studies mentioned that the varied biological activities might be due to the synergistic action of its components (Ahangari et al., 2018; Bueno-Silva et al., 2013). One of the most biologically active flavonoids of propolis is caffeic acid phenethyl ester (CAPE). CAPE has been studied extensively as the most important individual component of propolis. The existing studies focused on its potential therapeutic properties such as antibiotic, antioxidant, anti-inflammatory, anti-oxidative stress, antitumor, antidiabetic, anti-neurodegeneration, and anti-anxiety properties (Murtaza et al., 2014; Yordanov, 2019).

The main objective of this study was to investigate the antimicrobial and antioxidant properties of some natural products commonly consumed by humans around the world. Among these widely popular products are propolis and Arabic coffee. This study consisted of four parts: the first part aimed to investigate the effect of propolis ethanolic extract (PEE) on the planktonic growth and biofilm-forming abilities of some commercial probiotics. Whereas the second part focused on the effect of the biologically active propolis component CAPE on the growth, biofilm-forming abilities, and cell death induction in different *Candida* species and strains. In the third part the effect of CAPE on actin protein in eukaryotic cells was examined; and in the last part, which was a minor part, the antioxidant activities of the methanolic extract of Arabic coffee (*Coffea arabica*) beans and its subsequent fractions were estimated.

2. Literature review

2.1. The human microbiota

The human microbiota includes all microorganisms that live on the surface or in the deep layers of human tissues and body fluids. It involves about 10-100 trillion microbial cells hosted by each individual. The human microbiota consists of a wide variety of bacteria, archaea, fungi, and some other single-celled eukaryotes (Ursell et al., 2012). Those microorganisms that reside in our bodies did not come by random colonization, but are formed by interactions with the host and are therefore distinctive to each tissue site. Our evolution has occurred in the context of microbial colonization; therefore, we have many host functions that depend on cues derived from microorganisms (Hand, 2016).

The normal human microbiota is almost stable, with particular genera inhabiting different body regions during certain periods in life. Microbes of the normal microbiota may be beneficial for the host (compete with and may even attack invading pathogens, produce some nutrients for the host, necessary for proper digestion of carbohydrates, etc.), harmful to the host (by causing various health problems and diseases), or may live the host as commensals (live together with the host for a long time without any noticeable harm or benefit). Although most members of the normal human microbiota residing in the human body are harmless in healthy people, these microbes may cause disease in compromised individuals (Davis, 1996).

2.1.1. Normal human gut microbiota

The human gastrointestinal (GI) tract is colonized by a large number of microorganisms (Alfarrayeh et al., 2021; Lin and Zhang, 2017). The sum of bacteria, archaea, and eukarya that live in the GI tract is termed the ‘gut microbiota’ and has co-evolved with the host over a very long time to form a complex and mutually beneficial relationship. Gut microbiota has several health benefits for the host, such as the improvement of intestinal health, harvesting energy, competitive exclusion, and antimicrobial activity against pathogens, and immune modulation (Alfarrayeh et al., 2021; Thursby and Juge, 2017).

Culture-based studies showed that healthy adults almost share the same gut bacterial species which are called “core microbiota”. For instance, *Escherichia coli* can be present in most people. However, culture-independent sequencing studies have revealed a huge microbial diversity that is greatly variable over time and across populations (Lozupone et al., 2012). Each person harbors more than 1,000 species-level phylotypes: “clusters of sequences that have as much diversity in

their small subunit ribosomal RNA genes as named species". The majority of these phylotypes are bacterial species belonging to only a few phyla. In adult individuals, Bacteroidetes (mostly *Porphyromonas* and *Prevotella*) and Firmicutes (mostly *Ruminococcus* and *Clostridium*) often predominate the microbiota, whereas Actinobacteria (mostly *Bifidobacterium*), Proteobacteria (mostly *Escherichia coli*), and Verrucomicrobia, although present in many people, are generally minor components (Azad et al., 2018; Lozupone et al., 2012; Thursby and Juge, 2017). *Lactobacilli*, *Streptococci*, methanogenic archaea (mostly *Methanobrevibacter smithii*), eukaryotes (mostly yeasts), and viruses (mostly phages) are also present as members of gut microbiota (Azad et al., 2018; Lozupone et al., 2012).

The human gut microbiota is influenced by various factors, including diet, toxins, antibiotic therapies, and pathogens; with diet being a very important one (Fig. 1). Food components that are indigestible for human enzymes (for instance, fibers), serve as substrates for gut microbial metabolism. The fermentation of these food components by bacteria can produce a range of growth-promoting and growth-inhibiting factors for specific phylotypes. Moreover, the end products of bacterial metabolism, particularly vitamins and short-chain fatty acids, are very important for human health (Graf et al., 2015).

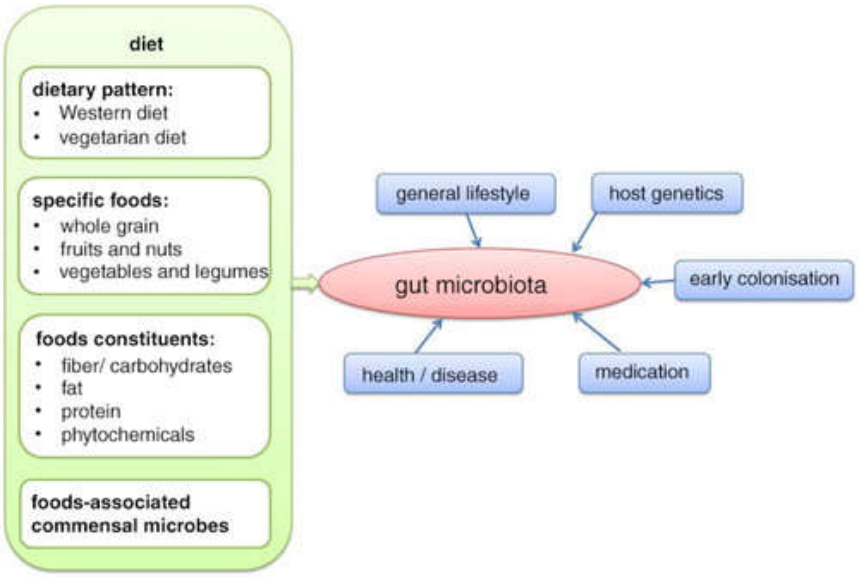

Fig. 1. Factors which influence the composition of the human gut microbiota with a special focus on diet (Graf et al., 2015).

Any alterations in the gut microbiota as a result of environmental factors, including diet, toxins, antibiotic therapies, and pathogens can lead to a condition known as dysbiosis (Carding et al., 2015). Dysbiosis may develop as temporary or chronic clinical symptoms, or it could be

asymptomatic but may increase susceptibility for many diseases, including intestinal, metabolic, and brain disorders (Blumstein et al., 2014). This problem can be solved by using probiotics, which can recolonize the gut, improve its normal microbiota and enhance overall health (Alfarrayeh et al., 2021; Gill and Guarner, 2004; Puebla-Barragan and Reid, 2019).

2.1.2. Probiotics

The term "probiotics" describes the microorganisms that, when administered in sufficient amounts, give health benefits to hosts. A good probiotic must be safe, free of vectors capable of transferring resistance to antimicrobial agents, non-pathogenic, and has no toxicity factors, and preferably of human origin. Moreover, a probiotic should show a high ability to survive intestinal conditions (very low pH, hydrolytic enzymes, bile salts, etc.). Furthermore, a probiotic must have obvious favorable effects on the host, a stimulatory effect on the immune system, and an antagonistic effect against pathogenic microorganisms (Plaza-Diaz et al., 2019).

Modulation of the gut microbiota can be done using probiotics, which may be used in the treatment of several health problems. For example, probiotic feeding with a high-fat diet revealed alteration of the gut microbiota members with a decrease in the gram-positive bacterial phyla Firmicutes and Actinobacteria in mice. Whereas, in a hyperlipidemia mouse model, the probiotic administration of *Lactobacillus* caused significant changes in the microbiota composition, including an increased abundance of Bacteroidetes and Verrucomicrobia and a reduced ratio of Firmicutes. This confirms that probiotic species may play important roles in maintaining the gut microbiota community in humans and animals (Azad et al., 2018). Due to this reason, probiotics are increasingly used in both veterinary and human medicine (Puebla-Barragan and Reid, 2019; Weese, 2003), and they are many commercial forms available as dietary supplements under several brand names in the markets.

Commercial forms of probiotics, which are available as dietary supplements under several brand names in the markets, may consist of bacteria or yeast. They are available as capsules, tablets, packets, or powders. Moreover, different fermented foods, such as yogurt and dairy drinks, contain high amounts of probiotics. Commercial probiotic products may contain a single microorganism or a mixture of several species. The most commonly used probiotics include lactic acid bacteria, specifically *Lactobacillus* and *Bifidobacterium* species. The yeast *Saccharomyces boulardii* also appears to have health benefits. It is very important to know that probiotic effects tend to be strain-specific, so a health benefit attributed to one strain is not necessarily applicable to another strain,

even within the same species. Therefore, generalizations about potential health benefits should not be made (Williams, 2010).

Recently, the National Institute of Health, USA, reported in a fact sheet intended for health professionals that the seven core genera of microbial organisms most commonly used in probiotic products are: *Lactobacillus, Bifidobacterium, Saccharomyces, Streptococcus, Enterococcus, Escherichia,* and *Bacillus* (National Institutes of Health, 2020).

2.1.2.1. Probiotics biofilms

Probiotics can grow in two forms in the GI tract: either freely swimming planktonic cells or as biofilms attached to the intestinal lining. Biofilms of probiotics are considered an advantageous property since they can resist environmental conditions, support longer persistence in the gut of the host and prevent colonization by pathogenic microorganisms by competitive inhibition or steric impediment and having the possibility of enhancing the immune response of the host cell (Alfarrayeh et al., 2021; Salas-Jara et al., 2016).

An essential feature of biofilms is the production of an extracellular polymeric substances (EPS), which provide protection against antimicrobial agents and enzymes, and offer a microenvironment for the metabolic interaction of the population. Communication between microorganisms within the biofilm is mediated by quorum sensing, "a process which may regulate gene expression". Mature biofilms mostly have greater resistance to antimicrobial agents and tolerance to gastric pH than a newly formed biofilms (Salas-Jara et al., 2016).

The biofilm formation in bacteria consists of five stages (Fig. 2): (I) Initial attachment; (II) irreversible attachment; (III) early development of biofilm architecture (microcolonies formation); (IV) maturation; and (V) dispersion. In stage (I), the cells start to adhere to the surface, this adherence is strongly dependent on the physiochemical properties of the surface of the bacterial cell and the surface on which the attachment occurs such as texture (rough or smooth), hydrophobicity, pH, and temperature. At this point, the adhesion is reversible since the microorganisms are not yet undergoing any differentiation process which involves a series of morphological changes leading to biofilm formation. In stage (II), the attachment becomes irreversible since it involves a shift from a weak interaction of the bacterial cell with the surface to a very strong binding with the production of EPS. Stage (III) involves the formation of microcolonies resulting from simultaneous accumulation and growth of microorganisms and it is the main stage at which EPS is formed. EPS support the bond between bacteria and the underlying

substratum and protect the colony from any environmental stresses. In stage (IV), the biofilm becomes mature and grows into an organized structure with a flat or mushroom-like shape. In the last stage, cells of the biofilm start to detach from the top of the biofilm and return to a planktonic form, spreading out. Detachment of single cells seems to be an active process permitting the colonization of new places (Salas-Jara et al., 2016).

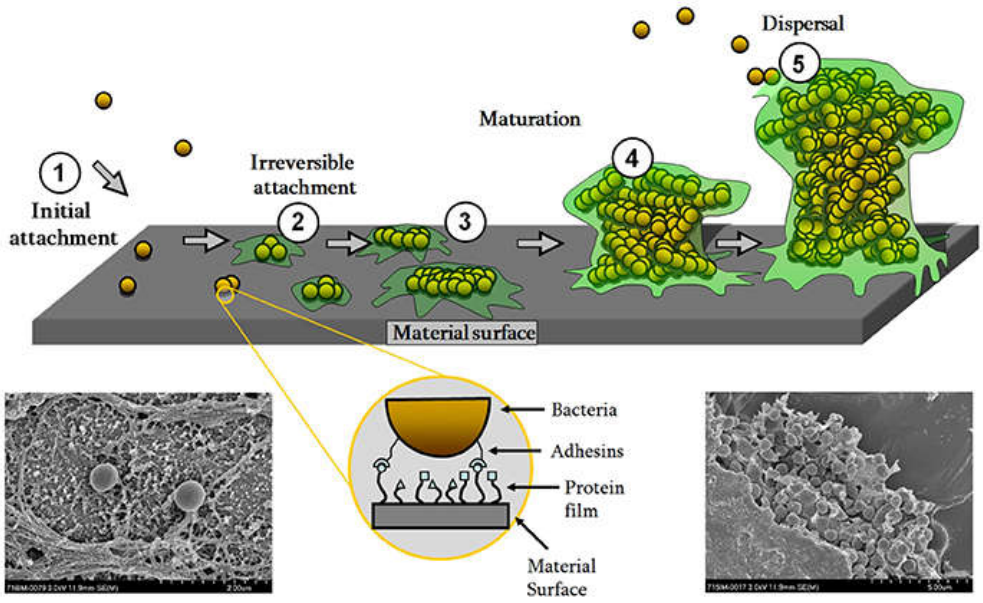

Fig. 2. Biofilm formation scheme with scanning electron micrographs of *S. epidermidis* single cells (lower left) or in biofilm community surrounded by EPS (lower right) on a titanium surface (Sabaté Brescó et al., 2017).

2.1.3. *Candida* species: general characteristics, biofilm structure, and apoptosis

2.1.3.1 General characteristics

The genus *Candida* refers to a yeast that is part of the healthy human microbiota. It lives in commensalism with the human, however, in some cases, *Candida* spp. tend to be opportunistic fungal pathogens, and it may cause candidiasis. A relatively minimal suppression of the immune system is needed to promote the switching of *Candida* from commensal to pathogenic lifestyle, and due to that reason *Candida* is considered as the most common cause of nosocomial fungal infections (Muñoz et al., 2020; Patil et al., 2015; Perlroth et al., 2007; Walsh and Dixon, 1996).

Candidiasis is among the most common human infections; its symptoms depend on the location of the infection. Most of the infections may lead to minor symptoms such as slight localized rashes, redness, itching, and discomfort, though symptoms can be severe or even mortal if left without treatment in immunocompromised individuals (Muñoz et al., 2020; Patil et al., 2015;

Walsh and Dixon, 1996). Mostly, candidiasis is attributed to *Candida albicans*, however; non-albicans *Candida* species (*Candida parapsilosis*, *Candida tropicalis*, and *Candida glabrata*) have been reported to cause between 30% and 54% of the *Candida* infections (Ghannoum, 2001; Silva et al., 2009).

The ability of *Candida* spp. to shift from commensal to pathogenic lifestyle is due to the presence of different virulence factors. Mainly, the capability of switching morphology between yeast and hyphal forms, and the ability to form biofilms are the major properties crucial to *Candida* spp. pathogenesis (Fig. 3). It is reported that most *Candida* infections are accompanied by biofilm formation on host tissues, organs, or abiotic surfaces such as urinary catheters, which result in high morbidity and mortality. As with all microbial biofilms, *Candida* biofilms are highly resistant to antimicrobial treatment. Thus, the effectiveness of the current therapeutic agents against *Candida* biofilms is considered low, with few exceptions (El-Houssaini et al., 2019; Tsui et al., 2016). The biofilms of *Candida* were 30 to 2000 times more resistant than planktonic cells to many antifungal agents, including amphotericin B, fluconazole, itraconazole, and ketoconazole (Seneviratne et al., 2008).

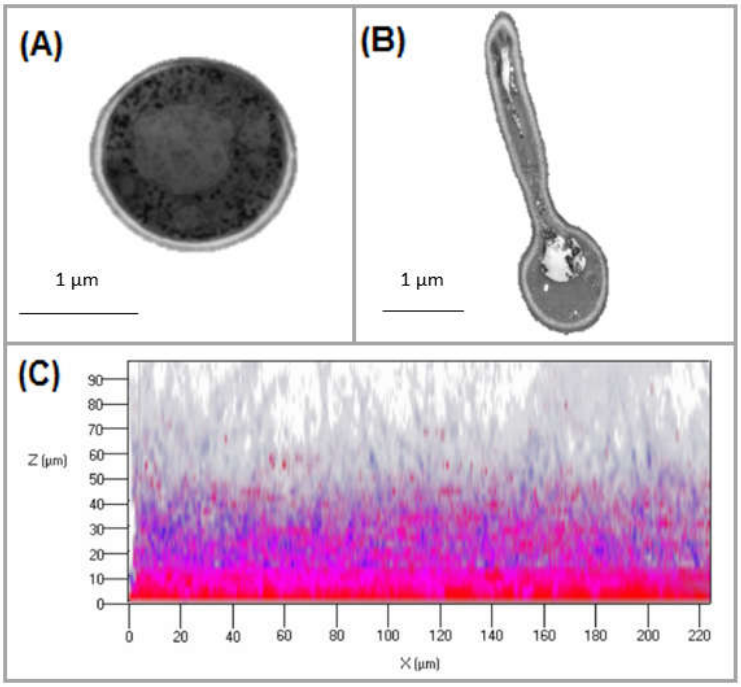

Fig. 3. Morphogenesis and biofilm formation in *Candida albicans*. (A) Transmission electron micrograph of a planktonic yeast cell (Ciociola et al., 2021). (B) Transmission electron micrograph of a germinating yeast cell with a germ tube. (C) Confocal scanning laser microscopy of *in vitro* grown *C. albicans* biofilm (Tsui et al., 2016).

2.1.3.2. *Candida* biofilm structure

The process of biofilm formation in *Candida* has been explained by Chandra and co-workers (2001) on polymethylmethacrylate strips. They demonstrated that the process of biofilm formation occurs fundamentally in three distinct developmental phases (Fig. 4A): early (0–11 h), intermediate (12–30 h), and maturation (38–72 h) phases. The early phase involves adherence of blastospores to the surface of the strips, development of distinct microcolonies, and appearance of thick tracks of fungal growth, as a result of cell growth and aggregation. By the intermediate phase, the formation of EPS (12-14 h) was predominant. EPS appeared as a hazy layer that covers the fungal microcolonies. During maturation, the production of EPS increased significantly and the biofilm appeared as a thick EPS layer in which the basal blastospore communities are embedded. *Candida* communities and the EPS in which they are encased comprise the biofilm (Chandra et al., 2001).

On a flat, hydrophobic surface, a standard biofilm reveals a double-phase distribution of elements. These two phases involve an adherent blastospore layer, in addition to a covering layer of hyphae submerged in EPS. Moreover, a transport system composed of water channels is present between hyphal cells. These water channels enable the transmission and circulation of nutrients from the surrounding environment through the biofilm to the lowest layers and also allow the removal of wastes (Fig. 4B) (Seneviratne et al., 2008).

The study of *in vivo* models of *Candida* biofilms showed similar architectural features to those described for *in vitro* systems. However, *in vivo* models showed faster biofilm maturation and the thickness of biofilms was more than those formed in the *in vitro* systems. The biofilms grown *in vitro* were 25-450 μm in thickness, whereas in the *in vivo* models, the thickness mostly exceeds 100 μm (Andes et al., 2004; Seneviratne et al., 2008).

Many extrinsic and intrinsic factors can affect the process of biofilm formation and the structure of the biofilm in *Candida*. Some of these factors have been widely studied such as the substratum to which the biofilm is attached, available carbon source, presence of saliva, oxygen availability, EPS, and *Candida* species (Cavalheiro and Teixeira, 2018; Seneviratne et al., 2008).

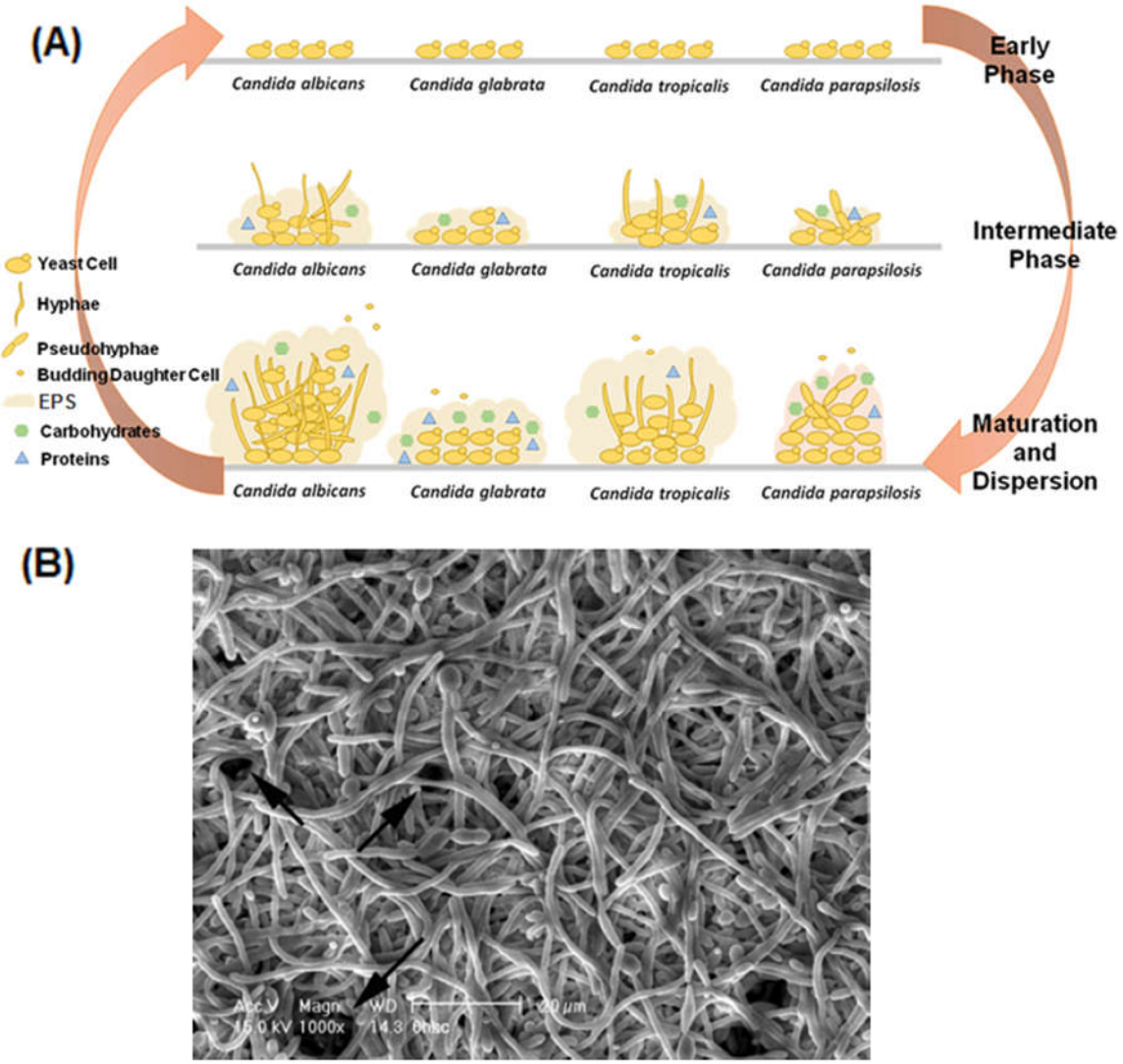

Fig. 4. (A) Comparative schematics of the three stages of biofilm formation by *C. albicans, C. glabrata, C. tropicalis*, and *C. parapsilosis*, highlighting the different capacities to produce extracellular matrix (ECM), the varying components present in the ECM, and the ability to exhibit different cell morphologies (Cavalheiro and Teixeira, 2018). (B) SEM image of *C. albicans* biofilm. The biofilm is composed of a mixture of blastospore, pseudohyphae, and hyphae embedded in EPS. Water channels are present among the cellular components and are indicated by arrows (Seneviratne et al., 2008)

2.1.3.3. Comparison of the biofilm-forming ability of different *Candida* species

As mentioned in the previous section, the species of *Candida* is an important factor that controls the biofilm-forming ability and the structure of the biofilm. The analysis of biofilm film formation in *C. albicans*, *C. parapsilosis*, *C. tropicalis*, and *C. glabrata* isolates of different origins have revealed some variations among the biofilms of different species. It has been found that

biofilms of *C. albicans* were more confluent than biofilms of other *Candida* species, showing different morphologic forms: oval budding, continuous septate hyphae and pseudohyphae, in infected tissues. On the surface of plastic coverslips, *C. albicans* biofilms exhibit a dense network of yeasts and filamentous cells embedded in a matrix of exopolymeric material (Fig. 4A) (Cavalheiro and Teixeira, 2018). Kuhn and co-workers (2002) investigated the differences between the biofilms of *C. albicans* and non-*C. albicans* species. They found that *C. albicans* can form more biofilms than less commonly pathogenic species, including *C. parapsilosis*, *C. glabrata*, and *C. tropicalis*. When compared to *C. albicans* strains, *C. parapsilosis* strains formed biofilms with both a different morphology, with the biofilms appearing to be devoid of extracellular matrix material and hyphae and irregularly grouped yeast cells, and much less thickness. Remarkably, the morphology of *C. parapsilosis* biofilms may resemble that of bacteria at times. Moreover, they reported that invasive *C. albicans* isolates, obtained from a patient with denture stomatitis, form more biofilm than noninvasive (commensal) isolates (Kuhn et al., 2002a). However, Lattif and co-worker reported that no significant differences were found between the surface topography, architecture, and the thickness of the biofilms formed by *C. metapsilosis* and *C. orthopsilosis* isolates as compared to *C. parapsilosis* (Lattif et al., 2010). Interestingly, Al- Fattani and co-workers (2006) demonstrated that biofilms of *C. tropicalis* produced large amounts of EPS, and these biofilms were totally resistant to both amphotericin B and fluconazole (Al-Fattani and Douglas, 2006). Moreover, Cavalheiro and Teixeira (2018) reported that in spite of the low content of carbohydrates and proteins in the EPS of *C. tropicalis* biofilms, they are more resistant to eradication than the biofilms of *C. albicans* (Cavalheiro and Teixeira, 2018).

2.1.3.4. Antifungal resistance through biofilm formation

The development of resistance to the antifungal agents in fungi is strongly correlated to the biofilm formation property. For example, in *C. albicans*, the resistance of 48h-old biofilms to all antifungal agents was shown to be 5 to 8-fold higher than planktonic cells (Cavalheiro and Teixeira, 2018; Hawser and Douglas, 1995). Increased resistance to different antifungal agents has also been reported in *C. parapsilosis* biofilms (Kuhn et al., 2002b). This high resistance of *Candida* biofilms might be due to several factors, including (I) the increased metabolic activity that occurs in the early stages of biofilm formation; (II) the presence of EPS which prevents the diffusion of the antifungal drugs into the biofilm; (III) the changes that occur in the gene expression during the biofilm formation process in *Candida*, including the upregulation of *Candida* drug resistance

(CDR) and multi drug resistance (MDR) genes that are responsible for azole resistance transporters which seems to be significant for the emergence of antifungal resistance in the early stages of the biofilm formation; (IV) the presence of persister cells within the biofilms of *Candida*. These cells are "dormant, non-dividing cells that have a high tolerance to antimicrobial drugs". This tolerance might be due to the dormancy of the cells, which permits the antifungal drugs to bind to their target molecules, but it is impossible to inhibit their function (Cavalheiro and Teixeira, 2018).

2.1.3.5. Apoptosis in *Candida*

Apoptosis, or programmed cell death, is a type of cell death that involves a series of molecular and physiological changes occurring in a cell and ultimately lead to its death. Apoptosis has been documented in several fungal species as a result of exposure to different stress conditions, like weak acid stress, oxidative stress, or ultraviolet radiation (Al-Dhaheri and Douglas, 2010). Phillips and co-workers (2003) have proved that treatment with antifungals, such as amphotericin B, can induce apoptosis in planktonic cells of *C. albicans* (Phillips et al., 2003). Fungal cells that undergo apoptosis reveal several apoptotic markers (Fig. 5), including phosphatidylserine externalization, DNA fragmentation (Al-Dhaheri and Douglas, 2010; Hamann et al., 2008), chromatin condensation, the release of cytochrome *C*, caspases activation (Tulha et al., 2012), shrinkage of the cell, and fragmentation into membrane-bound apoptotic bodies (Saraste and Pulkki, 2000).

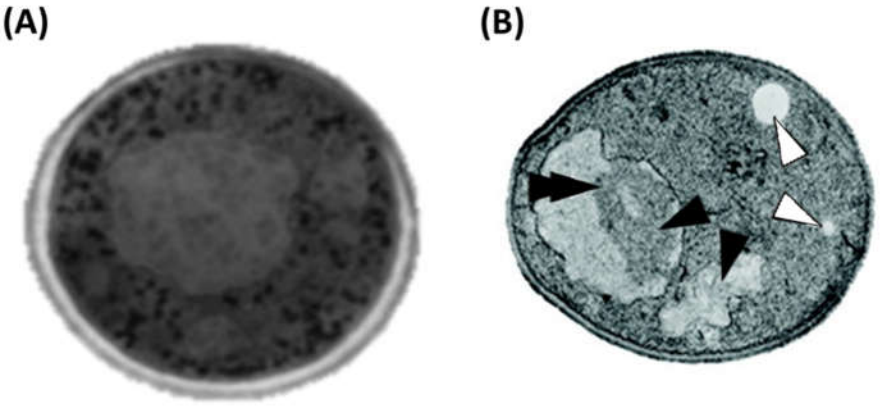

Fig. 5. Transmission electron micrographs of *C. albicans*; (A) control cell; (B) apoptotic cell with morphological apoptotic markers (nucleus fragmentation (black arrowheads), chromatin condensation (double arrowhead), peripheral vacuole formation (white arrowheads), shrinkage of the cell, less dense cytoplasm) (Phillips et al., 2003).

The apoptosis is executed in a highly organized way reflecting the existence of well-preserved molecular pathways (Saraste and Pulkki, 2000). Many pro- and anti-apoptotic regulators have been characterized in the molecular machinery of apoptosis in yeast. Numerous proteins that are responsible for the apoptotic machinery in the mammalian cell are conserved in yeast, including the yeast caspase (also called yeast metacaspase) (Yca1p), the apoptosis-inducing factor (Aif1p), yeast endonuclease G (Nuc1p), Cdc48p, the serine protease HtrA2/Omi (Nma111p), yeast apoptosis-inducing factor-homologous mitochondrion-associated inducer of death (Ndi1p), yeast survivin (Bir1p), and many others (Fig. 6) (Laun et al., 2012).

In mammalian cells, several cysteine-dependent aspartate-specific proteases (caspases) act as the primary drivers for apoptosis. More than two decades ago, in 2000, Uren and co-workers discovered the presence of a mammalian caspases homolog, called metacaspase, in plants, fungi, and protozoa (Uren et al., 2000). Two years later, the role of the Yca1p in yeast apoptosis was identified by Madeo and co-workers (2002). They reported that the overexpression of Yca1p increased the rate of cell death with distinctive apoptotic markers that need caspase activity. On the other hand, the absence of Yca1p prevents the induction of apoptosis in hydrogen peroxide-treated cells (Laun et al., 2012; Madeo et al., 2002). In 2009, a homolog of Yca1p, *Candida* metacaspase (CaMca1p), has been described in *C. albicans* by Cao and co-workers. They found that deletion of the CaMca1p gene leads to decreased caspase activity (Al-Dhaheri and Douglas, 2010; Cao et al., 2009).

The discovery of caspases and the identification of their properties led to the development of caspase inhibitors, such as carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]- fluoromethylketone (Z-VAD-FMK), which can be used to investigate whether the process of apoptosis occurring in the cell includes active caspases or not. The use of such inhibitors enabled the researchers to discover another type of apoptosis which is caspase-independent, in which the cells may undergo apoptosis showing typical apoptotic markers but without the activation of caspase (Xiang et al., 1996). Later on, further studies have shown that this phenomenon could be due to the release of the Aif1p from the mitochondria and its transfer to the nucleus controlled by its nuclear localization signal. To release the Aif1p, which is anchored to the inner membrane of the mitochondria, it must be cleaved by a calcium-dependent calpain protease (Sevrioukova, 2011).

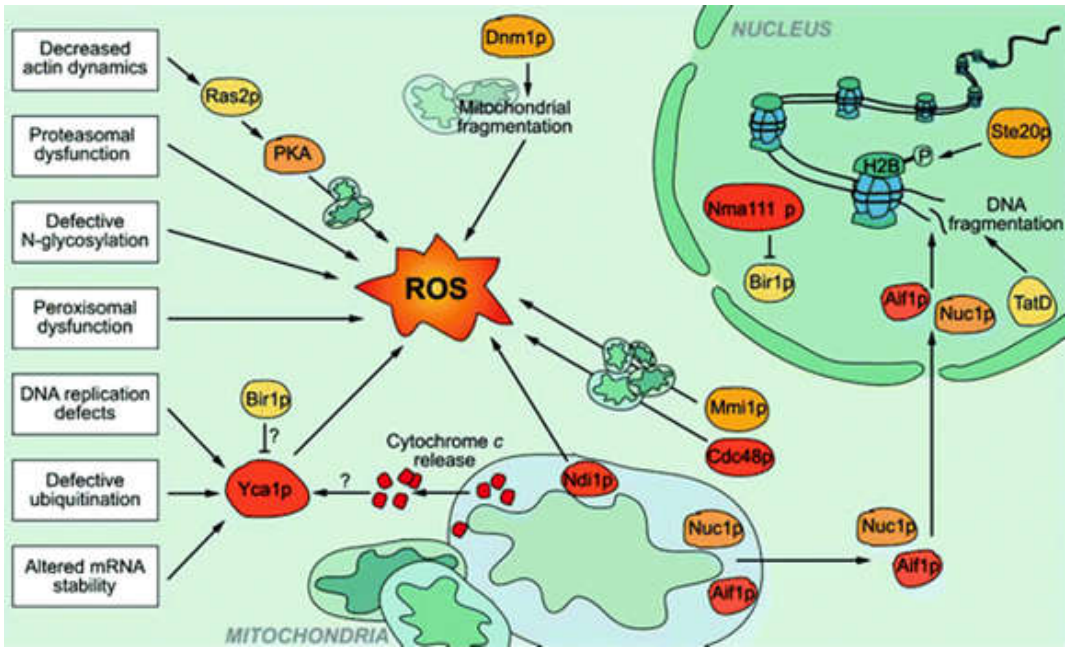

Fig. 6. The fundamental molecular machinery of apoptosis in yeast. Different proteins that act as the core of the apoptotic machinery in the mammalian cell are conserved in yeast, which includes the yeast caspase (Yca1p), the apoptosis-inducing factor (Aif1p), yeast endonuclease G (Nuc1p), Cdc48p, the serine protease HtrA2/Omi (Nma111p), yeast apoptosis-inducing factor-homologous mitochondrion-associated inducer of death (Ndi1p), yeast survivin (Bir1p), and many others (Laun et al., 2012).

2.2. Actin and fungal cytoskeleton

The cytoskeleton is present in all living organisms including prokaryotes and eukaryotes (Wickstead and Gull, 2011). It plays a very important role in contraction, cell motility, cytokinesis, movement of organelles and vesicles through the cytoplasm, establishment of the intracellular organization of the cytoplasm, establishment of cell polarity, and many other functions that are essential for cellular homeostasis and survival (Goodman and Zimmer, 2007).

Bacterial cytoskeleton consists of several actin homologs. Three of these homologs, whose three-dimensional structures are known, show significant structural similarity to eukaryotic actin in spite of a variable degree of sequence similarity. These bacterial actin-like cytoskeletal proteins include cell shape-determining protein MreB, plasmid segregation protein ParM, and actin-like protein MamK. More bacterial proteins of the actin superfamily are also existing, however, they are not known to be cytoskeletal elements such as cell division protein FtsA, the protein chaperone DnaK (Hsp70), and sugar hexokinases (Shih and Rothfield, 2006).

In eukaryotes, actin is one of the foremost abundant, essential, and conserved proteins across eukaryotic phyla (Pollard et al., 2000). It is present in the cell in two forms: (I) the monomeric, globular actin (G-actin), and (II) the polymeric, filamentous actin (F-actin). The structure of G-actin can be divided into two domains, a smaller one and a larger one. The smaller domain contains subdomains 1 and 2, while the larger one contains subdomains 3 and 4 (Fig. 7). The cleft between the two domains contains the primary cation and nucleotide-binding sites. Physiologically the bound divalent cation is Mg^{2+}, *in vitro* the cation is often Ca^{2+} to keep actin in monomeric form. The nucleotide-binding site binds ATP, ADP.Pi or ADP. F-actin (Fig. 8) is a right-handed double-stranded helix with a rapidly growing barbed end, which is the preferred site of ATP-bound G-actin incorporation (polymerization), and a pointed end, where the disassembly of actin occurs (depolymerization). Actin undergoes many cycles of rapid polymerization and depolymerization, allowing the cells to continuously remodel the actin cytoskeleton and providing the force for processes like cell movement and vesicle internalization. More than 100 highly conserved regulatory proteins are used by eukaryotic cells to preserve the pool of actin monomers, enhance nucleation and growth of actin filaments, stabilize and cross-link filaments into bundles, and control the actin filaments disassembly. The precise control and triggering of these regulatory proteins in space and time enables cells to form actin filaments at particular regions of the cytoplasm where they can perform their function in response to different stimuli (Mishra et al., 2014).

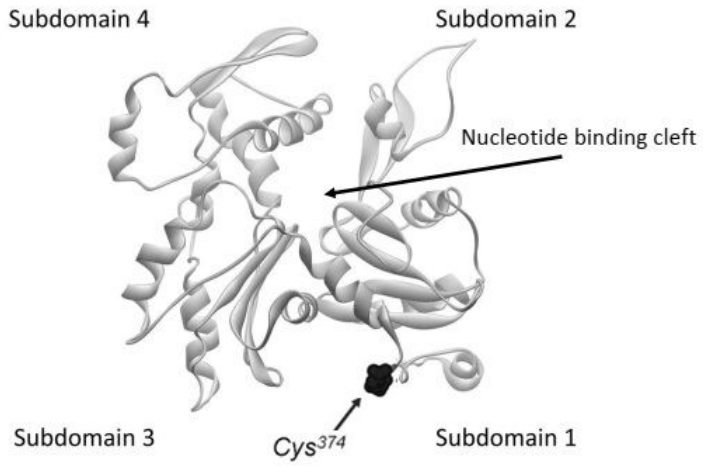

Fig. 7. The three dimensional structural model of the actin monomer (based on the pdb file *2ZWH*) indicating the four subdomains, the position of the nucleotide-binding cleft, and the position of the fluorescent label used in this study (Cys^{374}) (Ujfalusi et al., 2010).

Fig. 8. Actin filament as seen by electron microscope (Huxley, 1973).

The network of actin filaments in yeast cells is analogous to that present in metazoans, but less extensive (Mishra et al., 2014). For example, *Saccharomyces cerevisiae* has an actin cytoskeleton that consists of a set of protein elements very similar to those present in the actin cytoskeletons of higher eukaryotes including mammals (Akram et al., 2020). Yeast cells can form three different actin structures: cortical actin patches, actin cables, and the actomyosin ring (Fig. 9), as compared to more than 15 structures found in metazoan cells. Furthermore, yeasts have only one actin isoform and fewer regulatory proteins with little redundancy (Mishra et al., 2014).

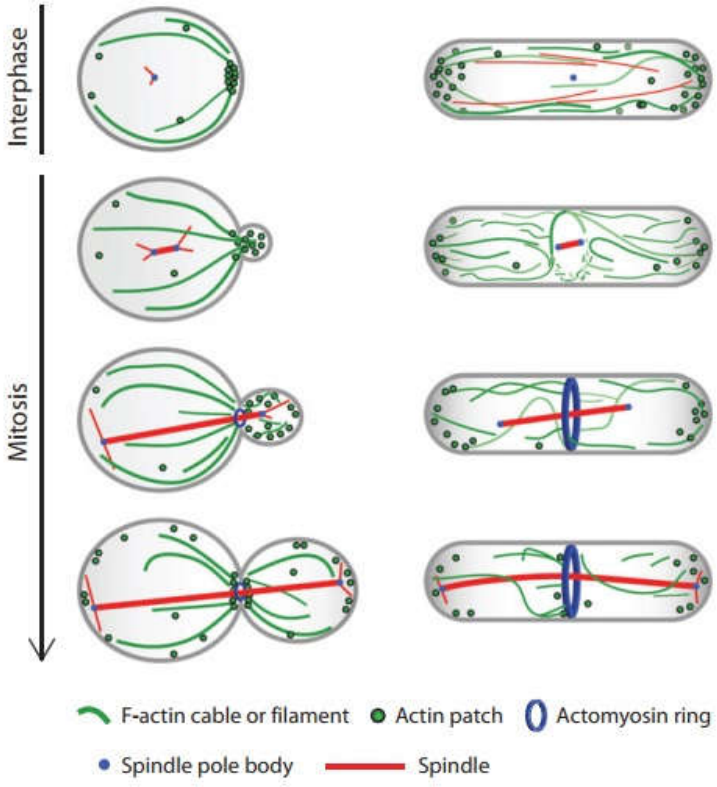

Fig. 9. Organization of actin during the cell cycle in budding yeast (left) and fission yeast (right) (Mishra et al., 2014).

Böhl and co-workers (2007) studied the effect of several flavonoids on actin polymerization. They found that flavonoids exert different and partially opposing biological impacts on actin; even though actin polymerization was inhibited by flavonols (kaempferol, fisetin, quercetin, genistein, and taxifolin), it was enhanced by the structurally related flavan epigallocatechin. Infrared spectroscopic investigations showed flavonoid-specific conformational changes in actin, which may lead to different biological effects on the actin (Böhl et al., 2007).

2.3. Natural products

Simply, natural products are any products that have natural origins. It can be (1) a complete organism; (2) part of an organism; (3) an extract of an organism or part of an organism, and exudates; (4) pure compounds isolated from an organism. Despite this, the term "natural products" is mostly used to describe the secondary metabolites produced by any living organism. Secondary metabolites are small molecules that are nonessential for the survival, growth, development, or reproduction of the organism. They usually include products of overflow metabolism that may occur due to nutrient limitation, or shunt metabolism produced during idiophase, defense mechanism, or regulator molecules. Secondary metabolites often play an essential role in plant defense against herbivores and other interspecies defense mechanisms (Sarker and Nahar, 2012).

Natural products have been the main source of most of the active components of medicines. Before the development of high-throughput screening and the post-genomic era; more than 80% of drug substances were natural products or inspired by a natural compound (Harvey, 2008). Between 1983–1994, about 39% of all 520 new approved medicines were natural products or based on natural origins, and nearly 60–80% of antibacterial and anticancer drugs were developed from natural products (Cragg et al., 1999). In 2000, approximately 60% of all medicines under clinical trials for the various types of cancer were natural products. Whereas, in 2012, nearly 40% of all modern medicines that were in use were developed from natural products (Sarker and Nahar, 2012). Dar and co-workers (2017) reported that about one-half of all medicines in use till 2017 consist of natural compounds or their derivatives. Moreover, natural products have also provided the molecular template or intellectual stimulus for the production of about half of all synthetically produced drugs (Dar et al., 2017).

Natural products and natural product-derived modern medicines are widely used in the "natural" pharmaceutical industry which is developing rapidly in Europe and North America,

Mexico, China, Nigeria, and many other developing countries. The acceptance of herbal medicines by people as food supplements, nutraceuticals, complementary and alternative medicine, has increased sharply in the last few years (Sarker and Nahar, 2012).

2.3.1. Propolis: general characteristics, botanical sources, chemical composition, and antimicrobial properties

2.3.1.1. General characteristics of propolis

Propolis (bee glue) is one of the most important honeybee products. It is prepared by honeybees as a resinous material collected from different plant leaves, buds, and exudates, partially digested by the salivary β-glycosidase and then combined with beeswax. Propolis is used by honeybees to fill the cracks in the honeycombs, to smooth the walls of the hive, to stabilize the humidity and temperature inside the colony throughout the year, and to protect the bees from predators (Bankova et al., 2000; Ristivojević et al., 2015). Propolis has a pleasant aromatic smell, and it may vary noticeably in color from yellow-green to red and dark brown according to its geographical origin and botanical sources (Wagh, 2013). Generally, propolis as a raw material consists of 50% plant resins, 30% wax, 10% essential and aromatic oils, 5% pollen, and 5% other organic and inorganic substances, including amino acids, vitamins, and minerals (Bankova et al., 2000; Ristivojević et al., 2015).

In the last two decades, many studies investigated the biological activities of propolis. These studies revealed that propolis has many pharmacological properties such as antimicrobial, antioxidant, antitumor (Bankova et al., 2000; Huang et al., 2014), anti-inflammatory (Wang et al., 2013), antifungal, antiulcer, antiparasitic (Ristivojević et al., 2015), and anti-human immunodeficiency virus activities (Ito et al., 2001). Due to these varied biological properties, propolis was intensively used in the food and cosmetic industries (toothpaste, soap, syrup, and candy) (Ristivojević et al., 2015). It is also used as a food preservative which, unlike the other preservatives, may have a generally favorable effect on health (Bahtiti, 2013). Furthermore, propolis is also used in traditional and modern medicine for the prevention and curing of colds, wounds and ulcers, rheumatism, sprains, heart disease, diabetes, and dental caries (Huang et al., 2014; Ristivojević et al., 2015).

2.3.1.2. Botanical sources of propolis

The chemical composition of propolis varies greatly depending on the species of bees, geographical and climatic factors, plant resources, and collecting seasons. The specificity of local

plant resources defines the chemical composition of propolis and its classification into a particular group. It has been reported that plant buds' resins such as *Poplar* sp. (*Populus alba*, *P. tremula*, and *P. nigra*) are considered the main resource of poplar-type propolis from temperate regions (Europe, North America, and non-tropical regions of Asia) (Ristivojević et al., 2015). Other less important sources of resins for poplar-type propolis are *Pinus* spp., *Prunus* spp., *Acacia* spp., *Betula pendula*, *Aesculus hippocastanum*, and *Salix alba* (Dezmirean et al., 2021). The main source of Brazilian propolis is *Araucaria angustifolia*, *Baccharis dracunculifolia*, and *Eucalyptus citriodora*; while in Venezuela and Cuba, the source of propolis is mainly some *Clusia* species. Finally, the red Mexican propolis usually comes from the genus *Dalbergia* (Salatino et al., 2005). The most common types of propolis and their botanical origins are shown in Table (1).

Table 1. Most common propolis types and their botanical origins (de Groot, 2013).

Propolis Type	**Geographic Origin**	**botanical origin**
Poplar	Europe, North America, Non-tropical regions of Asia, New Zealand, China	- *Populus* spp., of section *Aigeiros*, most often *P. nigra* L.
Green (alecrim) Brazilian	Brazil	- *Baccharis* spp., predominantly *B. dracunculifolia* DC
Birch	Russia	- *Betula verrucosa* Ehrh
Red propolis	Cuba, Brazil, Mexico	- *Dalbergia ecastaphyllum* and other *Dalbergia* species
Mediterranean	Sicily, Greece, Crete, Malta	- Cupressaceae (species unidentified, possibly *C. sempervirens*) and Pinaceae
Clusia	Cuba, Venezuela	- *Clusia* spp., including *C. major, C. minor*
Pacific	Pacific region (Okinawa, Taiwan, Indonesia)	- *Macaranga tanarius*

2.3.1.3. Chemical composition of poplar-type propolis

Understanding the chemical composition and biological properties of propolis is very important for use in the pharmaceutical and food industries. Poplar-type propolis is one of the most widely studied types of propolis and is best known for its chemical and pharmaceutical properties. Several studies describe the typical properties of poplar-type propolis in terms of its content of biologically active compounds (Ristivojević et al., 2015).

For a long time, it was thought that the chemical composition of all types of propolis is constant, and thus was not studied in respect of differences in the composition versus botanical

source or geographical origin. Most of the studies that investigated the biological activities of propolis have simply called the samples with the plain term, the "propolis samples", without referring to their botanical or geographical origin, or providing any information about the differences in chemical composition. However, with the great progress in the separation and purification methods such as high-performance liquid chromatography (HPLC), high-performance thin-layer chromatography (HPTLC), high-performance thin-layer chromatography (HPTLC), and gas chromatography (GC), in addition to structure elucidation techniques such as mass spectrometry (MS), and nuclear magnetic resonance (NMR), many compounds have been found in the different types of propolis and it soon became apparent that there were considerable differences in the chemical composition of propolis emerging from different botanical or geographical origins. At present, we can talk about several types of propolis such as Euro-Asian propolis (the temperate or poplar-type), African, or green Brazilian propolis, each of which has distinct chemical properties and distinct biomarkers (Ristivojević et al., 2015).

The chemical profile of poplar-type propolis is very complex, and more than 340 constituents have been characterized (De Groot et al., 2014; Ristivojević et al., 2015). These constituents could be classified under the following categories: (1) aliphatic hydrocarbons and wax esters; (2) alcohols; (3) aldehydes; (4) aliphatic acids (short-chain) and their esters; (5) aliphatic fatty acids (long-chain) and their esters; (6) amino acids; (7) aromatic acids and their esters; (8) chalcones and dihydrochalcones; (9) terpenoids; (10) flavones and flavonols; (11) glycerol derivatives; (12) flavanones; (13) sugars and sugar alcohols; (14) acetophenones and other ketones; (15) steroids; (16) miscellaneous ingredients of poplar-type propolis (De Groot et al., 2014). Some currently known ingredients of poplar-type propolis are listed in Table (2) in chemical categories.

Two major groups of aromatic acids can be found in the poplar-type propolis: derivatives of hydroxybenzoic acid, such as gallic, gentisic, protocatechuic, salicylic, and vanillic acids; and derivatives of hydroxycinnamic acid such as p-coumaric, caffeic, and ferulic acids. In addition to the free form, they could be found as benzyl-, methylbutenyl-, phenylethyl-, and cinnamyl- esters. In this context, we will focus on caffeic acid phenethyl ester (CAPE) which is a major constituent of temperate propolis with a wide range of biological activities (Huang et al., 2014).

Table 2. List of some chemical compounds commonly present in the poplar-type propolis (Masek et al., 2018; Ristivojević et al., 2015).

No.	Compounds	Geographical origin	Botanical origin
Aromatic acids			
1	trans-cinnamic acid	Croatia	
2	4-methoxycinnamic acid	Croatia	
3	p-coumaric acid	Croatia	
4	3,4-dimethoxycinnamic acid	Croatia	
5	trans-isoferulic acid	Croatia	
6	Ferulic acid	Croatia	
7	Caffeic acid	Croatia	
Esters			
8	3-methyl-3-buthenyl caffeate	Croatia	
9	2-methyl-2-buthenyl caffeate	Croatia	
10	3-methyl-2-buthenyl caffeate	Croatia	
11	Benzyl caffeate	Croatia	
12	Phenylethyl caffeate (CAPE)	Croatia	
Flavonols			
13	Quercetin	China, Serbia, Italy, Slovenia	
14	Kaempferol	Serbia, Italy, Slovenia	
15	Isorhamnetin	China, Serbia, Italy	
16	Kaempferide	Serbia, Italy, Slovenia	
17	Bis-methylated quercetin	Serbia, Italy	
18	Quercetin -methyl ether	China, Serbia, Italy	
19	Bis-methylated quercetin	Serbia, Italy	
Flavanonols			
20	Pinobanksin	China, Serbia, Italy, Slovenia, Germany	
21	Pinobanksin-5-methyl-ether-3-*O*-acetate	Serbia, Italy	
22	Pinobanksin-5-methyl-ether	Serbia, Italy	
23	Pinobanksin-3-*O*-acetate	China, Serbia, Italy, Slovenia, Germany	
24	Pinobanksin-3-*O*-propionate	Serbia, Italy	
25	Pinobanksin-3-*O*-butyrate (or isomer)	China, Serbia, Italy	
26	Pinobanksin-3-*O*-pentenoate (or isomer)	China, Serbia	
27	Pinobanksin-3-*O*-pentanoate (or isomer)	Serbia, Italy	
28	Pinobanksin-3-*O*-hexanoate (or isomer)	Serbia, Italy	
Flavones			
29	Luteolin	Serbia, Italy, Slovenia	
30	Apigenin	China, Serbia, Italy, Slovenia	
31	Chrysin	China, Serbia, Italy, Slovenia, Germany	
32	Methoxy-chrysin	Serbia, Italy	
Flavanones			
33	Naringenin	Serbia, Italy, Germany	
34	Liquiritigenin	Serbia, Germany	
35	Pinostrobin	Serbia, Germany	
36	Pinocembrin	China, Serbia, Italy, Slovenia, Germany	
Glycosides			
37	Apigenin-7-*O*-glucoside	Serbia	*P. nigra*
38	Quercetin-3-*O*-glucoside	Serbia, Europe, Asia, South America	

39	Isorhamnetin-3-*O*-rutinoside	Serbia, Crete	*P. nigra*
40	Kaempferol-*p*-coumaroyl rhamnoside	Portugal	
41	Quercetin-3-*O*-rutinoside	Portugal	
42	Quercetin-3-*O*-glucuronide	Portugal	
43	Kaempferol-3-*O*-rutinoside	Portugal	
44	Isorhamnetin-*O*-pentoside	Portugal	
45	Quercetin-3-*O*-rhamnoside	Portugal	
46	Isorhamnetin-*O*-glucuronide	Portugal	
47	Kaempferol-methyl ether-*O*-glucoside	Portugal	
48	Isorhamnetin-*O*-acetylrutinoside	Portugal	
49	Rhamnetin-*O*-glucuronide	Portugal	
50	Quercetin-dimethyl ether-*O*-rutinoside	Portugal	
51	Quercetin-dimethyl ether-*O*-glucuronide	Portugal	
52	Kaempferol-*O*-*p*-coumaroyl rhamnoside	Portugal	
53	Quercetin-7-*O*-glycoside	China	
54	Luteolin 7-*O*-glucoside	China	
55	Naringenin 7-rhamnoglucoside	China	
Phenolic glycerides			
56	2-Acetyl-1,3-dicaffeoyl glycerol	China, Germany, Serbia	*P. tremula* *P. nigra*
57	2-Acetyl-1-caffeoyl-3- coumaroyl glycerol	China, North-Russia, Swiss, Bulgaria, Turley, Germany, Poland, Belarus, Serbia	*P. tremula* *P. nigra* *P. sichuan,* *P.euphratica*
58	2-Acetyl-1-feruloyl-3- caffeoyl glycerol	China, North Russia, Swiss, Bulgaria, Germany	*P. tremula*
59	2-Acetyl-1-feruloyl-3- coumaroyl glycerol	Russia, Swiss, Bulgaria, Germany, Croatia, Poland	*P. tremula*
60	2-Acetyl-1,3-diferuloyl glycerol	China, North Russia, Swiss, Bulgaria, Turkey	*P. tremula* *P. nigra* *P. euphratica*
61	2-Acetyl-1-caffeoyl-3- cinnamoyl glycerol	China	
62	2-Acetyl-1-caffeoyl-3- cinnamoyl glycerol (isomer)	China	
63	2-Acetyl-1,3-dicoumaroyl glycerol	Netherlands, North-Russia, Swiss, Bulgaria, Turkey, Germany, Poland, Belarus Croatia, Serbia	*P. euphratica* *P. nigra*
64	2-Acetyl-1-coumaroyl-3- feruloylglycerol	Netherlands	
65	Acetyl coumaroyl glycerol	Turkey, Serbia	*P. euphratica* *P. nigra*
66	Coumaroyl glycerol	Turkey	*P. euphratica*
67	Coumaroyl glycerol (isomer)	Turkey	*P. nigra*
68	1,3-Dicoumaroyl glycerol	Belarus, Poland, Russia	*P. sichuan,* *P .tremula*
69	1-Coumaroyl-3-caffeoyl glycerol	Belarus, Poland, Russia, Serbia	*P. sichuan,* *P. tremula* *P. nigra*
70	Caffeoyl glycerol	Serbia	*P. nigra*
71	Tri coumaroyl glycerol	Serbia	*P. nigra*
72	Coumaroyl feruloyl glycerol	Serbia	*P. nigra*
73	Coumaroyl feruloyl glycerol (or isomer)	Serbia	*P. nigra*
74	Dicaffeoyl coumaroyl glycerol	Serbia	*P. nigra*
75	Dicaffeoyl feruloyl glycerol	Serbia	*P. nigra*

2.3.1.4. Antimicrobial properties of propolis

Propolis is a well-known and very important natural antibiotic (Dezmirean et al., 2021). It has been used for skin treatment and healing wounds and ulcers since ancient times. Despite this, it was only in recent decades that detailed studies of propolis' constituents and biological properties were carried out (Dantas Silva et al., 2017). In the last two decades, many studies have appeared investigating the valuable antibacterial and antifungal activities of propolis, due to the need for new remedies against contagious diseases, especially with the emergence and increase of antibiotic resistance in various pathogens (Silva-Carvalho et al., 2015).

2.3.1.4.1. Antibacterial activity

The antibacterial activities of propolis against numerous bacterial strains have been investigated by many studies, which supported the fact that Gram-positive bacteria are more susceptible to propolis than Gram-negative bacteria. Information from several studies indicates that propolis can inhibit bacterial motility and enzyme activity, show bacteriostatic activity against various bacterial genera, have bactericidal effects at high concentrations, and affects plasma membrane (Silva-Carvalho et al., 2015).

In a study that examined the effect of propolis ethanolic extract (PEE) on the physiology of *Bacillus subtilis*, *Escherichia coli*, and *Rhodobacter sphaeroides*; Mirzoeva and co-workers (1997) reported that propolis and some of its components, such as cinnamic acid and flavonoids, increased the permeability of the inner bacterial membrane to ions leading to the loss of the membrane potential and the inhibition of bacterial motility (Mirzoeva et al., 1997).

It has been suggested that the combination of propolis with other antibiotics would allow dose reduction of selected antibiotics, thus potentiating their effect (Silva-Carvalho et al., 2015). Scazzocchio and co-workers (2006) investigated the antibacterial activity of Italian PEE and its effect on the activity of some antibiotics against some clinically isolated Gram-positive bacterial strains. They found that Italian PEE has a significant antimicrobial activity towards all tested clinical strains. Moreover, it significantly increased the activity of ampicillin, gentamycin, and streptomycin and moderated the action of chloramphenicol, ceftriaxone, and vancomycin. No effect was observed on the action of erythromycin (Scazzocchio et al., 2006).

Another study done by Wojtyczka and co-workers (2013) assessed the *in vitro* antimicrobial properties of a Polish PEE alone and combination with antimicrobial drugs, against methicillin-sensitive *Staphylococcus aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) clinical

isolates. PEE sowed varying effectiveness against different *S. aureus* strains and strengthened the antimicrobial activity of cefoxitin, clindamycin, tetracycline, trimethoprim+sulfamethoxazole, tobramycin, linezolid, penicillin, and erythromycin against all tested strains. However, no synergism was seen in the case of ciprofloxacin and chloramphenicol (Wojtyczka et al., 2013).

In another study, the antibacterial activity of aqueous and organic extracts of French poplar-type propolis was tested against 36 Gram-negative and Gram-positive bacterial strains including *Staphylococcus aureus*. This study revealed that dichloromethane-based extracts have a selective Gram-positive antibacterial activity, especially against *S. aureus,* including MRSA and MSSA strains (Boisard et al., 2015). These results were in agreement with the findings of Velikova and co-workers (2000) who examined the antibacterial properties of different PEE from Bulgaria, Greece, Turkey, and Algeria against *S. aureus* and *E. coli*. All the samples showed good antibacterial activity against *S. aureus*, while in the case of *E. coli* the effect was either weak or lacking (Velikova et al., 2000).

Recently, Daraghmeh and Imtara evaluated the antibacterial properties of Palestinian propolis against multidrug-resistant clinical isolates, including *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Streptococcus faecalis*. The results showed that propolis samples have the same trend to inhibit the growth of Gram-positive strains better than Gram-negative ones (Daraghmeh and Imtara, 2020). On the contrary, Katircioglu and Mercan found that Turkish PEE has a strong inhibitory effect against some Gram-negative bacteria like *E. coli* (Katircioglu and Mercan, 2006).

Inadequate information is available about the action of propolis on the anaerobic bacteria. In Polish studies, it has been found that the anaerobic bacteria of the *Fusobacterium* genus were the most sensitive to low concentrations of PEE. However, bacteria of the genus *Actinomyces*, *Bacteroides*, *Clostridium*, *Peptococcus*, *Peptostreptococcus*, and *Propionibacterium* were only sensitive to the high concentrations of PEE (Przybyłek and Karpiński, 2019).

Various studies revealed that as the chemical composition of propolis varies from climate to climate and from region to region, the antibacterial activity also shows some differences. The antibacterial activity of propolis is higher for samples from a wet-tropical rain forest-type climate. Other studies showed the impact of the geographical origin of propolis on its antibacterial activities, for example, propolis samples from the north and center of Portugal have high activity against *S. aureus* (Silva-Carvalho et al., 2015).

2.3.1.4.2. Antifungal activity

Antifungal activity is also affected by the variation in the chemical composition of propolis. Many researchers studied the effect of propolis from various geographic origins against different fungal species, mainly of clinical importance (Silva-Carvalho et al., 2015).

Quiroga and co-workers (2006) tested the antifungal activity of partially purified PEE from the northwest of Argentina against yeasts, xylophagous, and phytopathogenic fungi. They reported that the partially purified propolis extract and its isolated compounds, pinocembrin and galangin, have the capacity of being used as antifungal agents (Quiroga et al., 2006). Later on, Falcao and co-workers (2014) examined the antifungal activity of Portuguese propolis and its floral sources *Populus x Canadensis* Moenchen (a hybrid species of *Populus*) and *Cistus ladanifer L*. against *C. albicans*, *Trichophyton rubrum*, and *Aspergillus fumigatus*. The results revealed that the plant extracts did not exhibit important antifungal activity compared to propolis ethanolic extracts, with exception of *T. rubrum*. However, propolis samples revealed a good antifungal effect, with the highest activity found against *T. rubrum* and the lowest against *A. fumigatus* (Falcão et al., 2014). Four propolis samples from Poland were also tested for antifungal activity by Szweda and co-workers (2015). The results demonstrated significant differences in the fungicidal activity of the four samples. Only one of them displayed high fungicidal activity against *C. albicans*, *C. glabrata*, and *Candida krusei* (Szweda et al., 2015). Different organic extracts of French propolis were also confirmed to be active against *C. albicans* and *C. glabrata* but only have a weak activity towards *Aspergillus fumigatus* (Boisard et al., 2015). Brazilian PEE was also examined for antifungal activity against 80 strains of *Candida*, including 20 strains of *C. albicans,* 20 strains of *C. tropicalis*, 20 strains of *C. krusei* , and 15 strains of *C. guilliermondii.* Every strain of *Candida* was shown to be sensitive to the ethanolic extract of propolis. *C. albicans* was the most sensitive species, while the most resistant species tested was *C. guilliermondii* (Ota et al., 2001). Moreover, Brazilian green and red propolis also exhibited antifungal activity against different species of *Trichophyton*, the causative agent of dermatophytosis, red propolis being more effective than the green one (Silva-Carvalho et al., 2015; Siqueira et al., 2009). Dota and co-workers (2011) also evaluated the antifungal properties of PEE and propolis microparticles, taken from a sample of Brazilian propolis, against clinical yeast isolates obtained from vaginal exudates of patients with vulvovaginal candidiasis. All yeasts were inhibited by PEE and propolis microparticles, with small variation (Dota et al., 2011). Besides antifungal activity, Brazilian PEE can inhibit biofilm

formation by *C. albicans* from vulvovaginal candidiasis (Capoci et al., 2015). Another study demonstrated the effect of propolis against the planktonic cells and biofilms of different *Candida* species. The results revealed higher effect on *C. albicans* and *C. parapsilosis* than on *C. tropicalis* cells. Additionally, propolis was also found to be effective in preventing *Candida* species biofilm's formation and have the ability to eradicate their mature biofilms, as well as reducing filamentation in *C. tropicalis* and *C. albicans* (Papp et al., 2021; Tobaldini-Valerio et al., 2016).

2.3.2 Caffeic acid phenethyl ester (CAPE)

CAPE is a major component of temperate propolis (poplar-type) with a wide range of biological activities, such as inhibition of nuclear factor κ-B; inhibition of cell division; termination of cell cycle and induction of apoptosis (Huang et al., 2014). The IUPAC name of CAPE is 2-phenylethyl (2E)-3-(3,4- dihydroxyphenyl) acrylate. It is also called phenylethyl caffeate or phenethyl caffeate. The chemical formula of CAPE is $C_{17}H_{16}O_4$. Its chemical structure is shown in Figure (10). This polyphenolic ester compound can be produced in the laboratory by reacting caffeic acid with phenethyl alcohols. CAPE is a polyphenolic compound consisting of hydroxyl groups within the catechol ring, which is crucial for many biological activities (Murtaza et al., 2014).

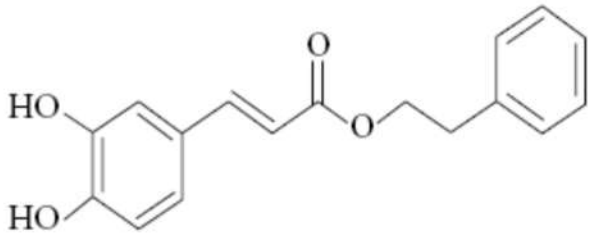

Fig. 10. Chemical structure of CAPE (Murtaza et al., 2014)

At present, CAPE has been given close attention for its important therapeutic effects in many diseases, including carcinomas, internal organ damage, metabolic diseases, inflammatory diseases, and microbial infections. CAPE will be a promising natural product for clinical application in the future (Lv et al., 2021). Its advantage is that it is devoid of some negative aspects of crude extracts of propolis, such as the inability to standardize, which is the keystone of implementing its therapeutic potential as a medicine (Yordanov, 2019).

2.3.2.1 Antimicrobial properties of CAPE

2.3.2.1.1 Antibacterial activity

Numerous studies demonstrated the antibacterial activity of CAPE against different bacterial species. A study conducted by Cui and co-workers (2013) offered important information for understanding the potential anti-*Helicobacter pylori* mechanism of CAPE. *H. pylori*, a major causative agent for GI sicknesses, has the enzyme *H. pylori* peptide deformylase that catalyzes the removal of the formyl group from the N-terminal of nascent polypeptide chains. Because of the great importance of the activity of this enzyme for the survival of *H. pylori*, it is considered a promising drug target for anti-*H. pylori* medicines. Results revealed that CAPE is a competitive inhibitor of peptide deformylase, as it blocks the substrate entrance and prevents the substrate from approaching the active site of the enzyme (Cui et al., 2013). Meyuhas and co-workers (2015) found that CAPE can significantly inhibit the growth of Gram-positive bacteria while having only a slight inhibitory effect on Gram-negative ones (Meyuhas et al., 2015). CAPE was also shown to be an effective inhibitor of bacterial motility. Even though the effect of CAPE was reversible, bacterial motility was recovered after a short time, maybe due to inactivation or detoxification of this compound (Mirzoeva et al., 1997). Recently, it has been found that CAPE is capable to inhibit the biofilm formation and development in *Streptococcus mutans*, which is related to dental caries (Lv et al., 2021). Moreover, CAPE can also inhibit the fundamental virulence factors of *S. mutans* associated with cariogenicity, including acid production, acid tolerance, and the ability to produce extracellular polysaccharides (Niu et al., 2020). The mechanism of CAPE action includes oxidative damages by increasing reactive oxygen species levels and altering glutathione levels (Collins et al., 2019). To overcome the low water solubility of CAPE, nanoparticles and CAPE-incorporated fibers were developed, both of which showed improved antibacterial activity (Lv et al., 2021).

2.3.2.1.2 Antifungal activity

According to the available literature, few studies have been found on CAPE's antifungal activity. In one of these studies, treatment with CAPE led to prolonged survival in a mouse model of disseminated candidiasis. Meanwhile, it can also prolong the survival of *C. albicans*-infected nematodes (*Caenorhabditis elegans*) and inhibit the *in vivo* filamentation of *C. albicans* (Breger et al., 2007; Lv et al., 2021). Two studies conducted by Sun and co-workers (2018) showed that CAPE has synergism with caspofungin and fluconazole against *C. albicans* and fluconazole-resistant *C. albicans*, respectively (Sun et al., 2018b, 2018a). The synergism with caspofungin was associated

with a loss of iron homeostasis induced by CAPE, leading to functional defects in the mitochondrial respiratory chain and energy depletion, which increases the susceptibility of *C. albicans* to caspofungin (Sun et al., 2018a). On the other hand, Barros and co-workers (2019) evaluated the antifungal effect of CAPE on the biofilms of *C. albicans.* CAPE exhibited good antifungal activity against the 40 clinical strains of *C. albicans* with MIC values varied from 16 to 64 μg/mL. Moreover, all CAPE concentrations tested (1X, 2X, and 5X MIC) significantly decreased the biomass of *C. albicans* biofilm. On the other hand, scanning electron microscope images at a concentration of 5x MIC CAPE displayed predominance of yeast form compared to the control group which revealed many hyphae (Barros et al., 2019). Moreover, Vita and co-workers (2014) found that one of the synthesized caffeic acid esters is more active than fluconazole in the inhibition of biofilm formation in *C. albicans*, as well as in the eradication of pre-formed biofilms (Vita et al., 2014).

2.3.3. Antioxidant properties of *Coffea arabica*

Natural products, including plants and plant extracts, are considered one of the main sources of natural antioxidants. Antioxidants help in the survival of plants, and they may also confer health benefits on people who consume various plant foods (Borchardt et al., 2008). An antioxidant is a molecule that can prevent the oxidation of other molecules; it can inhibit the oxidation process by reacting with free radicals, chelating catalytic metals, and also acting as oxygen scavengers (Tapan and Kaushik, 2015).

The reactive oxygen species (ROS) are continuously formed inside the human body. The ROS are detoxified through the antioxidants existing in the body. However, an increase in ROS production and/or insufficient antioxidant defense can undoubtedly affect and initiate oxidative damage to different biomolecules including proteins, lipids, lipoproteins, and DNA (Nirmalraj and Perinbam, 2015). This oxidative damage is a serious etiological factor involved in many chronic human diseases such as diabetes, cancer, atherosclerosis, arthritis, and neurodegenerative diseases, as well as in the aging process (Patel et al., 2010).

The antioxidants can be categorized into two groups, namely enzymatic and non-enzymatic. The enzymatic antioxidants are produced by our bodies, while most non-enzymatic antioxidants are either derived from natural plants or synthesized in the laboratory (Nirmalraj and Perinbam, 2015). The Arabic coffee (*Coffea arabica* L.) has been thought to be an important source of antioxidants for humans, because of the high total phenolic content and caffeine. The chemical

profile of Arabic coffee includes phenolic compounds and their derivatives (such as chlorogenic acids), alkaloids (mainly caffeine), diterpenoid alcohols (such as cafestol and kahweol), carbohydrates, lipids, and volatile and heterocyclic compounds (Affonso et al., 2016; Magharbeh et al., 2020).

Affonso and co-workers (2016) investigated the antioxidant activity of the aqueous extract of coffee bean residual press cake. They found that the aqueous extract has DPPH radical scavenging activity (%) similar to the positive control (Trolox) activity (Affonso et al., 2016), which confirms the widespread belief that coffee is an important source of antioxidants.

3. Aims of the study

In the first part of this study, we have studied the effect of PEE on the planktonic growth and biofilm-forming ability of five commercial probiotics (Normaflore® oral suspension, Enterol®, BioGaia® ProTectis® Baby, Linex® Forte, and Protexin® Restore). These probiotics were selected to represent the most common strains that have probiotic properties according to the National Institute of Health, USA, 2020, including fungal and bacterial species. Some of the selected probiotics were monostrain probiotics (containing one strain of a certain species), while the others were multispecies probiotics (containing more than one species). This part of the study aimed to answer the following questions:

- What is the effect of PEE on the planktonic growth of probiotics?
- How does PEE affect the biofilm-forming abilities of probiotics?
- What is the effect of PEE on the mature biofilms of Normaflore microbes (*Bacillus clausii*)?
- What is the possible mechanism of action of PEE on the biofilms of Normaflore microbes (*Bacillus clausii*)?

In the second part, we have selected one of the most bioactive components of propolis (CAPE) and studied its effect on planktonic growth, biofilm-forming ability, and cell death in different strains of *C. albicans*, *C. tropicalis*, *C. glabrata*, and *C. parapsilosis*. These *Candida* species were selected due to the high clinical importance as the main causative agents of candidiasis. The strains were selected to represent high biofilm-formers (*C. albicans* SZMC 1424, *C. tropicalis* SZMC 1366, *C. glabrata* SZMC 1374, and *C. parapsilosis* SZMC 8007) and non-biofilm formers (*C. albicans* ATCC 44829, *C. albicans* SZMC 1423, *C. tropicalis* SZMC 1512, *C. glabrata* SZMC 1378, and *C. parapsilosis* SZMC 8008). This part of the study was intended to answer the following questions:

- What is the effect of CAPE on the planktonic growth of different *Candida* species and strains?
- How does CAPE affect the biofilm-forming abilities of the biofilm-forming *Candida* species and strains?
- What is the effect of CAPE on the mature biofilms of different biofilm-forming *Candida* species and strains?

- Can CAPE induce apoptosis in *Candida* species? If yes, is it caspase-dependent or caspase-independent? And are there any differences in the apoptotic markers between the different *Candida* species?

In the third part of this study, we focused on the molecular interaction between CAPE and actin. This because some previous studies reported a probable interaction between flavonoids and actin. From this point, we started to think about the effect of CAPE on actin as a major cytoskeletal element of the yeast cell. Through this part of the study we aimed to answer the following questions:

- Is there any molecular interaction between CAPE and G- and F-actin?
- Does CAPE cause a detectable functional change in the conformation of G- and F-actin?

In the last part of the study, which was done in cooperation with Mu'tah University / Jordan, we wanted to study the antioxidant properties of a natural product which is widely consumed by Jordanian people which is the Arabic coffee beans (*Coffea arabica*). So that our aim in this part was to answer the following questions:

- Does the methanolic extract of Arabic coffee beans (*Coffea arabica*) possess antioxidant properties?
- If there are antioxidant properties, which fraction of the methanolic extract possesses the highest antioxidant activity?

4. Materials and methods

4.1. Materials used in the experiments

For our experiments, propolis sample collected from a local beekeeper in Pécs/Hungary, CAPE, glutathion, dithiothreitol (DTT), 2,2-Diphenyl-1- picrylhydrazyl (DPPH) (Sigma-Aldrich, Switzerland), Ethylene glycol-bis(2-aminoethylether)-N N N′N′-tetraacetic acid (EGTA), adenosine triphosphate (ATP), formalin (Sigma-Aldrich, USA), sodium dodecyl sulfate, crystal violet, peptone, yeast extract (Merck, Germany), agar-agar (Fluka, Switzerland), modified RPMI 1640 medium (containing 3.4% (w/v) MOPS, 1.8% (w/v) dextrose and 0.002% (w/v) adenine) (Sigma-Aldrich, Saint Louis, USA), potassium dihydrogen phosphate, disodium hydrogen phosphate (Reanal, Budapest, Hungary), dimethyl sulfoxide (DMSO), ethanol (VWR Chemicals, France), sodium chloride (VWR Chemicals, Debrecen, Hungary), glucose (VWR prolabo, Belgium), adenine, calcium chloride, magneisum chloride, potassium chloride (Scharlau Chemie S.A, Bercelona, Spain), Z-VAD-FMK (Biovision, Mountain View, CA), glutardialdehyde solution (Sigma-Aldrich, Frankfurt, Germany), osmium tetroxide (Sigma-Aldrich, Schnelldorf, Germany), propylene oxide, Durcupan (R) ACM components A/M, B, C, and D (Sigma-Aldrich, Schnelldorf, Germany), 0.45 µm filters, 0.22 µm vacuum filters (Merck Millipore, France), sterile 96-well microtiter plates for antibacterial and antifungal activity (Costar®, USA), and for biofilm assays (Sarstedt AG & Co. KG, Numbrecht, Germany, Catalog number: 83.3924.500), CF®488A Annexin V and propidium iodide (PI) Apoptosis Kit (Biotium, CA, USA), AnaeroGen™ sacs (Sigma-Aldrich, Japan), Tris-HCl, methanol (Chemolab Ltd., Hungary) ethyl acetate, acetone, and isopropanol were used. All chemicals in the study were of analytical or spectroscopic grade. Highly purified water was applied throughout the experiments.

4.2. Instruments used in the experiments

A microbiological incubator (Thermo Scientific Heraeus B12), Sanyo orbital incubator (Sanyo, Japan), Sanyo autoclave (Sanyo, Japan), Hitachi U-2910 UV/Vis spectrophotometer (Hitachi, Japan), WTW pH meter (inoLab, Germany), Multiskan EX plate reader (Thermo electron corporation, Shanghai, China), benchtop centrifuge (Hettich, USA), Ultramicrotome Reichert Jung Ultracut E (LabX, Canada), JEOL-1200EX Transmission electron microscope (TEM), Attune NxT flow cytometer (Thermo Fisher Scientific Inc., MA, USA), Orbital shaker (Thermo Electro Corporation, Shanghai, China), Shimadzu UV-2100 spectrophotometer (Nishinokyo Kuwabara-cho, Nakagyo-ku, Kyoto 604-8511, Japan), SETARAM µDSC-III differential scanning calorimeter (1198

avenue du Docteur Maurice Donat 06250 Mougins, France) and UV/VIS double beam Spectrophotometer SPUV-26 (Staffordshire, UK) were used throughout the experiments.

4.3. Test Microorganisms

For the first part of the study, the commercial probiotics were purchased from a pharmacy in Hungary and used for the investigations: Normaflore® oral suspension (*Bacillus clausii*), Enterol® (*Saccharomyces boulardii* CNCM I-745), BioGaia® ProTectis® Baby (*Lactobacillus reuteri* DSM 17938), Linex® Forte (*Lactobacillus acidophilus* LA-5 and *Bifidobacterium animalis subsp. Lactis* BB-12), and Protexin® Restore (*Lactobacillus paracasei, Lactobacillus rhamnosus, Lactobacillus acidophilus, Lactobacillus bulgaricus, Bifidobacterium breve, Bifidobacterium infantis,* and *Streptococcus thermophilus*).

For the second part, four species of *Candida* were used: *C. albicans*, *C. tropicalis*, *C. glabrata*, and *C. parapsilosis*. Nine strains were included, one of which was American type culture collection (ATCC) strain and the others were *Candida* isolates obtained from Szeged Microbial Collection (SZMC), University of Szeged, Hungary (Table 3). Those species were selected to represent some of the most important causative agents of candidiasis in different regions of Hungary including both high biofilm forming and non-biofilm forming strains. Four of these strains have high biofilm-forming ability (*C. albicans* SZMC 1424, *C. tropicalis* SZMC 1366, *C. glabrata* SZMC 1374, and *C. parapsilosis* SZMC 8007), while the others were non-biofilm forming strains (*C. albicans* ATCC 44829, *C. albicans* SZMC 1423, *C. tropicalis* SZMC 1512, *C. glabrata* SZMC 1378, and *C. parapsilosis* SZMC 8008) (Czuni et al., 2016). All strains were maintained at the Department of General and Environmental Microbiology, Institute of Biology, University of Pécs, Hungary.

4.4. Culturing media and growth conditions

Normaflore, BioGaia, Linex, and Protexin were grown in de Man, Rogosa, and Sharpe (MRS) broth (Sigma-Aldrich, Switzerland), while Enterol and all *Candida* strains were grown in yeast extract peptone dextrose (YPD) broth (1% yeast extract, 2% peptone, and 2% glucose in distilled water, pH 6.8). For solid agar plates, 2% agar-agar was added to the medium. The microbes of BioGaia, Linex, and Protexin were grown in an anaerobic atmosphere at 37 °C for 24 h in GasPak anaerobic system using AnaeroGen™ sacs. Normaflore and Enterol microbes were grown aerobically at 37 °C, 150 rpm. All *Candida* strains were grown aerobically at 35 °C, 150 rpm. All microorganisms were stored as frozen stock with 25% glycerol at -78 °C. Before each experiment,

the microorganisms were recovered on MRS or YPD plates. All stationary-phase and mid-exponential phase cultures were prepared according to the growth curves.

Table 3. *Candida* species examined in the study.

Species	Collection code	Biofilm-forming ability	Origin
C. albicans	ATCC 44829	Non biofilm former	Auxotrophic mutant isolated after N-methyl N'-nitro-N-nitrosoguanidine treatment of a wild-type strain of *C. albicans*.
C. albicans	SZMC 1423	Non biofilm former	Clinical sample/Debrecen, Hungary
C. albicans	SZMC 1424	High biofilm former	Clinical sample/Debrecen, Hungary
C. tropicalis	SZMC 1366	High biofilm former	Haemoculture/Debrecen, Hungary
C. tropicalis	SZMC 1512	Non biofilm former	- /Pécs, Hungary
C. glabrata	SZMC 1374	High biofilm former	Clinical sample/Debrecen, Hungary
C. glabrata	SZMC 1378	Non biofilm former	Clinical sample/Debrecen, Hungary
C. parapsilosis	SZMC 8007	High biofilm former	Clinical sample/Szeged, Hungary
C. parapsilosis	SZMC 8008	Non biofilm former	unknown

4.5. Effect of PEE on probiotics

4.5.1. Preparation of PEE

Poplar-type propolis sample was collected from a local beekeeper in Pécs/Hungary. It was extracted with ethanol 80% (v/v) in a water bath, at 70 °C, for 30 min and then filtered with a 0.22 μm vacuum filter to obtain its ethanolic extract (Alencar et al., 2007). The concentration of the stock solution was set to 222.2 mg/mL. In all experiments, the final concentration of the solvent (ethanol) was set to 1%.

4.5.2. Antimicrobial susceptibility testing of probiotics planktonic cells against PEE

Determination of minimal inhibitory concentration (MIC) by the broth microdilution method was performed based on the recommended protocol of the National Committee for Clinical Laboratory Standard Institute (CLSI, 2012, 2007, 2002). Each probiotic product has been handled as one unit including monostrain and multispecies products. In short, a standardized initial inoculum (0.5 McFarland) was used for all experiments. The tests were performed in sterile, flat-bottom 96-well microplates. Equal volumes of cell suspension and PEE solution were dispensed into the wells to get the final concentration ranging from 12.5 to 800 μg/mL. For Linex, BioGaia, and Protexin, treatment with glutathione (0-100 μg/mL) was also applied to confirm the effect of antioxidants on

the growth of probiotics that contain anaerobic and/or microaerophilic bacteria. For each experiment, negative controls (media and cell suspension without PEE addition) and blanks (media with PEE) were included. The plates were placed in an incubator at 37 °C, and after incubation for 24 h, the absorbance at wavelength 600 nm was measured using a Multiskan Ex plate reader. The MIC_{80} of PEE was defined as the lowest concentration with a growth reduction (80%) when compared to that of the negative control.

4.5.3. Probiotics biofilm-forming ability assay

In vitro biofilm formation by probiotics was assayed by the ability of cells to adhere to the wells of a 96-well tissue culture microplate. Biofilm formation assay was done as described by Stepanović and co-workers (Stepanović et al., 2007). To inoculate the biofilm-forming ability assay microplates, 0.5 McFarland standard equivalent cell number was applied to prepare stationary-phase probiotic culture. The stationary-phase culture was vortexed and thereafter diluted 1:100 using RPMI-1640 medium. The stock solution of PEE was used to prepare series of 2-fold dilutions. Equal volumes of these dilutions were added to equal volumes of the diluted cell suspensions to get the final concentrations ranging from 12.5 to 800 μg/mL. Negative controls and blanks were included in each experiment. Solvent concentration was always kept as 1%. The microplates were incubated at 37 °C for 24 h, afterwards, the liquid part was removed, and the remaining biofilms were repeatedly washed with Phosphate-Buffered Saline (PBS) (0.8% NaCl, 0.02% KCl, 0.18% $Na_2HPO_4.2H_2O$, 0.024% KH_2PO_4, pH 7.4). The biofilms were fixed with 2% formalin in PBS (v/v) and stained with 0.13% crystal violet (w/v) for 20 min at room temperature. The unincorporated crystal violet was removed and the wells were washed thoroughly and repeatedly with PBS buffer. Biofilm formation was quantified by adding 1% SDS (w/v) solution to each well to solubilize the stain overnight, and the absorbance (Abs) of the solution was measured at 600 nm using a Multiskan Ex plate reader. The cut-off values of absorbance (Abs_c) were established according to the formula (Stepanović et al., 2007):

$$Abs_c = \text{average } Abs_{blank} + (3 \times SD \text{ of } Abs_{blank})$$

where Abs_c is the cut-off value of absorbance, average Abs_{blank} is the average of three absorbance measurements of blank (media with the proper concentration of PEE), and SD is the standard deviation of three measurements of Abs_{blank}. Final absorbance values of the tested probiotics (Abs_f) were generated as the average of three measurements. Based upon the Abs_f values, the threshold of the strong and weak biofilms was determined, and the probiotics were classified into three categories:

non-biofilm formers ($Abs_f \leq Abs_c$), weak biofilm formers ($Abs_c < Abs_f \leq 2Abs_c$), and strong biofilm formers ($Abs_f > 2Abs_c$).

4.5.4. Normaflore biofilm eradication assay

The effect of PEE on mature biofilms was verified as described by Nostro and co-workers (Nostro et al., 2007). Briefly, for the inoculation of the assay microplates, stationary-phase culture was prepared by using 0.5 McFarland standard equivalent cell number and thereafter diluted 1:100 using RPMI-1640 medium. Microplates containing diluted probiotic cell suspension were incubated at 37 °C for 24 h. After the biofilm maturation, PEE treatment was applied. Accordingly, the original RPMI-1640 culture was discarded, and replaced with PEE-containing RPMI-1640 medium with concentrations ranging from 12.5 to 800 µg/mL. Negative controls and blanks were included in each experiment. After 24 h of incubation at 37 °C, the growth of free-living cells was estimated by measuring the absorbance of the liquid part of the media at 600 nm using a Multiskan Ex plate reader, and the remaining biofilms were washed, fixed, stained, and estimated as mentioned in the previous section. The biofilm eradication was estimated by comparing the absorbance values of the treated samples with that of the negative control.

4.5.5. Normaflore autoaggregation assay

Autoaggregation ability was investigated as described by Jeon and co-workers (Jeon et al., 2017). Briefly, stationary-phase cultured cells were collected by centrifugation (5000 rpm, 5 min), washed twice with PBS, and re-suspended in a final cell density equivalent to 1 McFarland standard in PEE-containing PBS in a concentration range from 12.5 to 50 µg/mL. Negative controls and blanks were included in each experiment. Solvent concentration was always kept as 1%. Absorbance was measured at 600 nm using a Multiskan Ex plate reader immediately at zero time and after 24 h of incubation at 37 °C, and the percentage of autoaggregation was calculated as follows:

$$\text{Autoaggregation (\%)} = (1 - (A_{24}/ A_0)) \times 100$$

where A_0 and A_{24} represent the absorbance at zero time and at 24 h, respectively.

4.5.6. Normaflore swarming motility assay

Swarming motility assay was done as described by O'May and co-workers (O'May et al., 2012). In short, basic MRS broth supplemented with 5 g/L of D-glucose and solidified with 0.5% agar (Fluka, Switzerland) were used to prepare swarm agar plates. PEE was added to the swarm agar to get final concentrations ranging from 12.5 to 50 µg/mL. Swarm agar plates were inoculated with a 5 µl aliquot of probiotics broth culture. Negative controls were included in all of the experiments.

Solvent concentration was always kept as 1%. After 24 h of incubation at 37 °C, the diameters of the swarming motility zones were measured and expressed as a percentage of the negative control.

4.6. Effect of CAPE on different *Candida* species

4.6.1. Preparation of stock solution of CAPE

The stock solution of CAPE was prepared by dissolving 10 mg of CAPE in 1mL of absolute ethanol to get a final concentration of 10 mg/mL. The stock solution was kept in the freezer at -20°C.

4.6.2. Antifungal susceptibility testing of *Candida* planktonic cells against CAPE

The broth microdilution method was performed to determine the minimal inhibitory concentration (MIC) based on the recommended protocol of the National Committee for Clinical Laboratory Standards Institute (CLSI, 2002). Briefly, a standardized initial inoculum (10^6 cell/mL) was used for all experiments. The tests were done in sterile, flat-bottom 96-well microplates. Equal volumes (100 µL) of cell suspension and CAPE-containing YPD medium were dispensed into the wells to get the final CAPE concentration ranging from 3.125 to 400 µg/mL. Micro-wells containing Caspofungin (CAS) (rather than CAPE) at concentrations ranging from 0.063 to 4 µg/mL were used as positive controls. For each experiment, negative controls (media and cell suspension without CAPE addition) and blanks (media with CAPE) were included. Solvent concentration was always kept as 1%. The plates were placed in an incubator at 35 °C, and after incubation for 48 h, the absorbance at 600 nm was measured using a Multiskan Ex plate reader. The MIC_{80} of CAPE was defined as the lowest concentration with a growth reduction of 80% when compared to that of the negative control.

4.6.3. *Candida* biofilm susceptibility assay

Biofilm formation assay was performed using the crystal violet staining method as described previously (Stepanović et al., 2007). To inoculate the biofilm susceptibility assay microplates, 0.5 McFarland standard equivalent cell number was applied to prepare stationary-phase yeast culture. The culture was vortexed and thereafter diluted 1:100 using RPMI-1640 medium. The stock solution of CAPE (10 mg/mL) was used to prepare series of two-fold dilutions. Equal volumes (100 µL) of these dilutions were added to equal volumes (100 µL) of the diluted cell suspensions to get final concentrations ranging from 1.562 to 100 µg/mL. Negative controls and blanks were included in each experiment. Solvent concentration was always kept as 1%. The microplates were incubated at 35 °C for 48 h, afterwards, the liquid part was removed, and the remaining biofilms were repeatedly washed with PBS (pH 7.4). The biofilms were fixed with 2% formalin in PBS (v/v) and stained with

0.13% crystal violet (w/v) for 20 min at room temperature. The unincorporated crystal violet was removed and the wells were washed thoroughly and repeatedly with PBS buffer. Biofilm formation was quantified by adding 1% SDS (w/v) solution to each well to solubilize the stain overnight, and the absorbance of the solution was measured at 600 nm using a Multiskan Ex plate reader. The minimum biofilm inhibitory concentration (MBIC) was defined as the lowest concentration of CAPE that able to inhibit 90% biofilm formation.

4.6.4. *Candida* biofilm eradication assay

The effect of CAPE on mature biofilms was verified as described by Nostro and co-workers (Nostro et al., 2007). Briefly, for the inoculation of the assay microplates, stationary-phase yeast cultures were prepared by using 0.5 McFarland standard equivalent cell number and thereafter diluted 1:100 using RPMI-1640 medium. Microplates containing diluted cell suspension were incubated at 35 °C for 48 h. After the biofilm maturation, CAPE treatment was applied. Accordingly, the original RPMI culture was discarded, and replaced with a CAPE-containing RPMI medium with concentrations ranging from 1.562 to 100 µg/mL. Negative controls and blanks were included in each experiment. Solvent concentration was always kept as 1%. After 48 h of incubation at 35 °C, the liquid part of the media was discarded, and the remaining biofilms were washed, fixed, stained, and estimated as mentioned in the previous section. The biofilm eradication was estimated by comparing the absorbance values of the treated samples with that of the negative control.

4.6.5. Biosorption of CAPE by *Candida* cells

To determine the cellular biosorption of CAPE, YPD broth cultures of *Candida* strains were grown overnight at 35 °C and 150 rpm. The number of yeast cells was adjusted to 10^7 cells/mL in each case, and the cultures were treated with 100 µg/mL CAPE and incubated at 35°C and 150 rpm in an orbital shaker. Solvent concentration was always kept as 1%. Samples were taken at the time points 0, 5, 10, 15, 20, 30, 60, and 120 min after admission, centrifuged (5000 rpm, 5 min), and the absorbance of the cell-free supernatants was measured at 330 nm (absorption maximum of CAPE) using a Hitachi U-2910 UV/Vis spectrophotometer. A calibration curve of two-fold serial dilutions of CAPE from 100 to 0.781 µg/mL was constructed and used to evaluate the biosorption levels of *Candida* cells (Sun et al., 2015).

4.6.6. Cell death examination assay

YPD broth media were inoculated with *Candida* cells (10^6 cell/mL) from fresh YPD plate cultures and incubated at 35 °C with shaking at 150 rpm until reaching the mid-exponential phase

depending on their growth curves. Media containing sub-lethal concentrations (MIC_{80}) of CAPE were inoculated with 2.5×10^6 cells/mL of the mid-exponential phase cultures of different *Candida* species and strains and incubated at 35 °C with shaking for 3 hours. Untreated cell samples were included as negative controls in each experiment. Solvent concentration was always kept as 1% in all experiments. After the incubation period, cells were harvested and washed with PBS. CF®488A Annexin V and PI apoptosis kit was used according to the manufacturer's instructions to identify apoptosis and necrosis. In brief, *Candida* cells were re-suspended in 1X annexin V binding buffer at a concentration of 5×10^6 cells/mL. To the 100 µl of this solution, 5 µl of CF®488A Annexin V and 2 µl of PI working solution were added. Tubes were gently vortexed and incubated for 20 minutes at room temperature in the dark. After incubation, 400 µl of 1X annexin V binding buffer was added to each tube and analyzed using an Attune NxT flow cytometer. Annexin V is responsible for the detection of phosphatidylserine translocation from the inner to the outer leaflet of the plasma membrane, whereas PI is a membrane-impermeant DNA-binding dye that is usually used to selectively stain dead cells in a cell population. PI is excluded by living cells and early apoptotic cells but stains necrotic and late apoptotic cells with compromised membrane integrity. The flow cytometric analysis was able to show four distinct populations of cells: viable cells which have low CF488A-Annexin V and low PI signal; apoptotic cells that have high CF488A-Annexinv V and low PI signal; late-stage apoptotic (secondary necrotic cells) with compromised membranes exhibiting high CF488A-Annexin V and high PI signal; and necrotic cells with low CF488A-Annexin V and high PI signal

4.6.7. Caspase inhibitor assay

Caspase inhibitor assay was performed as described by Yue and coworkers (Yue et al., 2013) with some modifications. Briefly, cells were divided into two groups. The first group was pretreated for 1 h at 35 °C with the broad-spectrum caspase inhibitor, Z-VAD-FMK (final concentration 77 µM) before incubation with CAPE. The second group was used as a control (not treated with the caspase inhibitor). For microplate assays, cells were harvested by centrifugation, washed twice with PBS, and then re-suspended in YPD broth. The cell density was adjusted to 2×10^6 cell/mL. Equal volumes (100 µL) of cell suspension and CAPE-containing YPD medium were dispensed into the wells to get the final CAPE concentration equal to the MIC_{80} of each strain. For each experiment, negative controls (media and cell suspension without CAPE addition) and blanks (media with CAPE) were included. Solvent concentration was always kept as 1%. The plates were placed in an incubator

at 35 °C, and after incubation for 48 h, the absorbance at 600 nm was measured using a Multiskan Ex plate reader.

4.6.8. Ultrastructural examination of *Candida* species by TEM

Media containing sub-lethal concentrations of CAPE were inoculated with 2.5×10^6 cells/mL of the mid-exponential phase cultures of different species of *Candida* and incubated at 35 °C with shaking for 3 hours to induce apoptosis. After the incubation period, cells were harvested by centrifugation (5000 rpm, 5 min). Pellets were immediately washed and re-suspended with modified PBS (a mixture of 50 mM K_2HPO_4 and KH_2PO_4 (pH 7.0), supplemented with 0.5 mM $MgCl_2$), and incubated at room temperature for 15 minutes to achieve equilibrium. Then, the samples were fixed overnight in 2.5% glutaraldehyde fixative buffered with modified PBS. Samples were then washed 4 times with modified PBS, and after short centrifugal sedimentation (2 minutes, 1000 rpm) preparation continued with a 2 % osmium tetroxide post-fixation on ice for 2 h. Cells were then washed twice with distilled water for 15 minutes and stained '*en bloc*' in 1% aqueous uranyl acetate for 30 minutes. After two further washing steps with distilled water and short sedimentation, cells were dehydrated in 70, 96, and 100% ethanol for 15 minutes each, subsequently. Cells were treated with propylene oxide twice for 10 minutes each time, then infiltrated for 1 h in propylene oxide/ Durcupan epoxy resin mixture (1:1) at room temperature. After 1 h, cells were transferred to fresh epoxy resin drops for another 1 hour. The resin was then changed and samples were left overnight in the fresh resin drops at room temperature. The next day, the resin was changed twice while incubating at 40 °C for 2 h, subsequently. Finally, samples were encapsulated in fresh epoxy resin and left at 56 °C for a two-day long polymerization. Serial ultrathin sections were cut with Reichert Ultramicrotome, collected onto 300 mesh Nickel grids, counterstained on drops of uranyl acetate and Reynolds solution of lead citrate, washed thoroughly in sterile distilled water, and examined with a JEOL-1200 EX TEM at 80 KeV (Phillips et al., 2003).

4.7. Effect of CAPE on actin

4.7.1. Actin preparation

The skeletal α-actin was prepared and provided from acetone-dried muscle powder of rabbit skeletal muscle by Dr. Zoltán Ujfalusi (Department of Biophysics at the Medical School, University of Pécs) according to the method of Spudich and Watt (Spudich and Watt, 1971). Actin was labeled with N-(1-pyrene) Iodoacetamide based on the method of Criddle and co-workers (1985).

The G-actin actin was stored in buffer A (4 mM Tris-HCl (pH 7.3), 0.2 mM ATP, 0.1 mM $CaCl_2$, 0.5 mM DTT, and 0.005 % NaN_3 at 4 °C), the concentration was measured and calculated at 290 nm, with a Shimadzu UV-2100i spectrophotometer using the absorption coefficient of 0.63 mL/(mg*cm) and a relative molecular mass of 42,300.

4.7.2. Anisotropy measurements

In steady-state anisotropy measurements, the anisotropy of unlabeled actin was monitored using the intrinsic fluorescence of tryptophan in the absence or presence of CAPE. Pyrene fluorescence was not applicable for these measurements as the spectra of the label overlap with the spectra of CAPE. Measurements were carried out using 10 μM actin. When applicable, CAPE at 50 μg/mL was added to actin monomers. The sample was excited with plane-polarized light at 290 nm, and the degree of polarization of the emitted fluorescence was detected at 336 nm (Ujfalusi et al., 2012).

4.7.3. Actin polymerization assay

The polymerization assay is based on the fact that the fluorescence intensity of pyrene-bound actin increases by a factor of ~25 upon polymerization. In these experiments, 5% of the total G-actin population was pyrene-labeled and that was used to monitor the time dependence of the formation of actin filaments. Briefly, monomeric calcium-actin was in buffer A after preparation. The bound calcium was replaced with magnesium by adding 200 μM EGTA and 50 μM $MgCl_2$ and incubating the samples for 5–10 min. The magnesium-actin was polymerized with 2 mM $MgCl_2$ and 100 mM KCl in either the absence or the presence of different concentrations of CAPE (0.5, 5, 10, 20, 30, 40, and 50 μg/mL). The actin concentration was set to 109 μg/mL. The excitation and emission wavelengths were 365 and 407 nm, respectively, whereas the PMT Voltage was 840 V and the optical slits were set to 3 nm in both the excitation and the emission paths (Bugyi et al., 2006). Time-dependent curves were analyzed with OriginPro 2016 software.

4.7.4. Differential scanning calorimetry

The thermal denaturation of the two kinds of α-actin (G and F) was examined between 20 and 100°C using a SETARAM μDSC-III differential scanning calorimeter in the absence or the presence of 25 μg/mL CAPE. The heating and cooling rates were 0.3 and 1 K/min, respectively. Conventional Hastelloy batch vessels were used with an 850 μl sample volume. The concentration of G- and F-actin was 46 μM. The experimental buffer without the protein was used as a reference. The sample and reference vessels were balanced with a precision of ±0.05 mg. The data were analyzed with the OriginPro 2016 software, and the denaturation peaks or melting temperature (T_m) values (the point

of 50% denaturation) of G-actin and F-actin in absence or presence of CAPE were determined. (Bugyi et al., 2006; Papp et al., 2005).

4.8. Antioxidant activity of Arabic coffee (*C. arabica*) beans

4.8.1. Plant material

The Arabic coffee (*C. arabica*) beans were collected entirely from the local Jordanian market within the Karak governorate in December 2018. The collected beans were then dried at 45 ºC overnight and afterward ground to powder.

4.8.2. Preparation of the plant extract and subsequent fractions

The preparation of plant extract and subsequent fractions was done in cooperation with the Faculty of Pharmacy at Mu'tah University, Jordan. Through the reflux method, 50 g of the dried ground beans were exhaustively defatted with 400 mL n-hexane for 8 hr. The defatted beans were soaked again in 400 mL methanol for 8 hr. The total methanol extract was concentrated till dryness under vacuum. The methanol extract residue was then fractionated against three different solvents namely, ethyl acetate, acetone, and isopropanol using the Soxhlet apparatus. The different fractions together with the residual methanol extract were filtered out, concentrated, and dried under reduced pressure. Afterward, they were all re-suspended in distilled water, filtered through a 0.45 µm filter, and used to prepare 1 mg/mL solution from each fraction in distilled water.

4.8.3. Antioxidant activity assay

The antioxidant activity was determined using DPPH radical scavenging assay. Serial concentrations of the methanolic extract or its subsequent fractions were added to 5 mL of 0.004% DPPH in methanol and incubated at room temperature for 30 min. Then the absorbance was measured against methanol at 517 nm. Inhibition of free radical scavenging activity was calculated using the following equation:

$$\text{Inhibition (\%)} = 100 \times (\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}})/\text{Abs}_{\text{control}}$$

where "Abs control" is the absorbance of the control reaction (containing all reagents except the tested compound) and "Abs sample" is the absorbance of the tested compound with all other reagents. Extract concentration providing 50% inhibition (IC_{50}) was determined for the methanolic extract or its subsequent fractions (Tepe et al., 2005).

4.8. Statistical analysis

All assays were carried out in triplicate, and data were expressed as Mean ± standard deviation (SD). For data processing and visualization of the results, OriginPro 2016 and MS Office Excel 2016

were used. Statistical analysis was performed either with one-sample or two-sample t-tests using Past3.21 software.

5. Results

5.1. Effect of PEE on free-living probiotic cells

To collect basic information about the effect of PEE on the viability of free-living (planktonic) probiotic cells in five different probiotic products, the susceptibility test was conducted. The results revealed that PEE has different effects on the viabilities of the different probiotics (Fig. 11). Due to the known antimicrobial activity of PEE, it can reduce the planktonic growth of probiotics in each case. However, lower concentrations of PEE improved the viability of Linex, BioGaia, and Protexin microbes which are mostly containing anaerobic and/or microaerophilic bacteria (Fig. 11). Similarly, the viability of those microbes was also improved when different concentrations of the antioxidant glutathione treatment was applied (Fig. 12).

Based on the performed experiment, MIC_{80} values have been determined. As Figure (11) demonstrates, different probiotics have varied MIC_{80} values in the range of 100 – 800 µg/mL. The lowest MIC_{80} value was found for Linex and Enterol (100 µg/mL), while it was doubled for Protexin and Normaflore (200 µg/mL). However, BioGaia, which contains *L. reuteri* DSM 17938, has the highest MIC_{80} value (800 µg/mL), and this might be due to the antibiotic-producing properties of this strain.

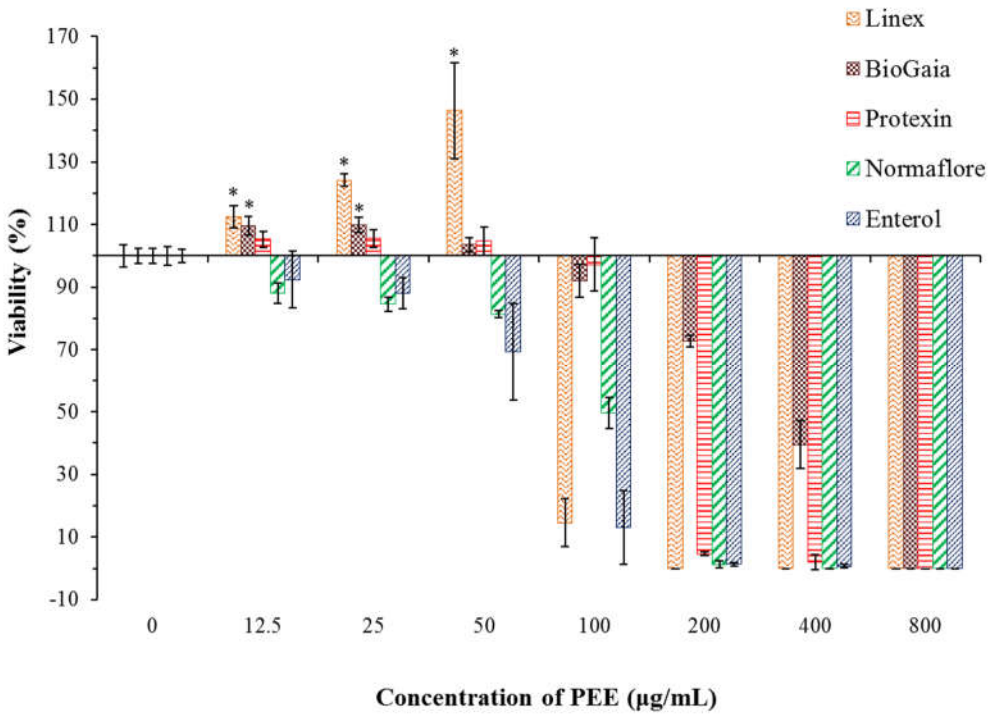

Fig. 11. Viability of planktonic form of probiotics in the presence of different concentrations of PEE. Data are shown as Mean ± SD from three independent experiments. *$p<0.05$ indicates a significant increment of the viability compared to the negative control (0 µg/mL).

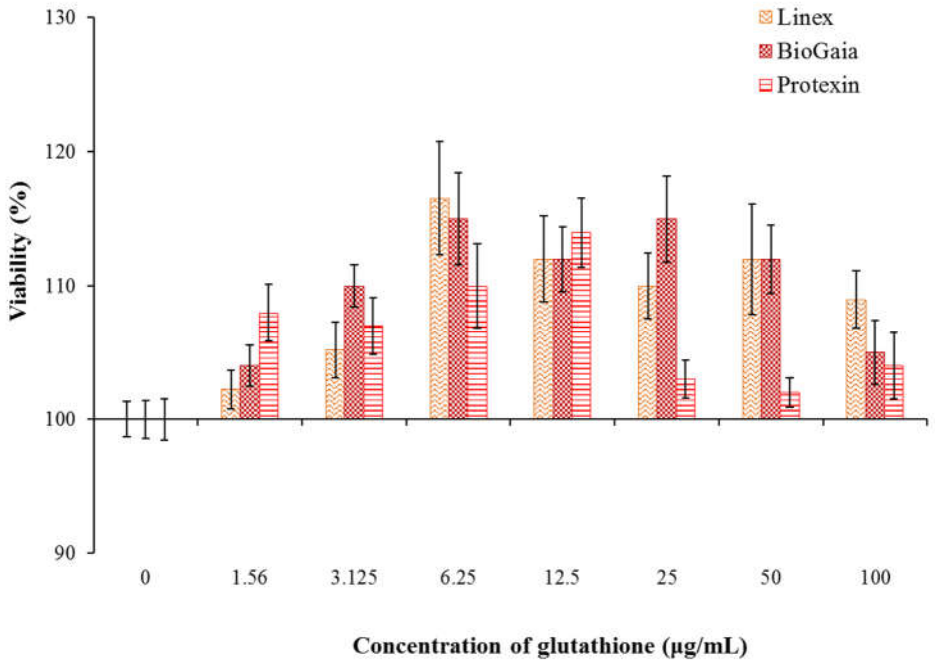

Fig. 12. Viability of planktonic form of probiotics which are mostly containing anaerobic and/or microaerophilic bacteria in the presence of different concentrations of glutathione. Data are shown as Mean ± SD from three independent experiments.

5.2. Effect of PEE on the biofilm-forming ability of the probiotics

The importance of biofilm-forming ability related to probiotics is unquestionable. Each applied probiotic was tested, and the results revealed that Protexin and Enterol microbes were non-biofilm formers under the applied conditions. On the other hand, the microbes of BioGaia, Linex, and Normaflore can form weak biofilms (Table 4).

The weak biofilm-forming probiotics have various responses to the PEE treatments. BioGaia microbes were highly sensitive to the PEE treatment, the lowest concentration of PEE (12.5 µg/mL) was enough to inhibit its biofilm-forming ability. However, the same concentration of PEE has a slight positive effect on the biofilm-forming ability of Linex microbes, but it still falls to the weak biofilm category. Interestingly, the biofilm of Normaflore microbes showed a unique property not only to tolerate higher concentrations of PEE but moreover, it has been enhanced and shifted from weak to strong biofilm at 12.5, 25 and, 50 µg/mL concentrations (Table 4).

Table 4. Effect of PEE on the biofilm-forming abilities of probiotics.

PEE Conc. (μg/mL)	Protexin			Enterol			BioGaia			Linex			Normaflore		
	Abs_f	Abs_c	Biofilm	Abs_f	Abs_c	Biofilm	Abs_f	Abs_c	Biofilm	Abs_f	Abs_c	Biofilm	Abs_f	Abs_c	Biofilm
0	0.086	0.094	NB	0.073	0.095	NB	0.080	0.079	WB	0.130	0.109	WB	0.129	0.067	WB
12.5	0.131	0.172	NB	0.100	0.149	NB	0.128	0.142	NB	0.224	0.147	WB	0.333	0.123	SB
25	0.145	0.200	NB	0.109	0.143	NB	0.128	0.161	NB	0.153	0.168	NB	0.398	0.156	SB
50	0.164	0.203	NB	0.124	0.170	NB	0.144	0.183	NB	0.152	0.197	NB	0.398	0.191	SB
100	0.189	0.247	NB	0.140	0.174	NB	0.166	0.203	NB	0.169	0.217	NB	0.311	0.267	WB
200	0.218	0.310	NB	0.173	0.215	NB	0.223	0.267	NB	0.212	0.251	NB	0.321	0.300	WB
400	0.198	0.228	NB	0.176	0.176	NB	0.218	0.221	NB	0.224	0.313	NB	0.298	0.252	WB
800	0.168	0.234	NB	0.164	0.186	NB	0.172	0.240	NB	0.175	0.254	NB	0.212	0.236	NB

NB: no biofilm ($Abs_f \leq Abs_c$), WB: weak biofilm ($Abs_c < Abs_f \leq 2Abs_c$), SB: strong biofilm ($Abs_f > 2Abs_c$).

5.3. Effect of PEE on mature biofilms of Normaflore

To get more inside to the unique and interesting response of Normaflore microbes to the PEE treatment, the effect of PEE on their mature biofilms was investigated. Application of different concentrations of PEE revealed that the mass of the mature biofilm was improved up to 400 µg/mL. Whereas biofilm eradication was observed at 800 µg/mL and the biofilm mass was reduced about 70% of the control (Fig. 13). Interestingly, the mature biofilms of Normaflore microbes were shifted from weak to strong at low concentrations of PEE (12.5 and 25 µg/mL). Concerning planktonic cells which were found in the suspension above the biofilm, PEE revealed a dose-dependent inhibitory effect on their growth (Fig. 13).

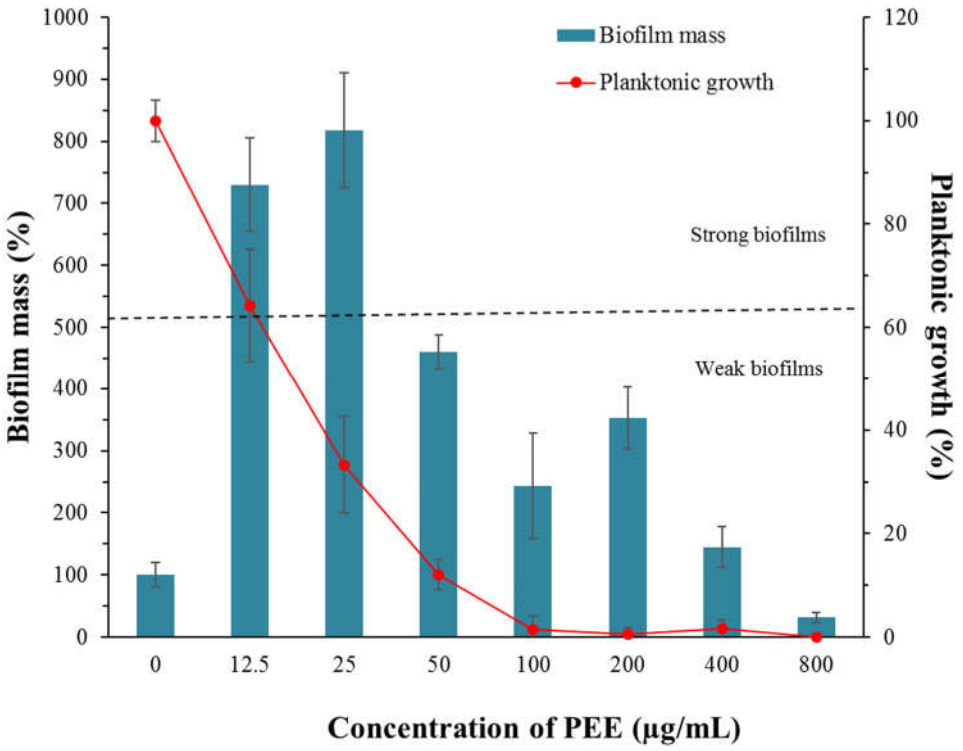

Fig. 13. Effect of different concentrations of PEE on mature biofilms and planktonic cells of Normaflore microbes. The dashed line indicates the threshold of strong and weak biofilms. Data are shown as Mean ± SD from three independent experiments.

5.4. Effect of PEE on autoaggregation in Normaflore

Autoaggregation is the process by which bacterial cells belonging to the same bacterial species recognize each other and form multicellular clumps (Trunk et al., 2018). Autoaggregation is known

to be positively correlated to biofilm formation ability (Sorroche et al., 2012). The results of the autoaggregation experiment revealed that PEE has a significant stimulatory effect on the autoaggregation ability of Normaflore microbes (Fig. 14). After 24 hours of incubation, the autoaggregation rate at 12.5, 25, and 50 µg/mL of PEE was about 9, 14, and 21% higher than the negative control (0 µg/mL), respectively. Concentrations above 50 µg/mL were excluded from this experiment because more than 50% of the cells were unviable at these concentrations (Fig. 11).

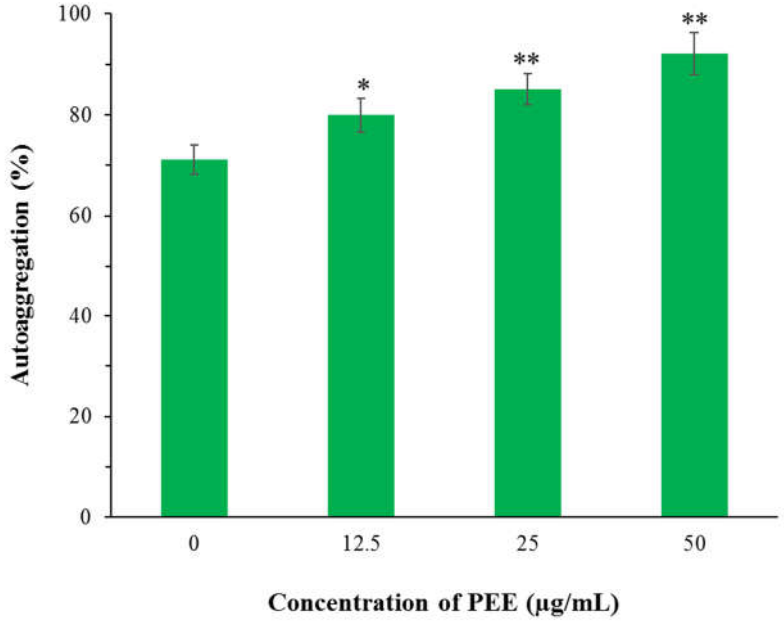

Fig. 14. Autoaggregation ability of Normaflore microbes after 24 h incubation in the presence of PEE. Data are shown as Mean ± SD from three independent experiments. *p<0.05 and **p<0.01 indicate significant differences compared to the negative control (0 µg/mL).

5.5. Effect of PEE on swarming motility in Normaflore

Swarming motility is the rapid and coordinated translocation of a bacterial population on a surface powered by rotating flagella (Kearns, 2010). The findings of this test showed that PEE has a significant inhibitory effect on the swarming motility of Normaflore microbes compared to the untreated group (Fig. 15). The rate of swarming motility decreased about 12, 22, and 33% when treated with 12.5, 25, and 50 µg/mL of PEE, respectively.

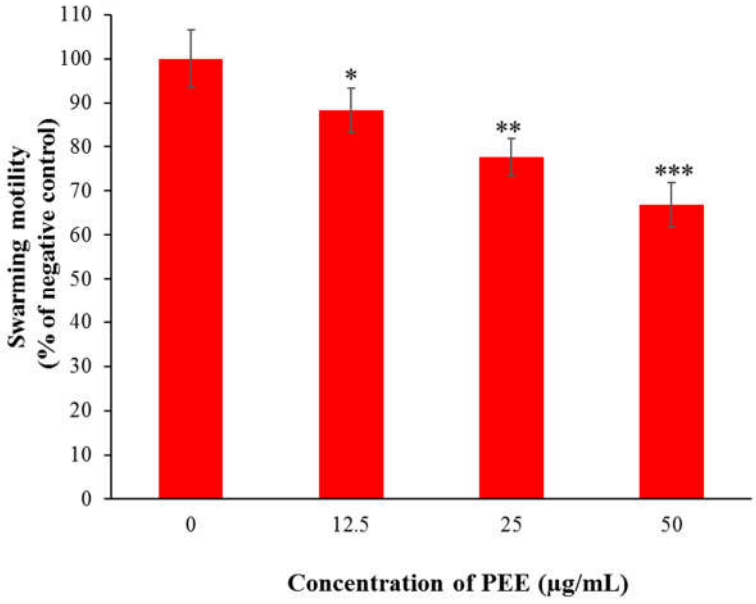

Fig. 15. Swarming motility of Normaflore microbes after 24 h incubation with PEE. Data are shown as Mean ± SD from three independent experiments. *p<0.05, **p<0.01 and ***p<0.001 indicate significant differences compared to the negative control (0 µg/mL).

5.6. Susceptibility of *Candida* planktonic cells to CAPE

The antifungal effect of CAPE on nine *Candida* strains was studied. The results of the MIC_{80} values for both CAPE and the positive control against different *Candida* species and strains are given in Table (5). It has been found that CAPE has a strain and dose-dependent effect. The MIC_{80} values were ranging from 12.5 to 100 µg/mL. The highest inhibitory effect was seen against *C. glabrata* SZMC 1378, *C. glabrata* SZMC 1374, and *C. parapsilosis* SZMC 8008 compared to the other strains. Whereas, the most resistant strain was *C. albicans* SZMC 1423.

5.7. Effect of CAPE on *Candida* biofilm-forming ability

The biofilm-forming ability is a very important property related to the pathogenicity of *Candida*. CAPE was used to determine whether different concentrations could inhibit the biofilm formation in four *Candida* strains that have high biofilm-forming abilities. The results of this experiment demonstrated that CAPE has a species and dose-dependent inhibitory effect on the biofilm formation in the four strains (Fig. 16). The MBIC values were 50, 50, 50, and 100 µg/mL for *C. albicans* SZMC 1424, *C. glabrata* SZMC 1374, *C. parapsilosis* SZMC 8007, and *C. tropicalis* SZMC 1366, respectively.

Table 5. MIC values of CAPE and CAS (positive control) against the different *Candida* strains.

Strain	MIC_{80} (μg/mL)	
	CAPE	**CAS**
C. albicans ATCC 44829	50	1
C. albicans SZMC 1423	100	1
C. albicans SZMC 1424 (B)	50	1
C. glabrata SZMC 1374 (B)	12.5	1
C. glabrata SZMC 1378	12.5	1
C. parapsilosis SZMC 8007 (B)	25	2
C. parapsilosis SZMC 8008	12.5	2
C. tropicalis SZMC 1366 (B)	50	2
C. tropicalis SZMC 1512	50	2

(B) indicates biofilm-forming strain

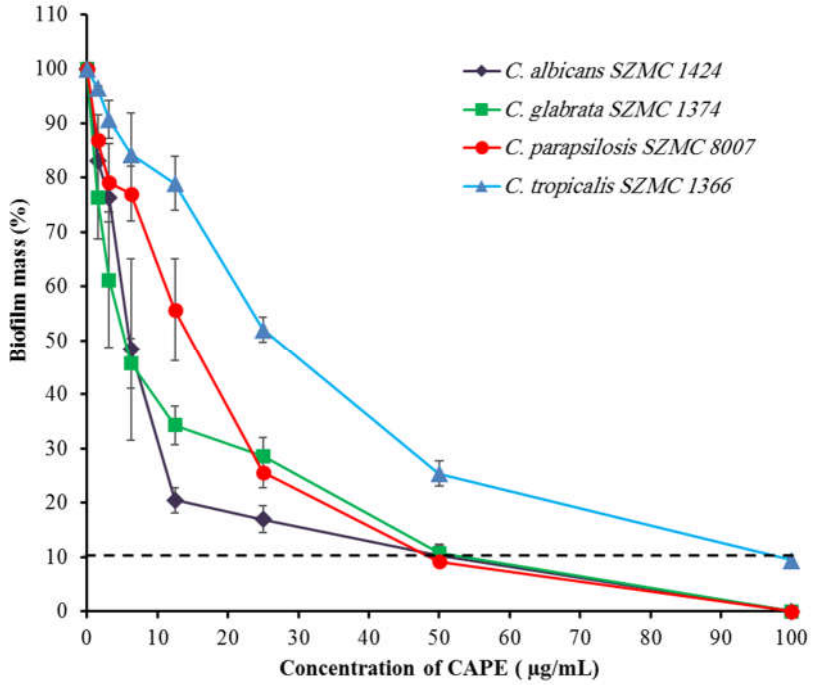

Fig. 16. Effect of different concentrations of CAPE on the biofilm-forming ability of four *Candida* species and strains. The dashed line represents the MBIC. Data are shown as Mean ± SD from three independent experiments.

5.8. Effect of CAPE on *Candida* biofilm eradication

The effect of CAPE on the mature biofilms of the four biofilm-forming *Candida* species was investigated. Application of different concentrations of CAPE on the mature biofilms of *C. albicans* SZMC 1424, *C. glabrata* SZMC 1374, *C. tropicalis* SZMC 1366, and *C. parapsilosis* SZMC 8007 resulted in partial eradication. The maximum eradication (19-49%) was achieved at 25 µg/mL for *C. albicans* SZMC 1424, *C. glabrata* SZMC 1374, and *C. parapsilosis* SZMC 8007 and at 100 µg/mL for *C. tropicalis* SZMC 1366. This indicates that the mature biofilms of *C. tropicalis* SZMC 1366 are the most resistant to CAPE. Moreover, the eradication process was no more dose-dependent above 25 µg/mL in *C. albicans* SZMC 1424, *C. glabrata* SZMC 1374, and *C. parapsilosis* SZMC 8007 (Fig. 17).

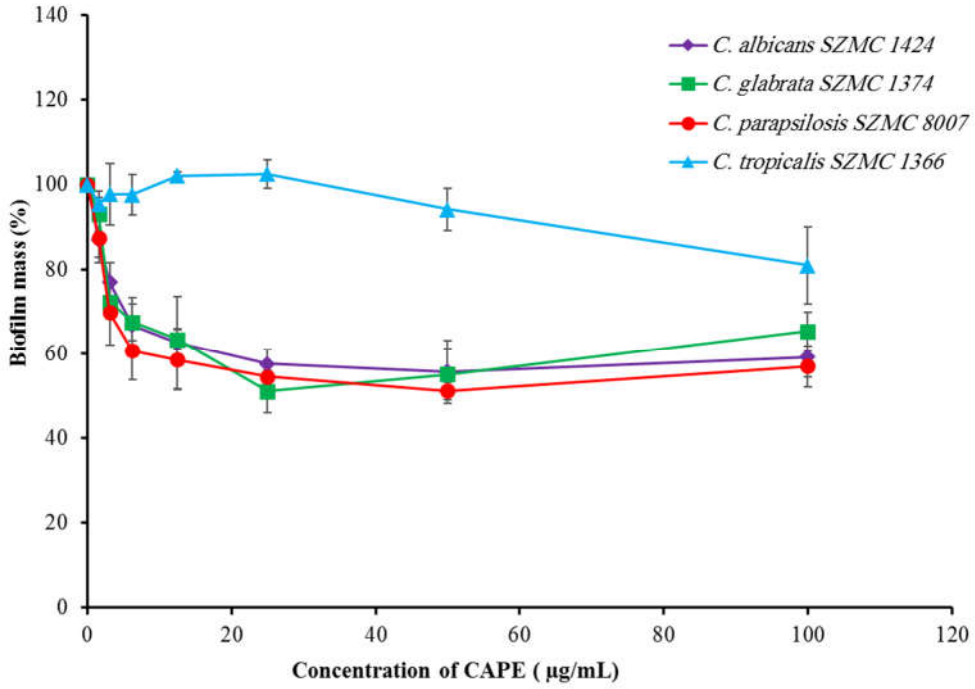

Fig. 17. Effect of different concentrations of CAPE on mature biofilms of four *Candida* species and strains. Data are shown as Mean ± SD from three independent experiments.

5.9. Biosorption of CAPE by *Candida* cells

Biosorption may be defined as "the removal/binding of desired substances from aqueous solution by biological material" (Michalak et al., 2013). The results revealed that the biosorption of CAPE by different *Candida* strains occurs very quickly, and the maximum biosorption was achieved within the first 30 to 90 minutes (Fig. 18). According to the amount of CAPE biosorbed, two groups

can be recognized: the first group was able to biosorb 53-63 µg/mL of CAPE and it includes *C. albicans* SZMC 1424, *C. parapsilosis* SZMC 8007, and *C. parapsilosis* SZMC 8008; while the second group was able to biosorb 74-86 µg/mL and it includes *C. albicans* ATCC 44829, *C. albicans* SZMC 1423, *C. tropicalis* SZMC 1366, *C. tropicalis* SZMC 1512, *C. glabrata* SZMC 1374, and *C. glabrata* SZMC 1378.

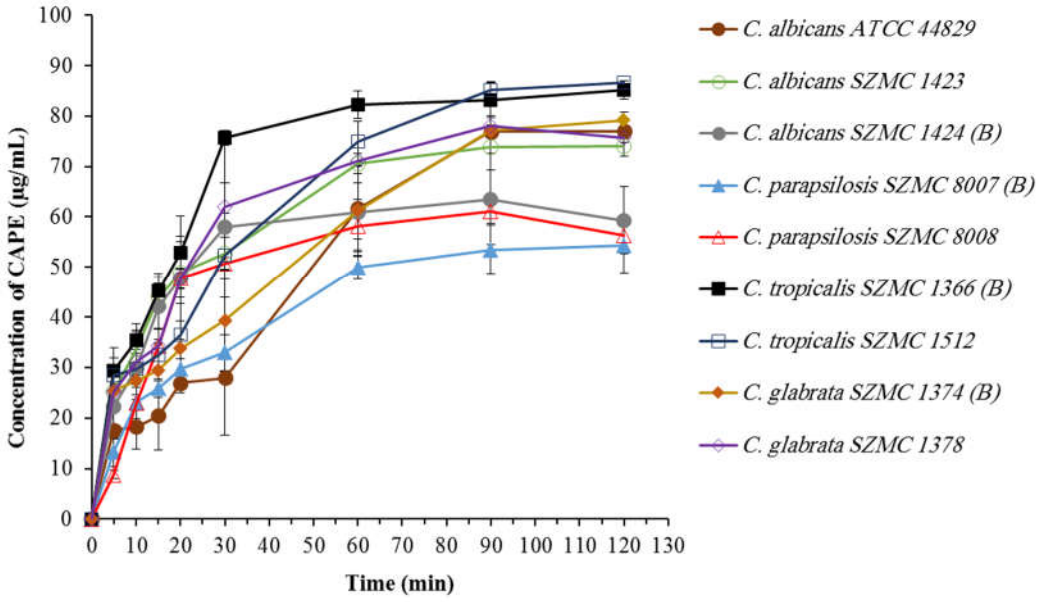

Fig. 18. Biosorption of CAPE by different *Candida* species and strains. Data are shown as Mean ± SD from three independent experiments. (B) indicates biofilm-forming strain.

5.10. Induction of apoptotic cell death in *Candida* spp. by CAPE

Cells of nine *Candida* strains treated with sub-lethal concentrations of CAPE were analyzed by double staining with CF®488A Annexin V and PI. The apoptotic cells with externalized phosphatidylserine were detected by CF®488A Annexin V, while necrotic cells were detected by PI staining. The results that are shown in Figure (19) demonstrate that CAPE can induce apoptosis in six of the tested strains at different levels. Among these strains, *C. albicans* SZMC 1423 and *C. albicans* ATCC 44829 revealed the highest percentage of early apoptotic cells (69.8 and 70.2%, respectively), whereas almost no apoptosis was seen in *C. glabrata* SZMC 1374, *C. parapsilosis* SZMC 8008, and *C. glabrata* SZMC 1378 (apoptotic cells ≤ 2%). On the other hand, no necrosis was seen in any of the tested strains (necrotic cells ≤ 1%).

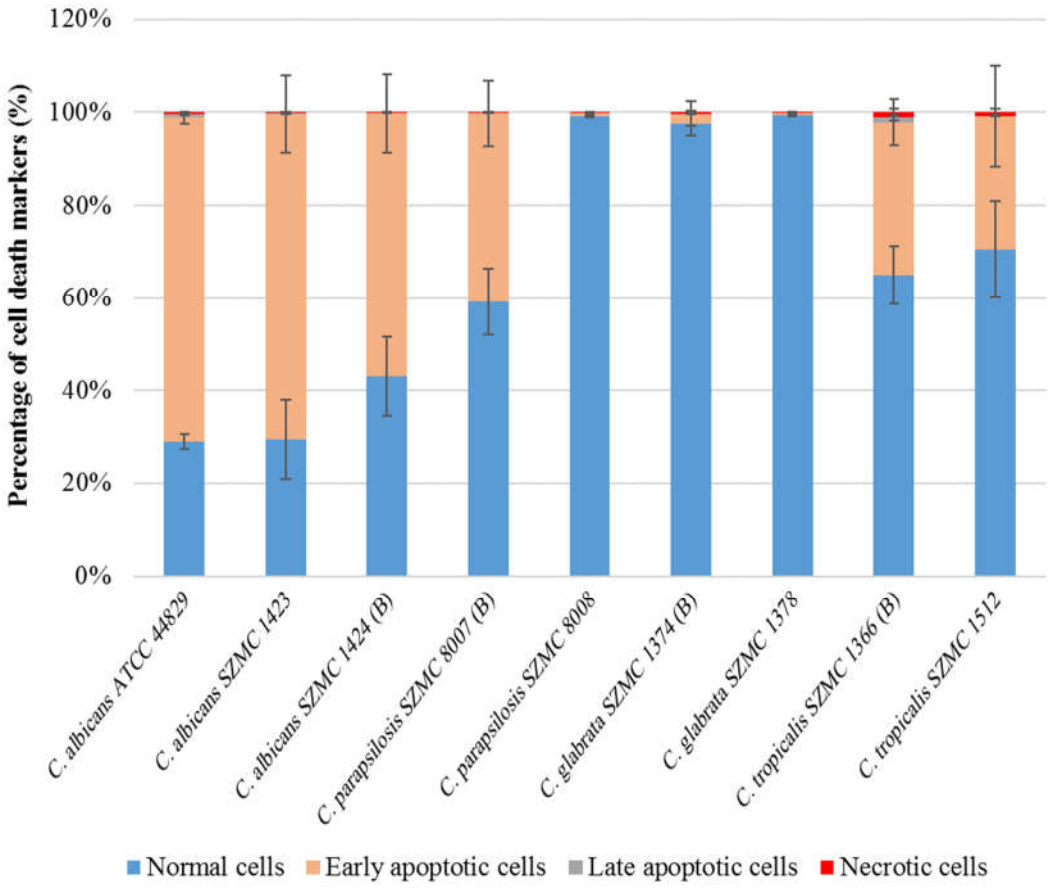

Fig. 19. Cell death induced by CAPE treatment in nine *Candida* strains as determined by annexin V and PI staining. Data are shown as Mean ± SD from three independent experiments. (B) indicates biofilm-forming strain.

5.11. Effect of caspase inhibitor on the growth of CAPE-treated *Candida* cells

To investigate whether yeast caspase Yca1p is involved in CAPE-induced apoptotic cell death, pre-incubation with pan-caspase inhibitor Z-VAD-FMK was applied for 1 h. The growth of the sub-lethal CAPE concentration-treated *Candida* strains that revealed apoptosis was analyzed with and without pre-incubation with Z-VAD-FMK. As shown in Figure (20), a significant increase in the viability was observed in *C. albicans* ATCC 44829, *C. albicans* SZMC 1424, *C. tropicalis* SZMC 1366, and *C. tropicalis* SZMC 1512 that are pre-incubated with pan-caspase inhibitor Z-VAD-FMK. However, the viability of CAPE-treated *C. albicans* SZMC 1423 and *C. parapsilosis* SZMC 8007 was not affected with the pre-incubation with pan-caspase inhibitor Z-VAD-FMK.

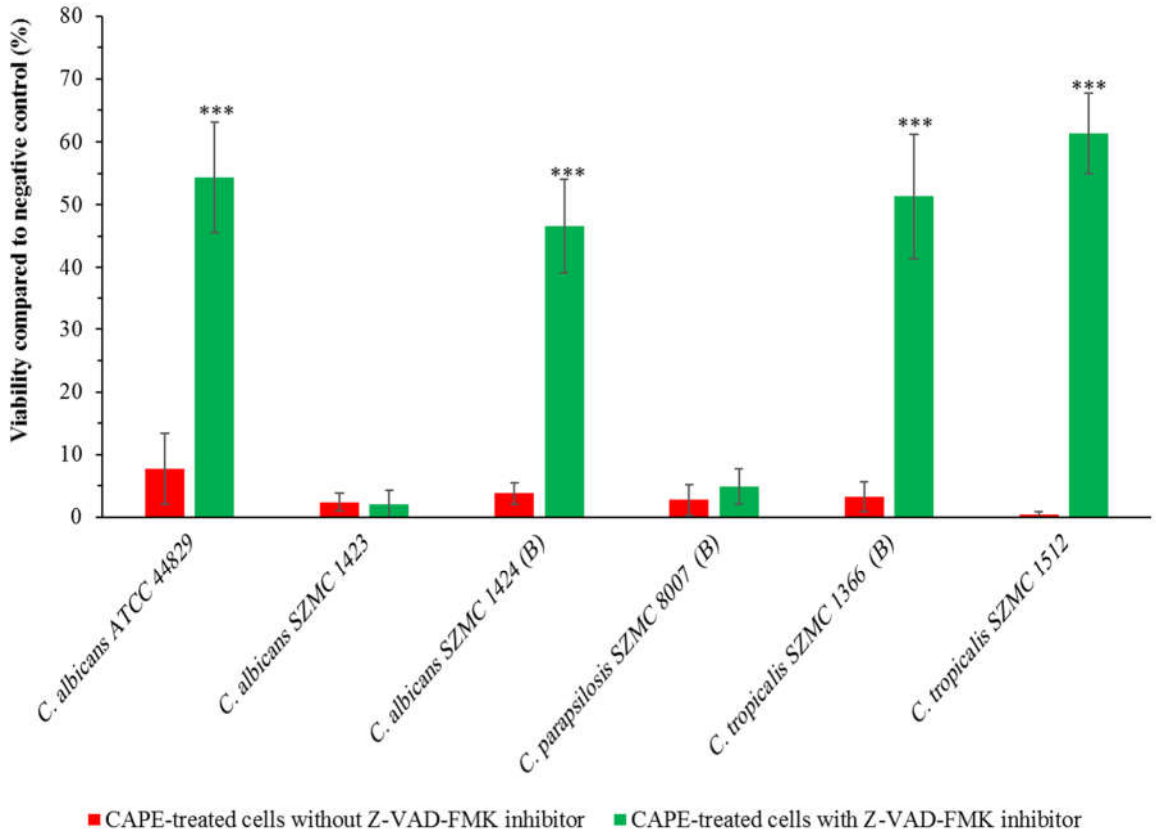

Fig. 20. Effect of pan-caspase inhibitor Z-VAD-FMK on the viability of six *Candida* strains treated with sub-lethal concentrations of CAPE. Data are shown as Mean ± SD from three independent experiments. ***$p<0.001$ indicates a significant increment of the viability compared to the viability without pre-incubation with pan-caspase inhibitor Z-VAD-FMK. (B) indicates biofilm-forming strain.

5.12. Visualization of apoptotic and necrotic markers

To visualize the changes in intracellular morphology of the cells after CAPE treatment, transmission electron microscopy was performed on *Candida* cells exposed to sub-lethal concentrations of CAPE. The TEM micrographs of *C. tropicalis*, *C. albicans*, and *C. parapsilosis* (Fig. 21, 22, and 23, respectively) mainly revealed typical hallmarks of apoptosis, including nuclear chromatin margination, nuclear blebs, condensation in the nucleus, vacuolization, plasma membrane detachment, huge lysosomes, cytoplasm fragmentation, cell wall distortion, and whole-cell shrinkage. However, very few cells displayed the signs of necrosis, such as membrane disintegration and loss of cytoplasm density. Whereas, the TEM micrographs of *C. glabrata* (Fig. 24) mainly revealed smaller necrotic signs.

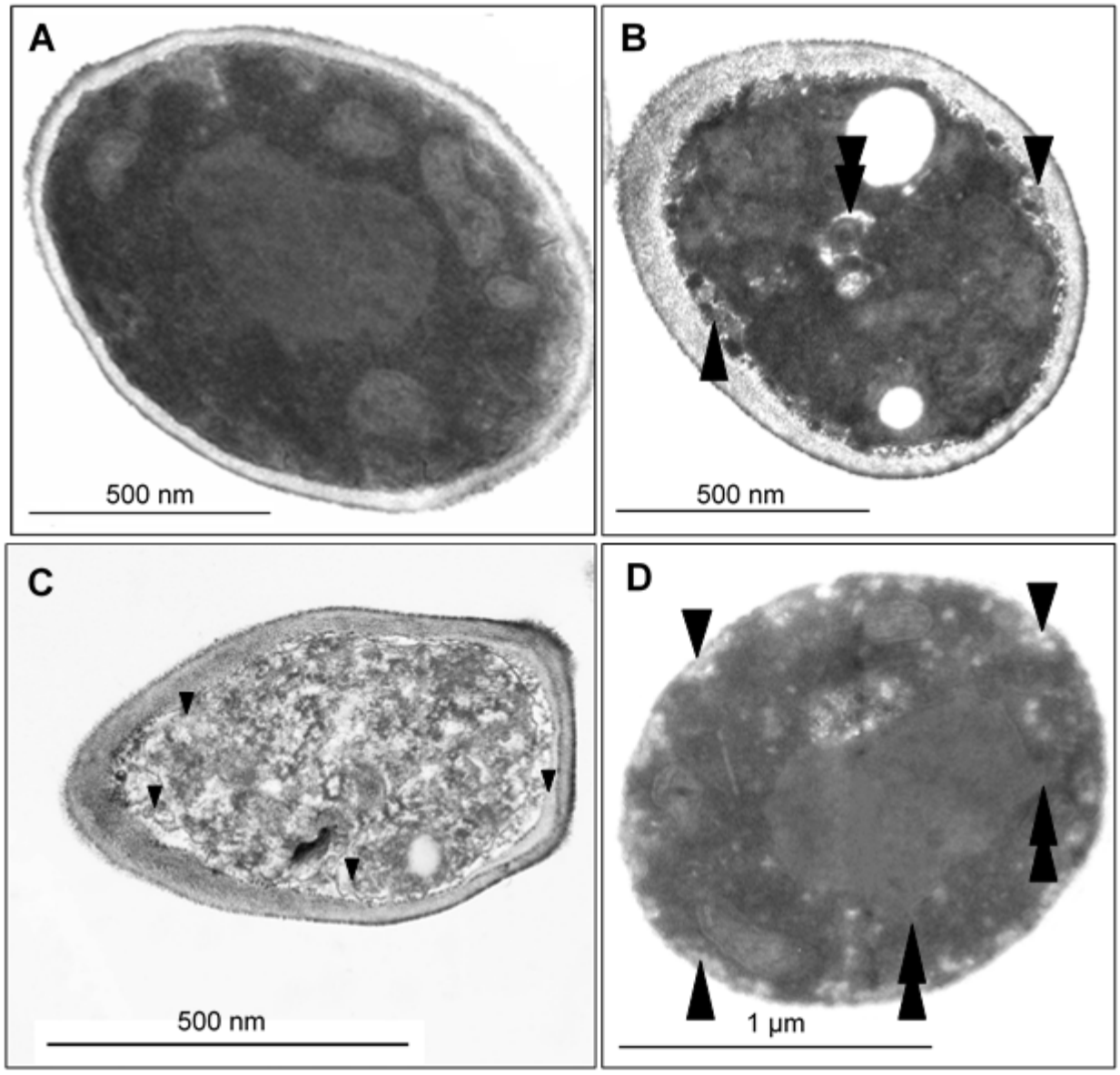

Fig. 21. (A) TEM micrographs of control *C. tropicalis* SZMC 1366 cell structure demonstrates intact membranes, small unevenly scattered condensed chromatin grains, and homogenous cytoplasm structure. (B, C, and D) TEM micrographs of *C. tropicalis* SZMC 1366 treated with a sub-lethal concentration of CAPE: (B) Late-stage disintegration with membrane fingerprints, vacuolization, plasma membrane detachment (arrowheads), and vacuole formation (double arrowheads). Fine granular homogenous cytoplasm organization disappeared, dense, compact cytoplasm with signs of fragmentation, rounded cell shape, and whole-cell shrinkage. (C) Necrotic cell with membrane ruptures (arrowheads) and loss of cytoplasm density. (D) Several peripheral vacuoles show plasma membrane involvement (arrowheads). Nuclear bleb formation (double arrowheads).

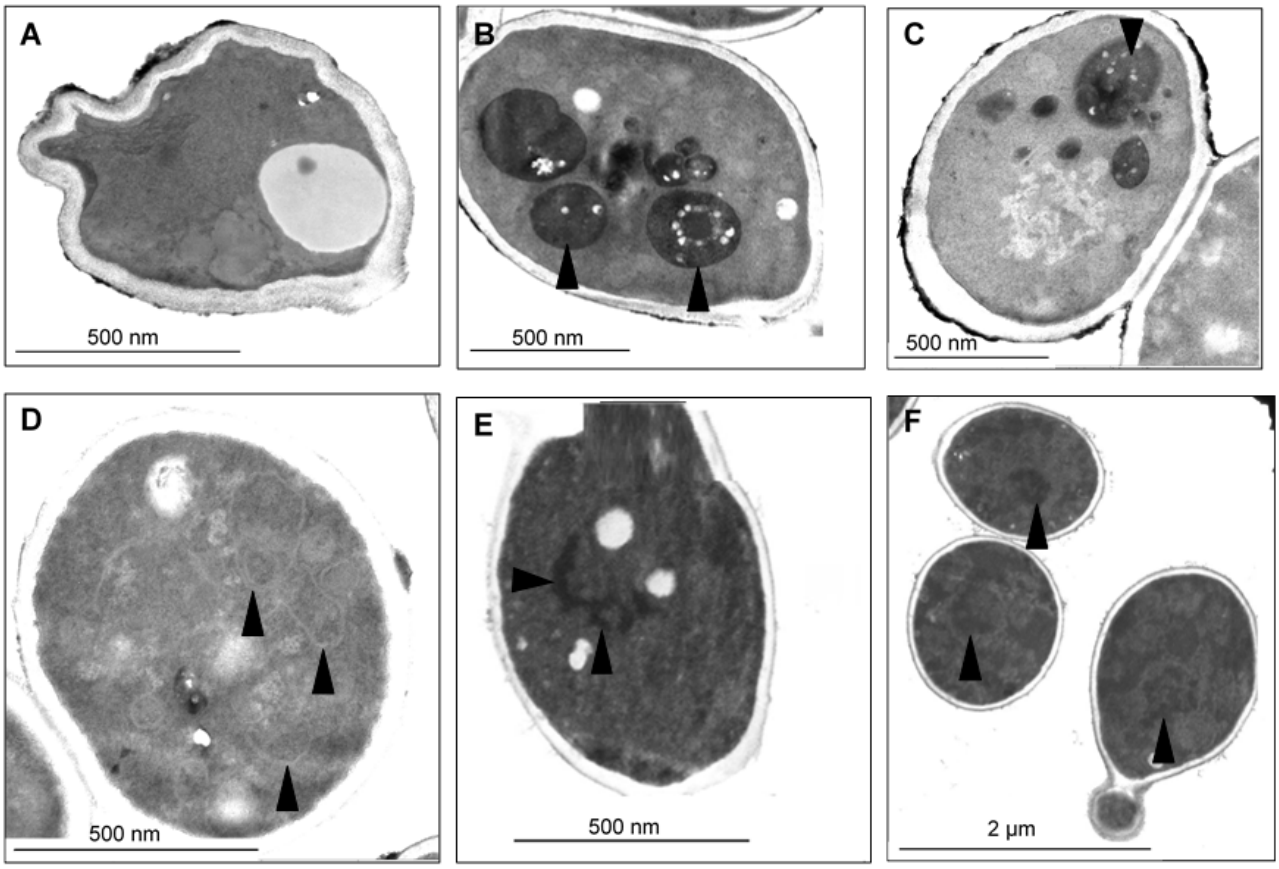

Fig. 22. TEM micrographs of *C. albicans* SZMC 1423 treated with a sub-lethal concentration of CAPE exhibit different markers of cellular deterioration. (A) Severe cell wall distortion. (B) and (C) Appearance of huge lysosomes was rather frequently detected (arrowheads). (D) Isolation membranes precondition of cytoplasm fragmentation (arrowheads). (E) and (F) Nucleus fragmentation and marginal condensation (arrowheads).

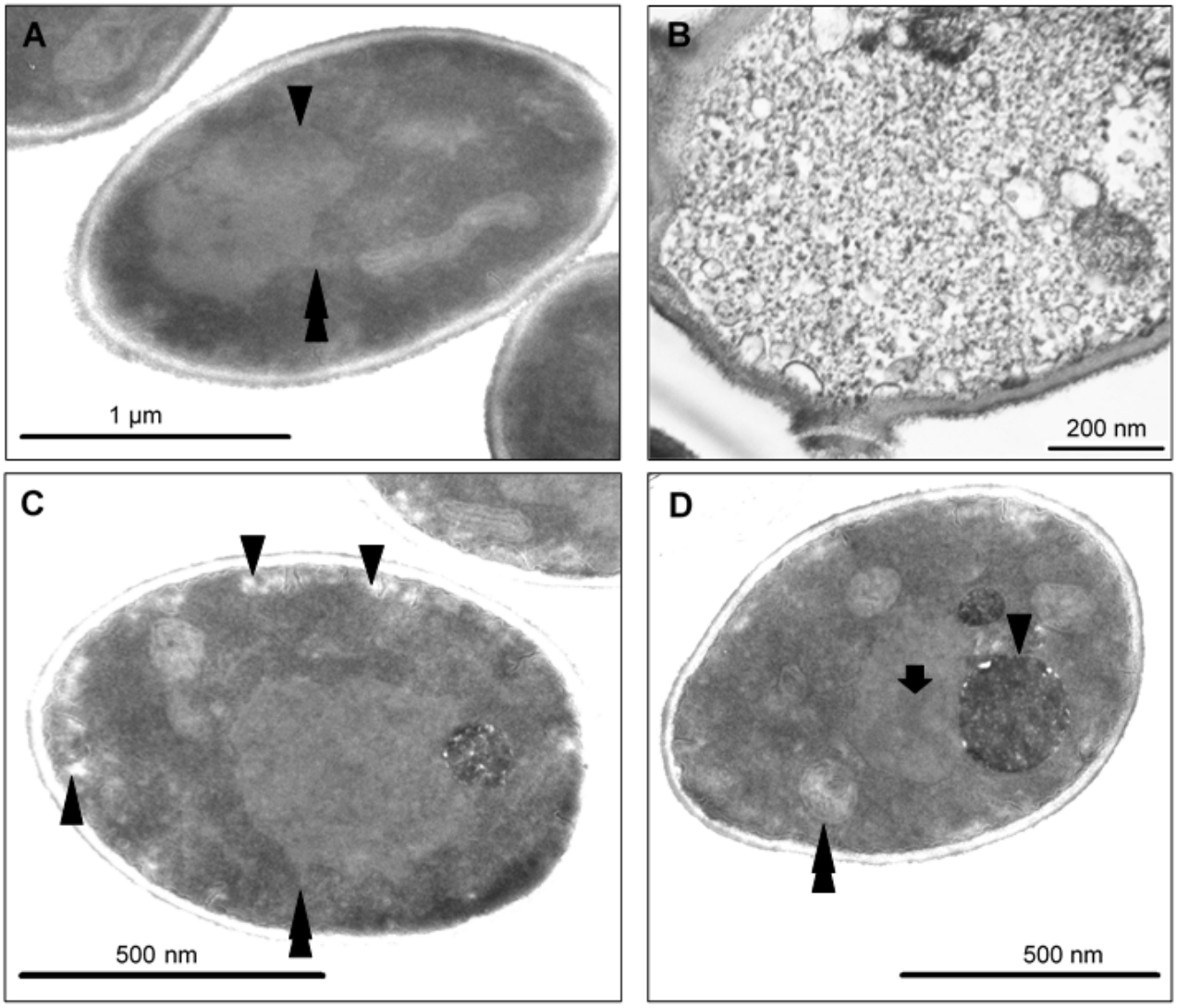

Fig. 23. TEM micrographs of *C. parapsilosis* SZMC 8007 treated with a sub-lethal concentration of CAPE. Both apoptotic and necrotic cell structural changes were observed in samples. (A) Nuclear chromatin margination and condensation (arrowhead) and blebs (double arrowhead) detached from the nucleus are typical apoptotic hallmarks. (B) Few necrotic cells were also present. Note membrane disintegration, obvious vacuolization, and loss of cytoplasm density. (C) Peripheral vacuole formation refers to Golgi fragmentation and cell membrane separation from the cell wall (arrowheads). Nuclear blebs (double arrowhead). (D) Nuclear condensation (arrow) and extremely large lysosomal bodies (arrowhead). Note swollen mitochondria (double arrowheads).

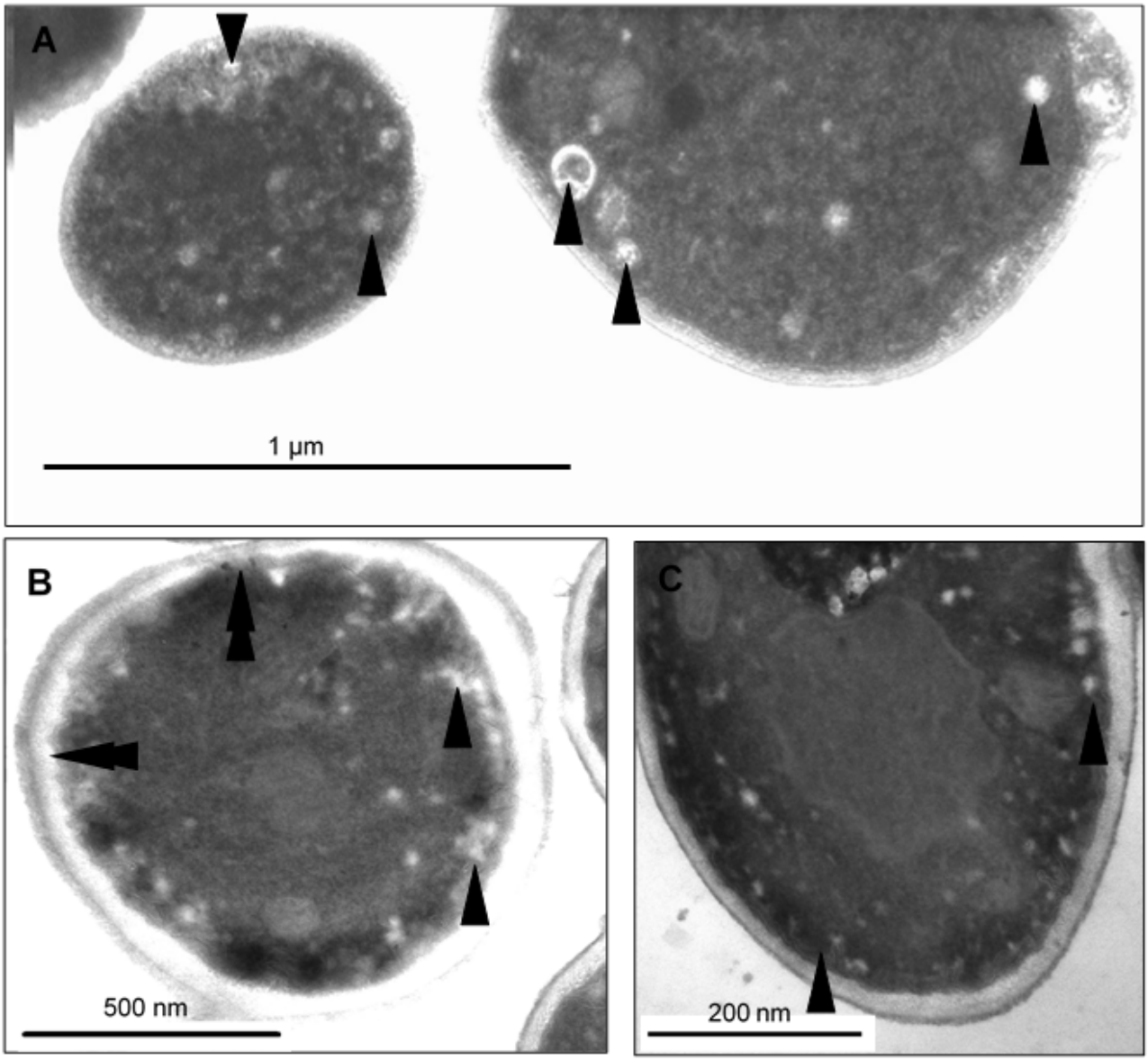

Fig. 24. TEM micrographs of *C. glabrata* SZMC 1374 treated with a sub-lethal concentration of CAPE. Mainly smaller necrotic signs were detected. Arrowheads denote small, mainly peripheral vacuoles typical of all samples. Cell wall disintegration was also observed (double arrowhead).

5.13. Effect of CAPE on the steady-state anisotropy of actin

The kinetics of the conformational change in G-actin induced by 50 µg/mL CAPE was investigated by monitoring the change in the steady-state anisotropy. Steady-state anisotropy experiments provide information on the flexibility of the actin in the cuvette, where a higher anisotropy value represents a stiffer protein matrix. It was found that the steady-state anisotropy of G-actin was not altered in the presence of 50 µg/mL CAPE, thus the flexibility of the protein matrix did not change which indicates no considerable effect for CAPE on G-actin (Fig. 25).

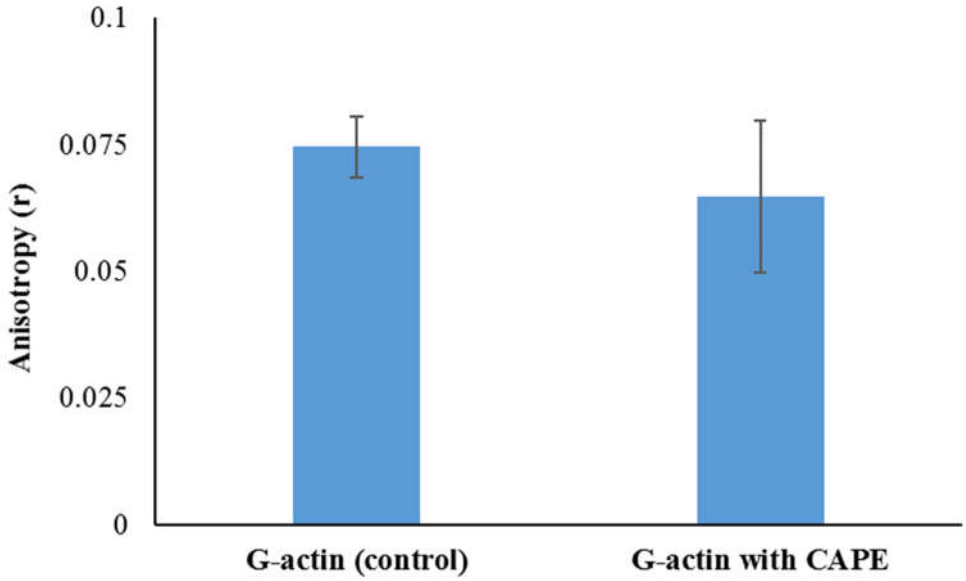

Fig. 25. The steady-state fluorescence anisotropy of G-actin in the absence and presence of 50 µg/mL CAPE.

5.14. Effect of CAPE on actin polymerization

The first stage of actin polymerization (the initial nucleation phase) has already been started before sample was placed into the fluorimeter. The elongation phase was observed in the first 15 min, then the polymerization process gradually slows down as the actin polymerization reaches a dynamic equilibrium state. To compare the polymerization rates, all of our measurement series were normalized, i.e. the measured fluorescence intensity values were transformed in the range 0-1 (Fig. 26). After normalization, the elongation rate was obtained from the slopes of the normalized pyrene intensity versus time curves for the first 15 minutes. The slope of the control was 0.00156, whereas the slopes of the test samples were between 0.00134–0.00277. According to these slope values, there

are no significant differences in the fluorescence signal measured in the absence and presence of different concentrations of CAPE.

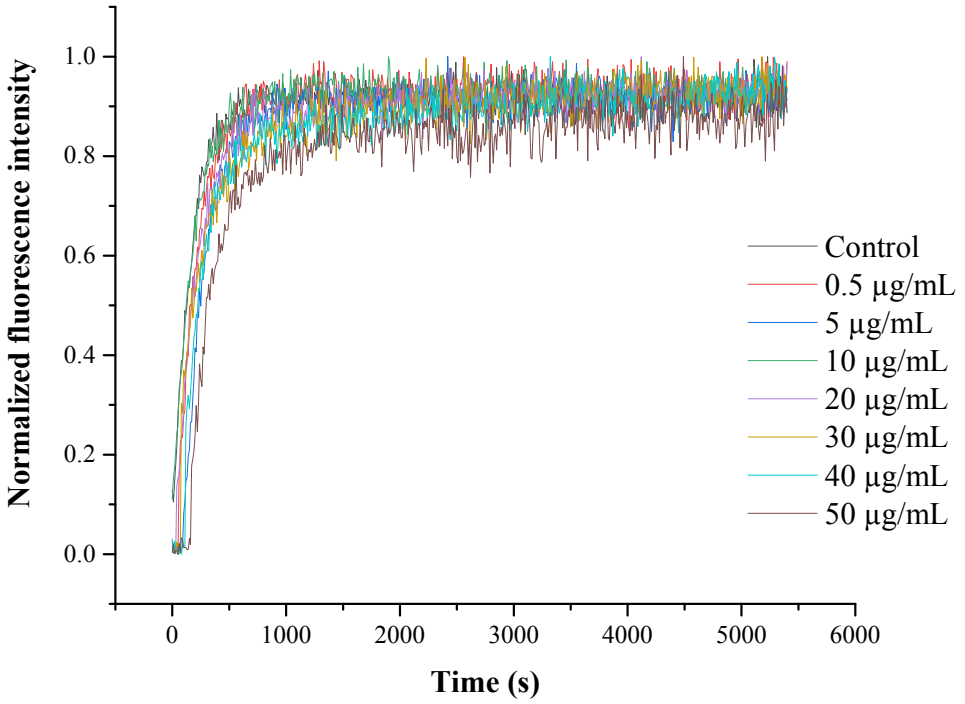

Fig. 26. Actin polymerization in the absence and presence of different concentrations of CAPE, normalized data.

5.15. Effect of CAPE on the thermal stability of actin

To check whether CAPE can affect the thermal stability of actin, the heat denaturation of G- and F-actin was measured using differential scanning calorimetry. The experiments were carried out in the absence or presence of 25 µg/mL CAPE. The denaturation peaks or T_m values appeared as an endotherm peak in the DSC curve since protein denaturation is an endothermic reaction. The results revealed that the T_m values of G-actin and F-actin in absence of CAPE were 55.53 °C and 66.09 °C, respectively (Fig. 27). While the T_m values of G-actin and F-actin in the presence of CAPE were 56.08 °C and 65.89 °C, respectively (Fig. 27). These results indicated that there is no significant effect for CAPE on the thermal stability of both G- and F-actins.

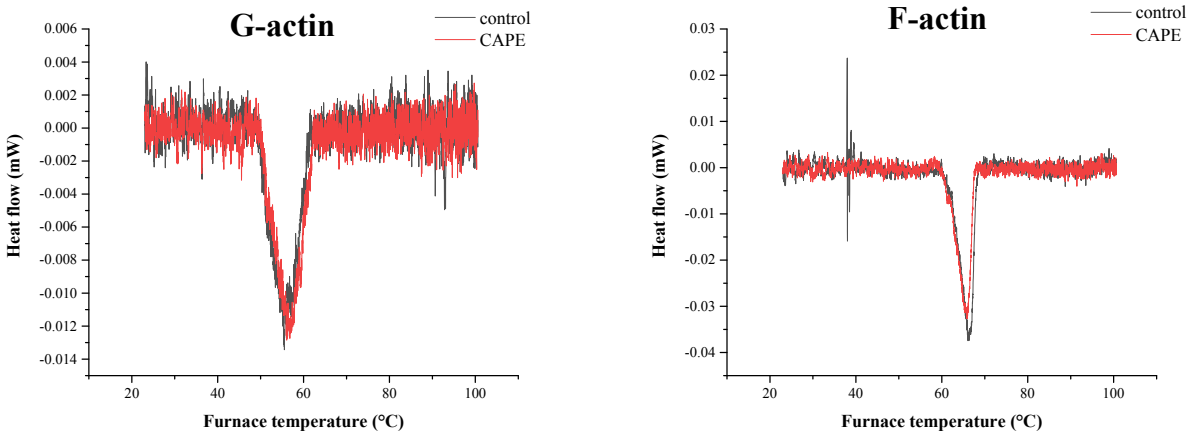

Fig. 27. Thermal stability of actin in the absence and presence of 25 µg/mL CAPE.

5.16. Antioxidant activity of Arabic coffee beans (*C. arabica*) extracts

The antioxidant activity of Arabic coffee beans (*C. arabica*) was determined *in vitro* by the ability of the extract to scavenge the DPPH radical. The IC_{50} values of the methanolic extract and its subsequent fractions are shown in Figure (28). The IC_{50} values ranged from 25.08 to 337.32 mg/mL. The best free radical scavenging activity (reflected by the smallest IC_{50} value) was exerted by the total methanolic extract followed by the acetone fraction. Whereas the lowest antioxidant activity was observed in the isopropanol fraction.

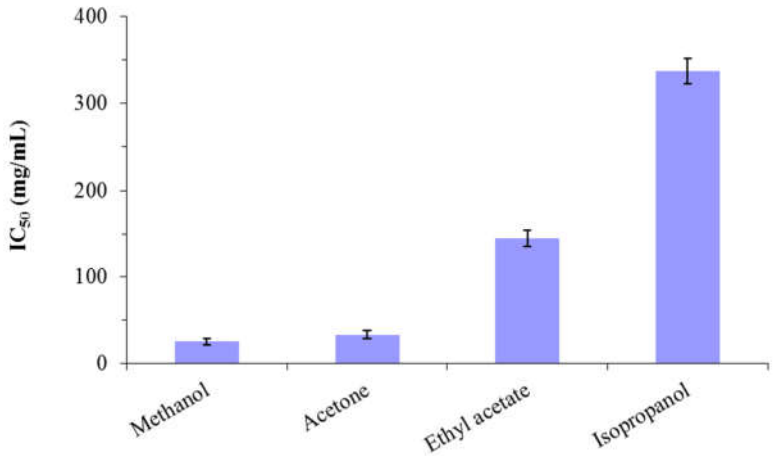

Fig. 28. IC_{50} values of the methanolic extract of Arabic coffee beans and its subsequent fractions. Data are shown as Mean ± SD from three independent experiments.

6. Discussions

The value of natural products in discovering new drugs has been and continues to be enormously important, mainly due to their unparalleled inherent chemical structural diversity, drug-like properties, and proven credentials (Sarker and Nahar, 2012). In recent years, propolis and its bioactive component CAPE were among the most important natural products that have attracted researchers' attention for their therapeutic properties (Papp et al., 2021). Many studies have reported the biological activities of propolis and CAPE as antibacterial and antifungal agents (Afrouzan et al., 2018; Al-Ani et al., 2018; Cui et al., 2013; Dantas Silva et al., 2017; Freires et al., 2016; Meyuhas et al., 2015; Niu et al., 2020; Papp et al., 2021; Veloz et al., 2019).

In the first part of this study, we focused on the effect of PEE on the planktonic growth and biofilm-forming abilities of some commercial forms of probiotics. Several studies revealed the importance of probiotics for the treatment of dysbiosis after GI infections, antibiotics treatment, or complementary therapies with natural antimicrobial substances (Ducatelle et al., 2015; McFarland, 2014; Wischmeyer et al., 2016). To the best of our knowledge, this is the first study that evaluates the *in vitro* effects of PEE on the planktonic growth of commercial forms of probiotics and their biofilm-forming abilities. Five commercial probiotics were used in this study to estimate their viability and biofilm-forming ability with and without PEE treatment. The importance of this study comes from the fact that propolis has antimicrobial properties (Boyanova et al., 2005; Stepanović et al., 2003; Uzel et al., 2005), and this is not only against the pathogenic bacteria, but it might have adverse effects on the growth of the intestinal microbiota and probiotic microorganisms ingested for their benefits (Haddadin et al., 2008). The antimicrobial properties of PEE are attributable to the presence of phenolic compounds, terpenes, caffeic, ferulic and coumaric acids, esters, and flavonoids (Al-Waili, 2018; Inui et al., 2014; Veiga et al., 2017).

The findings of this study revealed that PEE has a concentration-dependent inhibitory effect on the tested probiotics with variations between the different probiotic products. However, the growth of the probiotics that contain anaerobic and/or facultative anaerobic bacteria (Linex, BioGaia, and Protexin) has been enhanced at low concentrations of PEE. This prebiotic effect may be attributable to the antioxidant properties of PEE as a result of high total flavonoid and polyphenol content (Buratti et al., 2007; Laskar et al., 2010; Mohammadzadeh et al., 2007; Naima Benchikha et al., 2014). It has been reported previously that antioxidants can support the growth of anaerobic bacteria (La Scola et al., 2014). This suggestion is confirmed by the results obtained from the treatment with different

concentrations of the standard antioxidant glutathione, which similarly showed the improvement of the growth of the aforementioned probiotics (Fig 11 and 12). A similar prebiotic effect has been reported by Cho and co-workers (2016) on *Lactobacillus pentosus* SC60 (Cho et al., 2016), and also by Aabed and co-workers (2019) on *Lactobacillus paracaseii* and Protexin® (Aabed et al., 2019). Aabed and co-workers (2019) attributed the prebiotic effect of propolis to the free radical scavenging and antioxidant activity of the chemical components of propolis, particularly the phenolic compounds (Aabed et al., 2019).

BioGaia microbe (*L. reuteri* DSM 17938) has the highest ability to survive and tolerate high concentrations of PEE giving an advantage for BioGaia over the other probiotics. The PEE resistance of *L. reuteri* may be due to its antibiotic-producing ability. *L. reuteri* has been shown to produce the antimicrobial compound reuterin that has antibacterial activity against many bacterial species (Talarico et al., 1988). Although *L. reuteri* itself is less susceptible to reuterin than other bacteria, it can accumulate this compound and generate oxidative stress in its cells (Salminen et al., 2004). The production of reactive oxygen species (ROS) via the binding of reuterin with free thiol groups of proteins and small molecules can result in an enhanced imbalance in cellular redox status (Engels et al., 2016). Although the presence of ROS can limit the growth ability of *L. reuteri*, it is supposed that administration of a low concentration of PEE can act as a ROS scavenger enhancing its resistance and cell proliferation. Whereas, higher concentrations of PEE lead to decreased cell survival due to the suppressive antimicrobial effect of its flavonoids and polyphenols (Bouarab-Chibane et al., 2019; Fujimoto and Masuda, 2012; Górniak et al., 2019; Xie et al., 2015).

The biofilm-forming ability of probiotics plays an important role in the successful colonization and the effectiveness of the treatment of dysbiosis. This property allows them to withstand the environmental conditions, leading to the colonization and sustainability of their population (Salas-Jara et al., 2016). The five tested probiotics in this study could be divided into two categories: non-biofilm formers (Protexin and Enterol) and weak biofilm formers (BioGaia, Linex, and Normaflore). Application of PEE treatment caused various effects on the weak biofilm forming probiotics. The weak biofilms of BioGaia and Linex microbes were suppressed by low concentrations of PEE (12.5 and 25 µg/mL, respectively), while in the case of Normaflore only the highest concentration (800 µg/mL) was able to inhibit the biofilm formation. The antibiofilm properties of PEE could be related to its flavonoids and polyphenols content. In addition to their destructive activity on bacteria, flavonoids and polyphenols can suppress the biofilm formation process by altering bacterial

adhesion, motility (Górniak et al., 2019), and the regulatory mechanisms of the bacterial population such as quorum sensing or other universal regulator systems (Slobodníková et al., 2016). However, the interesting effect of low concentrations of PEE on the biofilm of Normaflore microbes, where the biofilm was enhanced and shifted from weak to strong, might be attributable to the enhancement of autoaggregation (Sorroche et al., 2012) and/or the inhibition of swarming motility (O'May et al., 2012). The results of the autoaggregation test showed a positive correlation between autoaggregation and the biofilm-forming ability of Normaflore microbes. A similar direct relationship has been reported by Sorroche et al (Sorroche et al., 2012) in *Sinorhizobium meliloti*. According to their suggestions, the same physical adhesive forces are responsible for both biofilm-forming ability and autoaggregation (Sorroche et al., 2012). On the other hand, PEE treatment inhibited the swarming motility of Normaflore microbes indicating an inverse relationship with biofilm-forming ability. A similar inverse relationship has been documented by several previous studies (Chakroun et al., 2018; O'May et al., 2016, 2012). It is assumed that once the bacteria start the attachment in the biofilm formation process, and due to the PEE-induced autoaggregation and the inhibition of surface-associated swarming motility, the attachment of more planktonic cells will lead to the formation of microcolonies, that can later lead to the formation of mature biofilm (Caiazza et al., 2007; O'May and Tufenkji, 2011).

Concerning the biofilm eradication experiment, Normaflore microbes were not only able to resist PEE but the biofilm status was shifted from weak to strong at low concentrations (12.5-25 µg/mL). This considerable increment in the biofilm mass might be due to the prebiotic effect of PEE. Moreover, it is proposed that the proliferation of the planktonic cells released from the mature biofilm, with enhanced autoaggregation at low concentrations of PEE, allows more cells to be reintroduced and increases the biofilm mass. At higher concentrations (50-400 µg/mL), where the planktonic cells were no more viable, only the prebiotic effect of PEE was responsible for the increment of the biofilm mass. However, only the highest concentration of PEE (800 µg/mL) was able to eradicate the biofilm mass. The eradication process may occur due to the disturbance of bacterial adhesion and quorum sensing (Górniak et al., 2019), the interaction with the extrapolymeric substances of the biofilm, and the killing mechanism of bacteria inside the biofilm, leading to detachment of the biofilm from the substratum (Chen et al., 2018).

The second part of the study concentrated on the antifungal activity of the biologically active propolis component CAPE against *C. albicans* and non-albicans *Candida* species. *Candida* spp. are

still considered the most important opportunistic pathogens that cause fungal infections worldwide. They are among the fourth to sixth most common nosocomial bloodstream isolate, according to estimates. Although *C. albicans* was the most frequent species isolated during candidemia, a greater role of non-albicans *Candida* spp. has been observed in recent years (Sutcu et al., 2016).

The antifungal activity of CAPE in combination with some antibiotics against *C. albicans* has been studied by previous studies. However, to the best of our knowledge, no published articles investigating the antifungal activity of CAPE as a single molecule against *C. albicans* or non-albicans *Candida* species were found in the literature except one conference abstract published in 2019, which studied the effect of CAPE on *Candida* biofilms (Barros et al., 2019).

The findings of our study revealed that CAPE has a high ability to inhibit the planktonic growth and biofilm-forming ability and moderate ability to eradicate the mature biofilms of the different strains of *Candida*. It has been reported that phenolic compounds, including CAPE, are effective inhibitors of iron absorption (Sun et al., 2018a). Sun and co-workers (2018) proposed that the antifungal mechanism of CAPE may include intracellular iron starvation, due to its ability to form insoluble complexes with iron ions which leads to the prevention of iron absorption by cells. Other studies attributed the antifungal activity of CAPE to its action on RNA, DNA, and cellular proteins which are probable targets of this compound (Murtaza et al., 2014). On the other hand, Su and co-workers (1995) suggested that the cytotoxicity of CAPE could be related to its apoptotic effect on the cells (Su et al., 1995). This suggestion is supported by the results of the apoptosis experiment in this study which revealed the ability of CAPE to induce apoptosis in most *Candida* strains including *C. albicans* ATCC 44829, *C. albicans* SZMC 1423, *C. albicans* SZMC 1424, *C. parapsilosis* SZMC 8007, *C. tropicalis* SZMC 1366, and *C. tropicalis* SZMC 1512. However, *C. parapsilosis* SZMC 8007, *C. glabrata* SZMC 1374, and *C. glabrata* SZMC 1378 did not exhibit apoptotic cell death, which indicates that different species of *Candida*, and in one case different strains of the same species, have different cell death responses to CAPE. On the other hand, TEM images of CAPE-treated *Candida* cells showed the typical hallmarks of apoptosis in most *Candida* species. Notably, the apoptotic hallmarks were almost the same in different *Candida* species. Similar apoptotic hallmarks have been reported by several previous studies on *C. albicans*. De Nollin and Borgers (1975) reported the alterations of the surface micromorphology in *C. albicans* after treatment with miconazole. Shrinkage of protoplasm, abnormal cell, and nuclear morphology, and vacuolization were also observed in plantaricin peptide-treated cells of *C. albicans* (Sharma and Srivastava, 2014).

Distortion of cell walls and membranes, which caused the alterations of the surface micromorphology, could be explained due to a change in permeability of the cell membrane which could cause the osmotic imbalance leading to alterations and indentations of the cell wall in collapsed cells (De Nollin and Borgers, 1975).

We also investigated the mechanism involved in CAPE-induced apoptosis in *Candida* spp. Application of the broad-range pan-caspase inhibitor Z-VAD-FMK significantly reduced the CAPE-induced apoptosis in *C. albicans* ATCC 44829, *C. albicans* SZMC 1424, *C. tropicalis* SZMC 1366, and *C. tropicalis* SZMC 1512. Such results suggest that this compound induced Yca1p-dependent apoptosis in these strains. Since CAPE can increase the permeability of plasma membrane to ions (Mirzoeva et al., 1997), it can cause depolarization in mitochondria. This could lead to the release of cytochrome c and other pro-apoptotic factors into the cytosol, which in turn leads to the activation of yeast metacaspase Yca1p resulting in the activation of caspase cascade inducing apoptosis (Lone et al., 2020). However, the pre-incubation of CAPE-treated *C. albicans* SZMC 1423 and *C. parapsilosis* SZMC 8007 with pan-caspase inhibitor Z-VAD-FMK did not affect their viability, which means that the CAPE-induced apoptosis in these strains was Yca1p-independent. This suggests that it could be due to the release of the apoptosis-inducing factor Aif1p from the mitochondria triggered by CAPE. These results support the suggestion that CAPE not only has species and strain-dependent cell death responses in *Candida*, but also could induce apoptotic cell death through different mechanisms.

In the third part of this study, we examined the effect of CAPE on actin. Böhl and co-workers (2007) reported that flavonoids may have different and partially opposing biological effects on actin. They attributed these different effects to the flavonoid-specific conformational changes in actin, which may cause different biological effects on the actin function (Böhl et al., 2007). Moreover, Weyant and co-workers (2000) found that CAPE can cause a rearrangement of the actin cytoskeleton in colon cancer cells (Weyant et al., 2000). In our case, no significant effect was observed for CAPE on actin function through anisotropy, polymerization, and thermal stability investigations. This could indicate that no direct molecular interaction has occurred between CAPE and actin. However, at the same time, we cannot rule out the possibility of interference between CAPE and actin-binding proteins which needs further investigations.

In the last part of this study, we investigated the antioxidant properties of Arabic coffee (*C. arabica*) beans extracts. Arabic coffee beans contain many components that could exhibit antioxidant

properties such as polyphenols, flavonoids, and hydroxycinnamic acids (caffeic, chlorogenic, coumaric, ferulic, and sinapic acids) (Affonso et al., 2016; Złotek et al., 2016). The Arabic coffee beans extract can reduce the free radical of DPPH to the yellow-colored diphenylpicrylhydrazine. This antioxidant capacity could be attributable to the bioactive components in Arabic coffee beans, such as phenolic compounds, caffeine, and hydroxycinnamic acids, that can donate hydrogen to the free radical to eliminate odd electron which is responsible for radicals' reactivity. The highest antioxidant capacity which was exhibited by the methanolic extract of the coffee beans could be due to the presence of all bioactive compounds that are present in its fractions. On the other hand, the antioxidant capacity of the different fractions has been found to increase with decreased solvent polarity. Our findings are in agreement with the findings of Affonso and co-workers (2016) who reported a high antioxidant activity of green coffee beans, and the findings of Yashin and co-workers (2013) who found that coffee has the highest antioxidant activity among 12 beverages used in their study.

7. Conclusions

From this study, we can conclude the following new results:

1. PEE has a probiotic product and concentration-dependent effect on the viability and biofilm-forming ability of the probiotics *in vitro*. PEE, in certain cases, can act as a prebiotic at low concentrations, however, at higher concentrations, it may inhibit the planktonic growth and biofilm-forming ability of the probiotics. At low concentrations, PEE can enhance the growth of the biofilms of Normaflore microbes (*B. clausii*), which could be due to the enhanced autoaggregation and the inhibited swarming motility. While, at high concentrations, PEE can eradicate the biofilms of Normaflore microbes (*B. clausii*). Therefore, more attention should be paid for the selection of the appropriate probiotics used for the treatment of dysbiosis, and on the other hand for the simultaneous application of PEE. The present observations showed limitations for the co-application of PEE and probiotics and adumbrated a potential double-face action of PEE on the natural gut microbiota.
2. CAPE has a concentration and strain-dependent antifungal effect on the viability and biofilm-forming ability of the different *Candida* species. Moreover, it has a moderate activity to eradicate the mature biofilms of biofilm-forming strains of *Candida*. CAPE has a species and strain-dependent cell death response in *Candida*. Therefore, CAPE could be considered as a promising drug nominee for antifungal therapy.
3. CAPE has no significant effect on the anisotropy, polymerization, and thermal stability of actin protein.
4. Methanolic extract of *C. arabica* beans and its acetone fraction have relatively high antioxidant properties as compared to the ethyl acetate and isopropanol fractions. Thus, they are considered as interesting sources for natural antioxidant compounds that have many applications in food and pharmaceutical industries.

8. Summary in English

The main objective of this study was to investigate the antimicrobial and antioxidant properties of some natural products commonly consumed by humans around the world. It consisted of four parts; in the first part, the effect of PEE on planktonic growth and biofilm forming abilities of five commercial probiotics (Enterol, Protexin, Normaflore, BioGaia and Linex) was investigated. Planktonic forms of probiotics showed varied susceptibilities with MIC values in the range of 100-800 µg/mL of PEE. However, low PEE concentrations significantly enhanced the planktonic growth of Linex and BioGaia microbes. Biofilm studies revealed that Enterol and Protexin were non-biofilm formers, while BioGaia, Linex and Normaflore showed weak biofilms, which were inhibited by 12.5, 25, and 800 µg/mL of PEE, respectively. PEE revealed double-face effect on the biofilms of Normaflore and Linex, which were enhanced at low concentrations of PEE and inhibited at higher concentrations. Interestingly, Normaflore biofilms were shifted from weak to strong biofilms at low PEE concentrations (12.5, 25, and 50 µg/mL). In the second part of the study, the effect of CAPE on planktonic growth, biofilm-forming abilities, mature biofilms, and cell death in nine different strains of *C. albicans*, *C. tropicalis*, *C. glabrata*, and *C. parapsilosis* was also investigated. The results showed that CAPE has a strain and dose-dependent effect on *Candida*, and the MIC values were between 12.5-100 µg/mL. Similarly, CAPE was able to inhibit the biofilm-forming abilities in a dose-dependent manner, and the MBIC values were between 50-100 µg/mL. However, CAPE showed only weak to moderate biofilm eradication ability on the mature biofilms on the different strains (19-49% of the original biofilm). Besides, CAPE was also able to induce apoptosis in six of the tested strains at different levels including *C. albicans* SZMC 1423, *C. albicans* ATCC 44829, *C. albicans* SZMC 1424, *C. parapsilosis* SZMC 8008, *C. tropicalis* SZMC 1366, and *C. tropicalis* SZMC 1366, whereas almost no apoptosis was seen in *C. glabrata* strains and *C. parapsilosis* SZMC 8008. The TEM images of *C. tropicalis*, *C. albicans*, and *C. parapsilosis* displayed typical apoptotic hallmarks including chromatin margination, nuclear blebs, condensation in the nucleus, vacuolization, plasma membrane detachment, huge lysosomes, cytoplasm fragmentation, and whole-cell shrinkage, while *C. glabrata* mainly revealed smaller necrotic signs. In conclusion, CAPE has a concentration and strain-dependent effect on the viability, biofilm-forming ability, and cell death response in the different *Candida* species. The third part of the study was intended to examine the molecular interaction between CAPE and actin protein. The results revealed no significant effect for CAPE on the steady-state anisotropy, polymerization, and thermal stability of actin. In the last part

of the study, the *in vitro* antioxidant activity of Arabic coffee beans methanolic extract and its subsequent fractions was also determined using DPPH method. The best antioxidant activity was exerted by the total methanolic extract followed by the acetone fraction. Whereas the lowest activity was observed in the isopropanol fraction. the antioxidant capacity of the different fractions has been found to increase with decreased solvent polarity. In conclusion, PEE has a double-face *in vitro* effect on the planktonic growth and biofilm formation of some probiotics. Therefore, more attention should be paid for the use of PEE. On the other hand, CAPE was found to be a promising natural antifungal agent against different *Candida* species. Finally, methanolic extract of Arabic coffee beans is an interesting source for natural antioxidant compounds that could be used in food and pharmaceutical industries.

9. Összefoglalás (summary in Hungarian)

A jelen tanulmány fő célja az volt, hogy megvizsgáljuk egyes természetes anyagoknak az antimikrobiális és antioxidáns tulajdonságait, amelyeket világszerte gyakran fogyasztanak az emberek. A vizsgálat négy részből állt. Az első részben a propolisz etanolos kivonatának (PEE) a kereskedelemben kapható probiotikumok (Enterol, Protexin, Normaflore, BioGaia és Linex) mikroorganizmusainak planktonikus növekedésére és biofilm képző képességére gyakorolt hatását vizsgáltuk. A probiotikumok planktonikus formái változó érzékenységet mutattak, a minimális gátló koncentráció (MIC) értékek a 100-800 µg/ml PEE tartományba estek. Az alacsony PEE-koncentrációk azonban jelentősen fokozták a Linex és BioGaia planktonikus mikroba formáinak a növekedését. A biofilm vizsgálatok azt mutatták, hogy míg az Enterol és a Protexin kezelés nélkül nem képeznek biofilmet, addig a BioGaia, a Linex és a Normaflore gyenge biofilm képzők, amelyeket 12,5, 25, illetve 800 µg/ml PEE gátolt. A PEE kettős hatását tapasztaltuk a Normaflore és a Linex biofilmjein, amelyek alacsony PEE-koncentrációknál fokozták, míg magasabb koncentrációknál gátolták a biofilm képződést. Érdekesnek bizonyult, hogy a Normaflore készítmény erős biofilmet képzett alacsony PEE koncentrációk mellett (12,5, 25 és 50 µg/ml). A vizsgálat második részében a koffeinsav-fenetilészternek (CAPE) *Candida albicans, C. tropicalis, C. glabrata és C. parapsilosis* 9 különböző törzsének a planktonikus növekedésére, biofilm képző képességére, érett biofilmre és a sejtpusztulásra gyakorolt hatásait vizsgáltuk. Az eredmények alapján a CAPE törzs- és dózisfüggő citotoxikus hatását mutattuk ki, ahol a MIC értékek 12,5-100 µg/ml között változtak. Hasonlóképpen, a CAPE dózisfüggő módon gátolta a biofilm képződést, az MBIC-értékek 50-100 µg/ml közé estek. A CAPE azonban csak gyenge vagy mérsékelt biofilm bontási képességet mutatott a különböző törzsek érett biofilmjein (az eredeti biofilm 19–49%-a). Ezen kívül a CAPE hat vizsgált törzsben (*C. albicans SZMC 1423, C. albicans ATCC 44829, C. albicans SZMC 1424, C. parapsilosis SZMC 8008, C. tropicalis SZMC 1366* és a *C. tropicalis SZMC 1366)* különböző mértékű apoptózist volt képes kiváltani, míg a *C. glabrata* törzsekben és a *C. parapsilosis SZMC 8008* törzsnél gyakorlatilag nem indukált apoptózist. A *C. tropicalis, C. albicans* és *C. parapsilosis* TEM képein tipikus apoptotikus jellegzetességek mutatkoztak, úgymint a kromatin marginációja, a sejtmag hólyagosodása és kondenzációja, vakuolizáció, a plazma membrán leválása, nagyméretű lizoszómák megjelenése, a citoplazma fragmentációja és a sejt zsugorodása, míg a *C. glabrata* esetében főképpen kisebb nekrotikus jeleket tapasztaltunk. Összegzésképpen elmondható, hogy a CAPE koncentráció- és törzsfüggő hatással van a különböző *Candida* fajok

életképességére, biofilm képző képességére és sejthalál-válaszára. A vizsgálat harmadik részében a CAPE-nek az aktin fehérjére gyakorolt hatását tanulmányoztuk. Az eredmények alapján a CAPE nem gyakorolt szignifikáns hatást az aktin egyensúlyi állapotú anizotropiájára, polimerizációjára és hőstabilitására sem. A vizsgálat utolsó részében az arab kávébab metanolos kivonatának és az abból származó különböző oldószeres frakcióinak az *in vitro* antioxidáns aktivitását határoztuk meg DPPH módszerrel. A legerősebb antioxidáns hatást a teljes metanolos kivonat, majd az aceton frakció fejtette ki, míg a legkisebb aktivitást az izopropanol frakciónál tapasztaltuk. Megállapítottuk, hogy a különböző frakciók antioxidáns kapacitása növekszik az oldószerek polaritásának csökkenésével. Összefoglalva, a PEE kettős hatást gyakorol *in vitro* egyes probiotikumok planktonikus növekedésére és biofilm-képződésére, ezért nagyobb figyelmet kell a PEE használatára fordítani. Másrészt a CAPE ígéretes természetes gombaellenes szer lehet különféle Candida fajok ellen. Végül az arab kávébab metanolos kivonata ígéretes természetes antioxidáns vegyületforrás, amely az élelmiszeriparban és a gyógyszeriparban is felhasználható lehet.

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11. List of publications

- **Publications related to thesis topic**

1. **Alfarrayeh, I**., Fekete, C., Gazdag Z., Papp G. (2020). Propolis ethanolic extract has double-face *in vitro* effect on the planktonic growth and biofilm formation of some commercial probiotics. *Saudi Journal of Biological Sciences*, 28(2021);1033-1039.
2. Magharbeh Mousa K., Al-Hujran Tayel A., Al-Jaafreh Ahmad M., **Alfarrayeh Ibrahim I.** and Ebada Sherif S. (2020). Phytochemical Screening and *in vitro* antioxidant and antiurolithic activities of *Coffea arabica*. *Research Journal of Chemistry and Environment*, 24(12); 109-114.

- **Conference proceedings related to thesis topic**

1. Ibrahim Alfarrayeh, Csaba Fekete, Zoltán Gazdag, Gábor Papp. Propolis ethanolic extract has double-face in vitro effect on the planktonic growth and biofilm formation of some commercial probiotics. In: Csiszár, B; Hankó, Cs; Kajos, L F; Kovács, O B; Mező, E; Szabó, R; Szabó-Guth, K (eds.). 9th Interdisciplinary Doctoral Conference 2020 Book of Abstracts. Pécs, Hungary: Doctoral Student Association of the University of Pécs (2020) 384 p. pp. 319-319., 1 p.
2. Ibrahim Alfarrayeh, Csaba Fekete, Zoltán Gazdag, Gábor Papp. Antifungal and antibiofilm effects of caffeic acid phenethyl ester on different Candida species. In: Csiszár, Beáta; Hankó, Csilla; Kajos, Luca Fanni; Mező, Emerencia (eds.) Medical Conference for PhD Students and Experts of Clinical Sciences: Book of Abstracts. Pécs, Hungary: Doctoral Student Association of the University of Pécs. (2020). 83 p. pp. 54-54. , 1 p..
3. Ibrahim Alfarrayeh, Csaba Fekete, Zoltán Gazdag, Gábor Papp. Antimicrobial and antibiofilm effects of propolis ethanolic extract on some commercial probiotics. In: Bódog, Ferenc; Csiszár, Beáta (eds.) VIII. Interdiszciplináris Doktorandusz Konferencia 2019: absztraktkötet=8th Interdisciplinary Doctoral Conference 2019: Book of Abstracts. Pécs, Hungary: Doctoral Student Association of the University of Pécs. (2019). 185 p. pp. 12-12., 1 p.
4. Ibrahim Alfarrayeh, Csaba Fekete, Zoltán Gazdag, Gábor Papp. Effect of propolis ethanolic extract on the growth and biofilm formation ability of some commercial probiotics. In: XVI. János Szentágothai Multidisciplinary Conference and Student

Competition – Abstracts. Pécs, Hungary: János Szentágothai Scholastic Honorary Society, Faculty of Sciences, University of Pécs (2019) pp. 252-252., 1 p.

- **Publications outside to thesis topic**

  No publications outside the thesis topic

- **Conference proceedings outside thesis topic**

  No conference proceedings outside the thesis topic