UNIVERSITY OF PÉCS

Doctoral School of Biology and Sportbiology

Antimicrobial and antibiofilm activities of Hungarian propolis on *Candida albicans* and *Staphylococcus aureus* and its mechanism of action

Ph.D. Thesis

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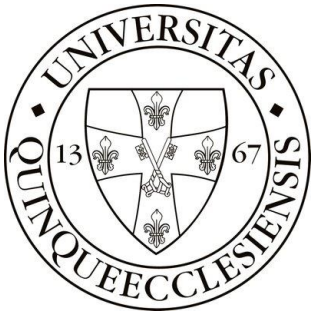

PÉCS, 2022

Table of Contents

List of abbreviations 4

1. Literature review 10

1.1. General knowledge of propolis 10

1.1.1. Historical overview 11

1.1.2. Types of propolis and plant origin 12

1.1.3. Chemical composition and standardization 13

1.2. Nosocomial infections 20

1.2.1. *Candida* infections 21

1.2.2. Staphylococcal infections 21

1.3. Therapeutic properties of propolis 22

1.3.1. Antifungal activity 23

1.3.2. Antibacterial activity 24

1.4. Mechanisms of action of propolis 25

2. Aims of the study 27

3. Materials and Methods 28

3.1. Microorganism and culture conditions 28

3.2. Raw material of propolis and the extractions method 28

3.3. Chemical characterization of propolis extracts 30

3.3.1. Determination of the total phenolic content (TPC) 30

3.3.2. Determination of total flavonoids content (TFC) 30

3.3.3. Spectrophotometric analysis of UV-visible spectra 31

3.3.4. Gas chromatography–mass spectrometry (GC-MS) 31

3.4. Antimicrobial effect of EEP 31

3.4.1. Antifungal susceptibility testing on *C. albicans* 31

3.4.2. Survival rate determination and adaptation assay 32

3.4.3. Antibacterial susceptibility testing of *S. aureus* 32

3.4.4. Chequerboard broth microdilution method 33
3.5. Effect of propolis extract on virulence factors 33
3.5.1. Effect of propolis extract on biofilm formation of *C. albicans* 33
3.5.2. Effect of propolis extract on biofilm formation of *S. aureus* 34
3.5.3. Effect of propolis extract on the eradication of mature biofilm of *C. albicans* and *S. aureus* 34
3.5.4. Effect of propolis extract on germ tube formation (GTF) 35
3.6. Mechanism of action of EEP on *C. albicans* 36
3.6.1. Cellular uptake of EEP 36
3.6.2. Effect of propolis on cell wall 36
3.6.3. Effect of propolis on cell membrane 36
3.6.4. DNA fragmentation and nuclei damage 37
3.7. Statistical analysis 37
4. Results 38
4.1. Chemical characterization of propolis samples 38
4.1.1. Determination of the total phenolics and flavonoids contents 38
4.1.2. Spectrophotometric analysis of UV-visible spectra 39
4.1.3. Chemical constituents of propolis extracts 39
4.2. Antimicrobial effect of propolis 42
4.2.1. Antifungal activity 42
4.2.2. Ethanolic extracts of propolis induced cytotoxicity and adaptation 42
4.2.3. Antibacterial activity 43
4.2.4. Killing effect of EEP in combination with antibacterial drugs 44
4.3. Effect of propolis extract on fungal and bacterial virulence factors 46
4.3.1. Biofilm formation of *C. albicans* 46
4.3.2. The eradication of *C. albicans* mature biofilm 47
4.3.3. Effect of propolis extract on germ tube formation (GTF) 47

4.3.4. Biofilm formation of *S. aureus* 49
4.3.5. The eradication of *S. aureus* mature biofilm 50
4.4. Mechanism of action of propolis 52
4.4.1. Propolis biosorption kinetics 52
4.4.2. Effect of propolis on cell wall and cell membrane 53
4.4.3. DNA fragmentation and nuclei damage 55
5. Discussion 57
5.1. Chemical characterization 57
5.2. Antimicrobial and anti-virulence activities 61
5.2.1. Antifungal activity 61
5.2.2. The antibiofilm and antigermination activities of propolis against *C. albicans* 62
5.2.3. Antibacterial activity 64
5.2.4. The antibiofilm activity of propolis against *S. aureus* 66
5.3. Mechanisms of action 68
6. Conclusion 70
7. Future perspectives 70
8. Summary in English 71
9. Összefoglalás (Summary in Hungarian) 72
10. Acknowledgments 73
11. Reference 74
12. List of publications and conferences 90
12.1. Article related to this thesis 90
12.2. Article not related to this thesis 90
12.3. Conference presentations related to this thesis 90
12.4. Conference presentations not related to this thesis 91
13. Appendix 92

List of abbreviations

ABTS	3-Ethylbenzothiazoline-6-Sulfonic Acid
AmB	Amphotericin B
ATCC	American Type Culture Collection
BC	Before Christ
CE	Catechin Equivalents
CFU	Colony Forming Units
CLSI	Clinical Laboratory Standards Institute
DAPI	4′,6-Diamidino-2-Phenylindole
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DW	Dry Weight
EDTA	Ethylenediaminetetraacetic Acid
EEP	Ethanolic Extract of Propolis
EEP1	Ethanolic Extract of Propolis of Pécs Sample
EEP2	Ethanolic Extract of Propolis of Szombathely Sample
GAE	Gallic Acid Equivalents
GC-MS	Gas Chromatography-Mass Spectrometry
gDNA	Genomic DNA
GTF	Germ Tube Formation
ICU	Intensive Care Unit
MBC	Minimal Bactericidal Concentration
MBEC	Minimal Biofilm Eradication Concentration
MBIC	Minimal Biofilm Inhibition Concentration
MDR	Multidrug-resistant
MFC	Minimal Fungicidal Concentration
MHB	Muller Hinton Broth
MIC	Minimum Inhibitory Concentrations
MM	*Candida* Minimal Medium
MRSA	Methicillin-Resistant *Staphylococcus aureus*
MSSA	Methicillin-Susceptible *Staphylococcus aureus*
NIs	Nosocomial Infections
OD	Optical Density

PBS	Phosphate-Buffered Saline
PI	Propidium Iodide
RFU	Relative Fluorescence Units
RI	Retention Index
RPMI	Roswell Park Memorial Institute Medium
SCC*mec*	Staphylococcal Cassette Chromosome *mec*
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
Spp.	Several Species
SZMC	Szeged Microbial Collection
TE	Tris-EDTA buffer
TFC	Total Flavonoids Content
TMS	Trimethylsilyl
TPC	Total Phenolic Content
Tris	Trisaminomethane
TSB	Tryptic Soy Broth
UV	Ultraviolet
UV-Vis	Ultraviolet-Visible
YPD	Yeast Extract Peptone Dextrose Medium
$\lambda_{ex/em}$	Excitation and Emission Spectra Wavelength

List of figures

Figure 1: *European honeybee specie (Apis mellifera L.) and illustration of a variety of raw propolis in different colours.* *11*

Figure 2: *The general composition of raw propolis and the typical components for each group; 1) resin and vegetable balsam (50%), 2) beeswax (30%), 3) essential and volatile oils (10%), 4) pollen (5%), and 5) other substances (5%) including amino acids, vitamins, enzymes, sugar, and minerals.* *15*

Figure 3: *Raw propolis samples collected from six different geographical regions of Hungary, Pécs (1), Szombathely (2), Szolnok (3), Csikóstőttős (4), Héhalom (5), and Somogybabod (6).* *29*

Figure 4: *The texture and colour of raw propolis samples from six regions in Hungary: Pécs (1), Szombathely (2), Szolnok (3), Csikóstőttős (4), Héhalom (5), and Somogybabod (6).* *29*

Figure 5: *Ethanolic extracts of propolis (EEP) of six samples; Pécs (1), Szombathely (2), Szolnok (3), Csikóstőttős (4), Héhalom (5), and Somogybabod (6).* *30*

Figure 6: *Absorption UV-Vis spectra of propolis extracts. All samples have the same pattern. Most of EEP samples showed strong absorption in the region between 250 and 350 nm, with a very intense peak at 293 nm and shoulders at 271 and 325 nm.* *39*

Figure 7: *Chemical composition of six ethanolic extracts of propolis (EEP) using GC-MS analysis. The columns show the relative concentrations (%) of chemicals grouped into 11 categories for each propolis sample.* *41*

Figure 8: *The survival rate of C. albicans ATCC 44829 on exposure to 6.25-400 µg/mL of ethanolic extracts of propolis (EEP) after 48 h incubation at 35°C, using the broth microdilution method. The absorbance was measured at 595 nm with a 96-well plate reader spectrophotometer.* *42*

Figure 9: *The survival rates of C. albicans ATCC 44829 in the presence of 200 and 400 µg/mL of EEP1 for 30 and 60 min (A), and the survival rates of pre-treated cells with 50 µg/mL EEP1 for 60 min (B). The cells were cultured on an agar plate for 48 h at 30°C, the determined number of colony-forming units was converted to the percentage of survival.* *43*

Figure 10: *Inhibitory effect of antibiotic drugs: (a) cefoxitin (0.25–16 µg/mL); (b) vancomycin (0.25–16 µg/mL); (c) oxacillin (0.125–8 µg/mL); and (d) EEP1 (12.5–100 µg/mL) on S. aureus; clinical isolates (SA H23 and SA H24), and reference strains methicillin-susceptible S. aureus*

ATCC 29213 (MSSA) and methicillin-resistant S. aureus ATCC 700699 (MRSA). According to the broth microdilution method, the culture was incubated at 35°C for 20–24 h. The absorbance of the growth was measured at 595 nm using a plate reader. 44

Figure 11: *The biofilm formation of C. albicans strains (ATCC 44829 and SZMC 1424) in the presence of 3.125-200 µg/mL of propolis extracts (EEP1 and EEP2) and the absence of propolis extracts (control) after 48 h incubation at 37°C, using crystal violet assay, the absorbance of biofilm biomass was measured by 96-well plate reader spectrophotometer. .. 46*

Figure 12: *Effect of propolis extracts (EEP1 and EEP2) at the concentrations of 3.125-200 µg/mL on preformed biofilms of C. albicans SZMC 1424. The cells were cultivated for 24 h at 37°C to form biofilms, then the mature biofilms were treated with propolis extracts for an additional 24 hours. Using the crystal violet assay, the absorbance of biofilm biomass was measured by 96-well plate reader spectrophotometer. 47*

Figure 13: *The effect of 50 and 200 µg/mL of propolis extracts (EEP1 and EEP2) on germ tube formation of C. albicans ATCC 44829 and C. albicans SZMC 1424. The cells were suspended in horse serum and incubated in an orbital incubator at 37°C for 60, 120, and 180 min, the germination was calculated by a light microscope. 48*

Figure 14: *The effect of EEP1 (12.5–200 µg/mL) on the prevention of biofilm formation of S. aureus SA H23 and SA H24 clinical isolates, S. aureus ATCC 29213 (MSSA), and S.aureus ATCC 700699 (MRSA) strains after 24 h incubation at 37°C. (ODc) the cut-off value of the optical density. (OD ≤ ODc) means no biofilm, (ODc < OD ≤ 2 ODc) means weak biofilm, (2 ODc < OD ≤ 4 ODc) means moderate biofilm, (4 ODc < OD) means strong biofilm. Asterisks indicate statistically significant differences between each treatment of EEP1 and in absence of EEP1 (* p <0.05, * p < 0.01, *** p < 0.001). 49*

Figure 15: *The effect of EEP1 on mature biofilm biomass of S. aureus SA H23, SA H24 clinical isolates, S. aureus ATCC 29213 (MSSA), and S. aureus ATCC 700699 (MRSA). The 24 h-old biofilm was treated with EEP1 (25–200 µg/mL) for 16 h at 37°C. Using the crystal violet assay, the absorbance of biofilm biomass was measured by a 96-well plate reader spectrophotometer. 50*

Figure 16: *The effect of EEP1 (25–200 µg/mL) on 24 h-old biofilms formed by: (a) clinical isolates of S. aureus SA H23, (b) SA H24, (c) S. aureus ATCC 700699 (MRSA), and (d) S. aureus ATCC 29213 (MSSA). The mature biofilms were treated with EEP1 for 16 h at 37°C. Using the double fluorescent staining, the metabolic activity of the cells (blue lines) and dead*

cells (black lines) were detected within mature biofilms, in proportion to resazurin and propidium iodide dyes, respectively. *51*

Figure 17: *The uptake of propolis extract (EEP1) by C. albicans ATCC 44829. The cells were incubated in an orbital shaker at 30°C at 150 rpm in the presence of 200 µg/mL EEP1, the extracellular concentration of the EEP1 was measured hourly for 4 h photometrically at 295 nm. The calibration curve (included in the graph) was determined under the same conditions.* *52*

Figure 18: *Effect of propolis extract (12.5-400 µg/mL of EEP1) on the cell wall integrity of C. albicans ATCC 44829, in the presence and absence of 0.8 M sorbitol. After the incubation of the cells for 48 h at 35°C, the absorbance was maseared using 96-well plate reader.* *53*

Figure 19: *The effect of propolis extract on the cell membrane of C. albicans ATCC 44829. (A) The leakage of intracellular substances absorbing at 260 nm was measured with 100, 200, and 400 µg/mL EEP1every hour for 6 h spectrophotometrically. (B) Effect of ergosterol on the survival rate in the presence of 200, 400, and 600 µg/mL of EEP1, and in the presence of 0.5, 1.0, and 1.5 µg/mL AmB (positive control).* *54*

Figure 20: *The quality assessment of genomic DNA extracted from C. albicans ATCC 44829 after a 1 h exposure to propolis extract (EEP1) was performed using the Agilent 4200 TapeStation system and the Agilent Genomic DNA ScreenTape assay. (A) The gel image; (L1) molecular weight markers, (L2) negative control, (L3) positive control (5 mM H_2O_2), (L4) 50 µg/mL EEP1, (L5) 200 µg/mL EEP1. (B) An overlay of the electropherograms.* *55*

Figure 21: *Show damage to the nucleus of C. albicans ATCC 44829 1 h after the exposure to propolis extract (EEP1) by DAPI staining; (1) Negative control, (2) Positive control (5 mM H_2O_2), (3) 50 µg/mL EEP1, (4) 200 µg/mL EEP1. The untreated cells showed homogeneous nuclei in shape and density. Arrows point to changes in the nucleus; (a) chromatin condensation, (b) enlarged nucleus, (c) irregular shape of the nuclear membrane, (d-f) shrunken and fragmented nuclei, and micronucleus. The cells were visualized by a fluorescence microscope.* *56*

List of tables

Table 1: *Chemical characterization of different types of propolis, geographic distribution, botanical origin, and biological activities. 16*

Table 2: *Expression of total phenolic contents (TPC) and total flavonoids contents (TFC) in milligrams of gallic acid equivalents per gram of propolis (mg GAE/g), and milligrams of catechin equivalent per gram of propolis (mg CE/g), respectively..... 38*

Table 3: *Retention time (RT), retention indices (RI), and the relative concentrations (percentage of the area under the peak) of the major chemical components (above 0.5% presence) in EEP samples. 40*

Table 4: *The MIC of EEP and antibiotics alone and in combination on S. aureus (at inoculum of 10^8 CFU/mL); clinical isolates (SA H23 and SA H24), and reference strains methicillin-susceptible S. aureus ATCC 29213 (MSSA) and methicillin-resistant S. aureus ATCC 700699 (MRSA), and the type of interaction according to fractional inhibitory concentration index (FICI), (N) not calculated. 45*

Table 5: *Summarizing the percentage DNA fragmentation of C. albicans ATCC 44829 after 1-hour exposure to 5 mM H_2O2 and propolis extract (50 µg/mL and 200 µg/mL EEP1) in the 250–3000 bp region using the Agilent Genomic DNA ScreenTape assay, the percentage of damaged nuclei by DAPI staining..... 56*

Table 6: *The determination of the total phenolics (TPC) and flavonoids contents (TFC) using the Folin-Ciocalteu and the aluminium chloride colorimetric methods, respectively, in propolis samples from the temperate and tropical regions. 58*

1. Literature review

1.1. General knowledge of propolis

Propolis often referred to as bee glue, is a resinous sticky material collected by honeybees (*Apis mellifera* L.). Generally, honeybees produce propolis by collecting lipophilic plant substances from buds, leaves, flowers, lattices, mucilage, branches, and barks. Usually, it is collected within a radius of 1 - 2 km from the hive. In the hive, the substances are partially digested by β-glycosidase, and mixed with some saliva and other secretions of the honeybees as well as with beeswax (Kuropatnicki et al., 2013b). Honeybees collect plant resin in sunny and warm weather, usually from May to November, but most frequently in the late summer, when the resin is still soft and readily available for collection (Ristivojevic et al., 2015). Propolis mainly protects beehives from adverse weather conditions or invaders. It is used by honeybees to line the inside of the hive, maintain an ideal temperature and moister, seal small cracks and holes, sterilize the queen-bee posture site, reduce the size of hive entrances, and embalm any dead animals or insects which are too large to be carried out to prevent the putrefaction. The uses of propolis are important in order to prevent hive infections and protect the bee community against diseases. Nowadays propolis is used in various fields, such as dermatology and cosmetic applications. In medicine, applications include treatment of cardiovascular and blood systems, respiratory system, dental care, cancer treatment, support and improvement of the immune system, digestive system, and liver protection. Propolis has the advantage of being used as a preservative in food technology due to its antioxidant and antimicrobial activities, and the residues of propolis appear to have an overall beneficial effect on human health (Siheri et al., 2017). In addition, the physical and chemical properties of propolis vary depending on the geographical region (Eroğlu and Yuksel, 2020). Propolis is a resinous substance with varying colours from yellowish-green to dark brown and reddish (Figure 1), depending on its botanical source, honeybee species, and age, it has a viscous consistency (Kuropatnicki et al., 2013b). Propolis is slightly water-soluble, viscous, soft, and sticky at temperatures of 25 to 45°C, and becomes hard and brittle at less than 15°C. It is a mixture with a pungent smell and a heat-insulating property (Kuropatnicki et al., 2013b; Wagh, 2013).

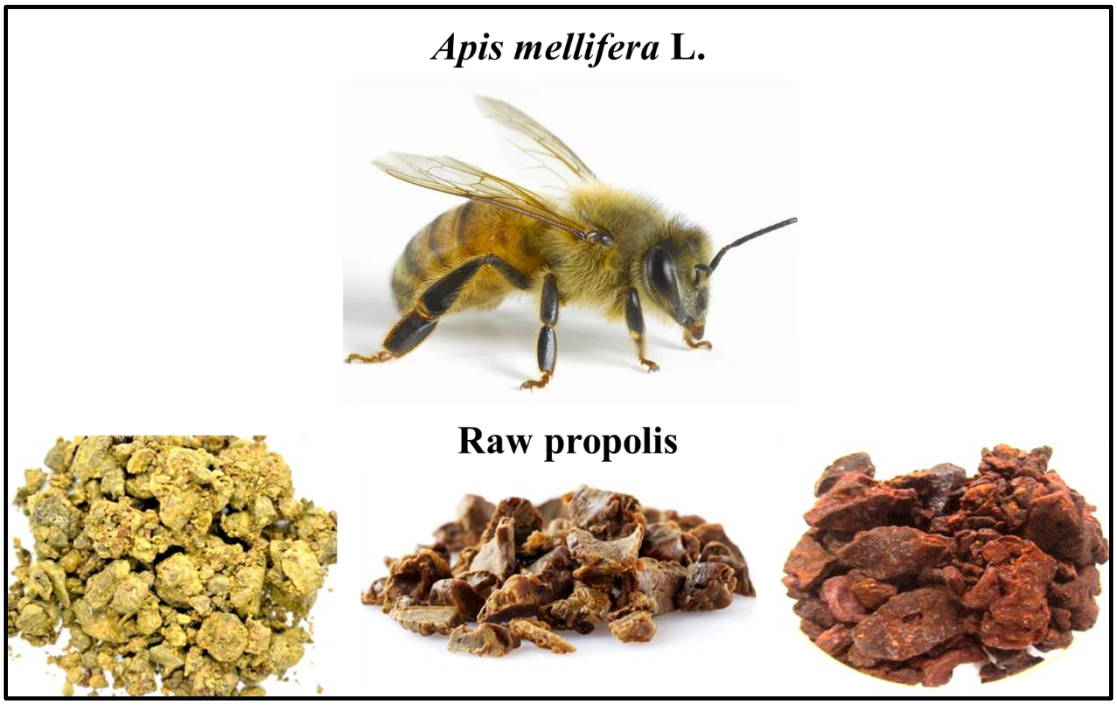

Figure 1: European honeybee specie (*Apis mellifera* L.) and illustration of a variety of raw propolis in different colours.

1.1.1. Historical overview

The ancient Egyptians, Greeks, and Romans civilizations were the first who used propolis, according to limited information obtained as a result of excavations, depictions, and cave paintings in different periods (Eroğlu and Yuksel, 2020). Propolis was one of the main ingredients used by ancient Egyptians in an embalming recipe for mummification of the dead bodies around 5500 BC and as a sedative and curative (Eroğlu and Yuksel, 2020), in which it served as a preservative agent. The internal and external use of propolis as a traditional medicine dates back to at least 2000 BC (Gupta and Stangaciu, 2014). It was mentioned in Herst, Ebers, Berlin, and Edwin Smith Egyptian papyri as a treatment for ulcers and sores (Ghisalberti, 1979). The term propolis is said to have been named as such by Aristotle Alexander's philosopher around 350 BC, it is derived from Hellenistic ancient Greek "pro" for (before, in front of, at the entrance to, or defence) and "police" for (community or city), so propolis means a substance in defences of the beehive (Castaldo and Capasso, 2002; Ghisalberti, 1979; Pasupuleti et al., 2017). Hippocrates and Pliny are said to have used propolis to cure bruises, wounds, sores, and ulcers. According to Pliny, propolis has the property of extracting stings and all foreign bodies from the flesh, dispersing tumours, allaying pains of the sinews, and cicatrizing ulcers of the most obstinate nature (Kuropatnicki et al., 2013b). Since the Second World War, the use of

propolis has gradually spread and now the list of its applications is almost endless. It includes not only wound healing but also the treatment for psoriasis, pubic itch, gingivitis, stomatitis, rheumatic disorders, and even sprains. Moreover, it is found in the composition of cosmetics and dietary supplements (Rojczyk et al., 2020). The Persian physician, Avicenna suggested the use of propolis for eczema, myalgia, and rheumatism. Roman and Greek Cypriots used propolis to form the contents of the perfume and the creams to nourish the skin. Research on the chemical properties of propolis began at the beginning of the 20th century. The study of propolis was initiated in the 1960s when scientist Dr. Stanisław Scheller observed the antibacterial properties of Polish propolis (Kuropatnicki et al., 2013a). Since the 1970s, scientists are very interested in propolis, several studies have been conducted to reveal the many benefits of this incredible substance. Propolis was gradually forgotten, but it returns to the fore in the past few decades, as an excellent alternative to antibiotics (Langenheim, 2003). Today it is still used in folk medicine but has also become a popular ingredient in health food and drinks or natural cosmetics (Bertrams et al., 2013). Researchers have been more interested in the chemical composition and biological properties of propolis in recent years.

1.1.2. Types of propolis and plant origin

Several studies have verified that the type of propolis varies from one sample to another depending on the geographical origins and the plant sources (Bankova, 2005). Table 1 summarizes the different types of propolis from the different geographical origins, the main constituents, its plant source, and the most characteristic effects. The best sources of propolis are collected from species of poplar, willow, birch, elm, alder, beech, conifer, and horse-chestnut trees (Silva et al., 2008). Poplar buds (*Populus alba, P. tremula,* and *P. nigra*) have been reported to be the main source of propolis from temperate zones, including Europe, North America, Asia, South America, New Zealand, Albania, Bulgaria, Greece, Hungary, Italy, Russia, Malta, the Netherlands, Poland, Turkey, Switzerland and the United Kingdom, with a minor contribution by secondary sources such as *Quercus*, *A. hippocastanum*, *Ulmus*, *Picea*, *Fraxinus*, *B. pendula*, *Salix alba*, and *Pinus*. The birch propolis type found in Russia, which has its origin in species such as *Betula verrucosa*, where the main compounds are flavones and flavonols differ from those found in poplar propolis (Bankova, 2005). Also, a Mediterranean propolis type was found in Greece, Cyprus, Egypt, Algeria, Morocco, and Malta, its main compounds are diterpenes most probably originating in coniferous plants of the genus *Cupressaceae* (Popova et al., 2010). Tropical propolis has a totally different compositional

pattern: the green propolis type, found in Brazil, has its main plant source on the leaves of *Baccharis* spp. and mainly contains prenylated phenylpropanoids (Righi et al., 2013). In Venezuela and Cuba, the main plant sources are the flower exudates of *Clusia* species, providing propolis rich in prenylated benzophenones. C-prenylflavonoids (or propolins) have been described in propolis from Pacific islands, where the resin sources are the fruit exudates of the tree *Macaranga tanarius* (Falcão et al., 2013a, 2013b). The detection of specific chemical markers is the most commonly adopted analytical strategy to group different propolis types. Flavonoids, phenolic acids, aromatic compounds and volatile propolis oils can be analysed with the development of separation and purification techniques such as high-performance liquid chromatography (Bruschi et al., 2003; Cuesta-Rubio et al., 2007), thin-layer chromatography (Milojković-Opsenica et al., 2016), liquid chromatography and gas chromatography coupled with powerful techniques such as mass spectrometry (Asgharpour et al., 2020; Cheng et al., 2013; Falcão et al., 2013b; Popova et al., 2010), and nuclear magnetic resonance (Cuesta-Rubio et al., 2007; Kasote et al., 2017). In addition, a simple technique of spectrophotometric registration of UV-Visible spectra was used to distinguish three types of propolis, according to the max absorption at 320 nm wavelength or 295 nm, and a plateau between 320 nm and 395 (Ristivojević et al., 2017). Brazilian propolis was classified into 12 types according to physicochemical properties and related to geographic locations; however, only three types of botanical origin were identified to be resins from *Populus* spp., *Hyptis divaricate*, and *Baccharis dracunculifolia* (Alencar et al., 2007; Silva et al., 2008). Recently, according to the thin-layer chromatography /high- performance liquid chromatography fingerprint, several authors confirmed the presence of two botanically different subtypes of European propolis defined as orange and blue (O-type and B-type) originating from *P. nigra* and *P. tremula*, respectively. On the other hand, G-type was characterized by deep green, light orange and blue bands (Ristivojevic et al., 2017). Propolis O-type is characterized by quercetin, while B-type corresponds mostly to caffeic acid, galangin, feruloyl, and *p*-coumaroyl derivatives. G-type corresponds to apigenin, apigenin-methyl-ether, or naringenin (Shawky and Ibrahim, 2018). However, some of the German propolis samples were classified as mixed type (Morlock et al., 2014).

1.1.3. Chemical composition and standardization

The chemical composition of propolis differs greatly among species of honeybees and depends on geographical and climatic factors, and collecting seasons (Bueno-Silva et al., 2017a;

do Nascimento et al., 2019). The specificity of local flora determines the chemical composition of propolis and its affiliation to a particular group (Falcão et al., 2014). The composition of propolis samples can vary considerably, even if they are collected at a relatively low distances from each other (Anđelković et al., 2017). Propolis is a very complex material. Generally, it is composed of 50% resin and vegetable balsam, 30% beeswax, 10% essential and aromatic oils, 5% pollen, and 5% other substances, including amino acids, vitamins, minerals, and organic components (Figure 2) (Robertson, 1990). Up to 2014, over 400 compounds have been identified in poplar propolis from many countries. They include flavonoids, phenylpropanoids, terpenoids, stilbenes, lignans, coumarins, and their prenylated derivatives. Some of them, such as phenolic glycerides and glycosides recently attracted more attention. Others such as acaricides, herbicides, and toxic metals might be indicators of environmental pollution and might originate from various anthropogenic sources. This list of compounds is still increasing (Ristivojevic et al., 2015). The propolis samples collected from different parts of the world were found to contain 824 components until 2018 (Šturm and Ulrih, 2019). Phenolic compounds, esters, flavonoids, terpenes, beta-steroids, aromatic aldehydes, and alcohols are the important organic compounds present in propolis. Twelve different flavonoids, namely, pinocembrin, acacetin, chrysin, rutin, luteolin, kaempferol, apigenin, myricetin, catechin, naringenin, galangin, and quercetin; phenolic acids, caffeic acid and cinnamic acid; and one stilbene derivative called resveratrol have been detected in propolis. It also contains important vitamins, such as vitamins B1, B2, B6, C, and E, and useful minerals such as magnesium, calcium, potassium, sodium, copper, zinc, manganese, and iron. A few enzymes, such as succinic dehydrogenase, glucose-6-phosphatase, adenosine triphosphatase, and acid phosphatase, are also present in propolis (Pasupuleti et al., 2017). The chemical composition of poplar-type of propolis can be classified into several categories, such as free aromatic acids, esters of these acids, flavonoids including flavones, flavanones, flavonols, and dihydroflavonols, chalcones and dihydrochalcones, terpenoids, acyclic hydrocarbons, alcohols, aldehydes, amino acids, aromatic hydrocarbons, fatty acids, ketones, sterols, sugars and sugar alcohols. The standardization of the chemical composition of propolis extracts is critically needed, to determine the appropriate type of propolis for a specific treatment, thus ensuring its efficacy, quality, and safety in use. Due to the large chemical heterogeneity of propolis, producing standardized and homogeneous extracts is a challenging task. The chemical characterization and standardization of propolis extracts require new, less time-consuming, and inexpensive techniques. Propolis can be standardized if we formulate different types of propolis according to their plant source and the corresponding chemical profile (Bankova, 2005). Poplar-type

propolis is one of the most intensively studied and the best-known type of propolis thus providing a good base for its standardization (Maraschin, 2016). The method of the extraction is an important step to benefit from the bioactive constituents of propolis, also is important for the standardization. The methods that we can use are: Maceration, ultrasound-assisted (with and without shaking), microwave-assisted, supercritical fluid, high-pressure, natural deep eutectic solvents, and solid-phase extraction. Thus, the extraction method can affect the chemical profile of propolis and affect its biological activity (Bankova et al., 2021; Devequi-Nunes et al., 2018; Pobiega et al., 2019a; Trusheva et al., 2007; Wagh, 2013). The chemical composition might vary according to the solvent used (water, ethanol, methanol, DMSO, glycol, etc). The concentration of solvent as well influences the activity of the extract (50% or 70% or 90% ethanol). In addition, the time and temperature of incubation with the solvent can vary within the same method. Many of the bactericidal components are soluble in water or alcohol. In general, alcohols and in particular ethanol are used as a universal extracting solvent for the active constituents of propolis (Ashry and Ahmad, 2012). However, it is not easy to select the best approach for extraction which is suitable for each type of propolis.

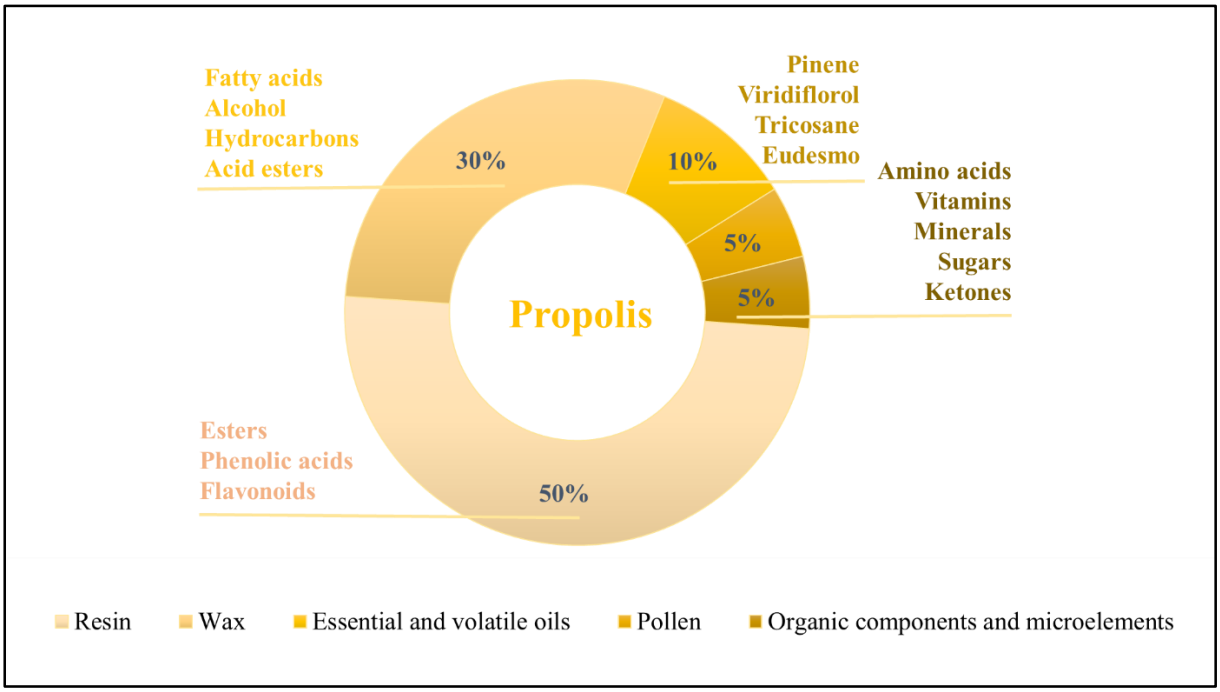

Figure 2: The general composition of raw propolis and the typical components for each group; 1) resin and vegetable balsam (50%), 2) beeswax (30%), 3) essential and volatile oils (10%), 4) pollen (5%), and 5) other substances (5%) including amino acids, vitamins, enzymes, sugar, and minerals.

Table 1: Chemical characterization of different types of propolis, geographic distribution, botanical origin, and biological activities.

Type	Region	Main compounds	Plant source	Activity	Cell used	Reference
Green propolis	Brazil Taiwan	Apigenin Artepillin C Caffeic acid Chrysin Cinnamic acid Ferulic acid Kaempferide Narigenin Pinobanksin Rutin	*Baccharis dracunculifolia* *Eucalyptus citriodora* *Araucaria angustifolia* *Mimosa tenuiflora*	Antibacterial Antibiofilm Antioxidant	*Bacillus Subtilis* *Escherichia coli* *Listeria monocytogenes* MRSA MSSA *Pseudomonas aeruginosa*	(Bezerra et al., 2020; Búfalo et al., 2009; Chen et al., 2018; Corrêa et al., 2020; Ferreira et al., 2017; Roberto et al., 2016)
				Antifungal	*Candida albicans* *Candida parapsilosis* *Candida tropicalis*	
				Anti-genotoxic	*Allium cepa*	
				Antitumour	HEp-2	
Red propolis	Brazil Cuba	Artepellin C Biochanin A Flavone Homopterocarpin Liquiritigenin Lupeol Medicarpin Methyl abietate Methyl *o*-orsellinate Naringenin Neovestitol	*Dalbergia ecastophyllum* *Clusia* spp. (*C. scrobiculata, C. minor, C. major,* and *C. rosea*)	Antibacterial Antioxidant	*Bacillus subtilis* *Enterococcus faecalis* *Enterococcus* spp. *Escherichia coli* *Klebsiella* spp. MRSA *Pseudomonas aeruginosa* *Streptococcus mutans*	(Alencar et al., 2007; Andrade et al., 2017; Cuesta-Rubio et al., 2007; Dantas Silva et al., 2017; Machado et al., 2016; Piccinelli et al., 2011; Regueira Neto et al., 2017; Rufatto et al., 2018)
				Antiparasitic	*Trypanosoma cruzi epimastigotes Y*	

		Pterocarpans Vestitol *β*-amyrin		Antitumour	HCT-116 SF-295 HL-60 OVCAR-8	
Brown propolis	Brazil Cuba	Artepillin C Baccharin Caffeic acids Chlorogenic acids Drupanin Kaempferide Kaempferol *p*-coumaric Phenylpropanoid Polyisoprenylated benzophenones Prenylated phenylpropanoids	*B. dracunculifolia* *C. rosea*	Anti-mycoplasma	*Mycoplasma* spp. (*M. bovis, M. gallisepticum, M. genitalium, M. hominis, M. hyorinis, M. penetrans,* and *M. pneumonieae*)	(Andrade et al., 2017; Cuesta-Rubio et al., 2007; Dantas Silva et al., 2017; de Oliveira Dembogurski et al., 2018; do Nascimento Araújo et al., 2020; Machado et al., 2016)
				Antibacterial Antibiofilm Antioxidant	*Enterococcus* spp. *Staphylococccus aureus*	
				Antiparasitic	*Trypanosoma cruzi epimastigotes Y* *Trichomonas vaginalis*	
				Antitumour	OVCAR-8	
Mediterranean propolis	Greek Cyprus Malta Sicily Bulgaria Turkey Greece Algeria	Communic acid Diterpenic acids Hydroxyditerpenic acid Imbricataloic Isoagatholal Isocupressic acid Pimaric acid	*Cupressus sempervirens* *Pinus species*	Antibacterial Antibiofilm Antioxidant	*Enterobacter cloacae* *Escherichia coli* *Klebsiella. pneumoniae* MRSA *Pseudomonas aeruginosa* *Staphylococcus epidermidis* *Streptococcus mutans* *Streptococcus viridans*	(El-Guendouz et al., 2016, p.; Graikou et al., 2016; Piccinelli et al., 2013; Popova et al., 2012, 2010; Velikova et al., 2000)

	Croatia Morocco	Pinocembrin		Antifungal	*Candida albicans* *Candida tropicalis* *Candida glabrata*	
Yellow propolis	Cuba Brazil	Acetyl triterpenes Flavanones Lanostane Lupane Oleanane Polymethoxylated Sterols Triterpenic alcohols Ursane	Undetermined	Antibacterial	*Staphylococcus aureus*	(Cuesta-Rubio et al., 2007; Machado et al., 2016; Márquez Hernández et al., 2010; Monzote et al., 2012)
				Antifungal	*Trichophyton rubrum*	
				Antiprotozoal	*Leishmania infantum* *Plasmodium falciparum* *Trypanosoma brucei* *Trypanosoma cruzi*	
				Antitumour	MRC-5 OVCAR-8	
Poplar propolis	Mostly from Eurasian regions*	Acetyloxycaffeate Caffeic acid Chrysin Dihydroflavonols Galangin Henolics Phenylpropanoids Pinobanksin Pinocembrin Prenyl caffeate Salicylic acid	*Populus* spp. (*P. nigra* L., *P. tremuloide, and P. alba* L.)	Antifungal	*Aspergillus fumigatus* *Candida glabrata* *Candida albicans* *Fusarium* spp.	(Boisard et al., 2020, 2015a; de Marco et al., 2017; Dezmirean et al., 2017; Popova et al., 2007; Ristivojević et al., 2020; Vardar-Ünlü et al., 2008; Wang et al., 2014)
				Antibacterial Antibiofilm Antioxidant	*Acinetobacter baumannii* *Bacillus cereus* *Enterococus* spp. *Escherichia coli* *Lactobacillus acidophilus* *Listeria* spp. *Mycobacterium smegmatis* *Pseudomonas aeruginosa* *Salmonella enteritidis* *Staphylococcus* spp. *Streptococcus* spp.	

		Anti-inflammatory	Murine macrophage RAW 264.7 HEK-293T and HEK-293

(MRSA) Methicillin-Resistant *Staphylococcus aureus.* (MSSA) Methicillin-Sensitive *Staphylococcus aureus.* (HEp-2) Human epidermoid carcinoma. (HCT-116) Colorectal carcinoma. (SF-295) Human glioblastoma. (HL-60) Human leukaemia. (OVCAR-8) Human ovarian carcinoma. (MRC-5) Human simian virus 40-immortalised lung fibroblasts. (HEK-293T and HEK-293) Human embryonic kidney cells.* England, France, Italy, Switzerland, Germany, Poland, New Zealand, Russia, Bulgaria, Macedonia, Estonia, Latvia, Lithuania, Slovakia, Slovenia, Serbia, Ukraine, Hungary, Syria, Turkey, Iran, Argentina, Canada, Chile, China, Korea, Uruguay, Uzbekistan, and the USA

1.2. Nosocomial infections

Nosocomial infections (NIs) are major threats to hospitalized patients, it also includes occupational infections that may affect staff. The NIs also referred to as healthcare-associated infections (HAI), including all infections acquired during or after the process of receiving health care between 48 hours after hospital admission and 3 days of hospital discharge. It may occur in different areas of healthcare delivery, such as in hospitals, long-term care facilities, and ambulatory settings (Sikora and Zahra, 2022). Infection occurs when a pathogen spread to a susceptible patient host. The etiology of HAI depends on the type of infection and the responsible pathogen; bacteria are the most common pathogens, followed by fungi and viruses (Edwardson and Cairns, 2019). The majority of the infections are associated with invasive procedures and surgery, and the use of invasive medical devices, such as endotracheal tubes, prosthetic devices, central venous catheters, invasive intracranial pressure monitoring, and urinary catheters. The Centers for Disease Control and Prevention broadly categorizes the types of HAI as follows: lower respiratory tract infections (hospital-acquired pneumonia, ventilator-associated pneumonia, and ventilator-associated tracheobronchitis central line-associated bloodstream infection, catheter-associated urinary tract infections, and skin and surgical site infection. These infections are associated with prolonged hospitalization, financial burden, and the development of multiple organ dysfunction, which can lead to serious problems like sepsis and even death. *Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli, Candida* spp., *Enterobacter* spp., *Enterococcus* spp., and *Klebsiella* spp. are the frequently isolated microorganisms from NIs in the Intensive care unit (ICU) (Sikora and Zahra, 2022). The emergence of multidrug-resistant (MDR) microorganisms is another complication associated with NIs. The World Health Organization now considers antimicrobial resistance a major threat to human health (Kollef et al., 2021). The notorious pathogens that are frequently reported include methicillin-resistant *S. aureus* (MRSA), vancomycin-intermediate *S. aureus*, vancomycin-resistant *S. aureus*, vancomycin-resistant *Enterococcus*, carbapenem-resistant *Enterobacteriaceae* spp., and MDR *P. aeruginosa* (Kollef et al., 2021). The carriers of MDR microorganisms are at greater risk for developing infections that are difficult to treat and associated with greater mortality and morbidity (Edwardson and Cairns, 2019).

1.2.1. *Candida* infections

Fungal pathogens are usually associated with opportunistic infections in immunocompromised patients and those with indwelling devices, such as central lines or urinary catheters. *Candida* species, such as *C. albicans, C. parapsilosis, C.glabrata* are the most commonly encountered fungal organisms associated with HAI. Altogether, *Candida* species make up the fourth most common pathogen across all types of HAI (Sikora and Zahra, 2022). The genus *Candida* refers to a fungus that forms part of the individual's microbiota, and is largely present in areas of mucous membrane such as the oral and vaginal cavity (Capoci et al., 2015). The ability for morphological transition between yeast cells and hyphal forms is an important virulence factor for candidiasis. *C. albicans* and other species are opportunistic pathogens which have been recorded as the most frequent cause of candidiasis (Gucwa et al., 2018) and candidemia (Mutlu Sariguzel et al., 2016). Furthermore, many hospital-acquired infections are associated with the ability of microorganisms to adhere to human cells (Capoci et al., 2015), and to form biofilms in implanted orthodontics, catheter materials, and other medical devices (Gucwa et al., 2018). Thus, the formation of biofilm by *C. albicans* is one of several virulence factors responsible for infectious disease, and increases the risk of periodontal disease (Siqueira et al., 2015), vulvovaginal candidiasis (Capoci et al., 2015), and the development of various mechanisms of resistance against antifungal agents (Bezerra et al., 2020).

1.2.2. Staphylococcal infections

S. aureus is a Gram-positive bacterium, a commensal bacteria that are found on the skin and mucous membranes of healthy humans and animals. It is also a ubiquitous opportunistic pathogen that is widely distributed in the environment and food. Pathogenic bacteria are uniquely adapted and endued with mechanisms for overcoming the body's defences and can invade parts of the body, such as the blood, where no bacteria are ordinarily found (Gould et al., 2012). *S. aureus* is a major concern due to its intrinsic virulence and ability to cause infection, as well as being a major cause of foodborne illness. Virulence factors for *S. aureus* include immune system evasion, adherence to host cells, host tissue damage, and toxin generation. In addition, the emergence of antimicrobial-resistant strains is a serious problem and needs attention to improve the development of new substances and prevention strategies (Mourenza et al., 2021). *S. aureus* is a common cause of a diverse spectrum of human infections, which can be extended from the superficial wound infections (skin and soft tissue infections)

to more serious life-threatening invasive diseases (Goudarzi, 2017). MRSA remains one of the principal multi-resistant pathogens causing serious community-onset infections, complicated skin structure infections and hospital-acquired infections (HA-MRSA), particularly bloodstream infections and ventilator-associated pneumonia (Gould et al., 2012). HA-MRSA infections often are associated with invasive devices, such as intravenous tubing or artificial joints, also during the surgical procedures. Treatment of patients usually includes the use of *β*-lactams antibiotics (methicillin, oxacillin, and cefoxitin etc.). Vancomycin (a glycopeptide drug) is the most common drug used to treat severe MRSA infections. The emergence of MDR strains carrying genes that are resistant to most of the currently available antimicrobial agents is a serious health care problem (David and Daum, 2017). Patients with MRSA were rated as 64% more likely to die than people with MSSA infection. Thus, MRSA is a major cause associated with morbidity and mortality, along with *Escherichia coli* (resistance to third-generation cephalosporins) (Gould et al., 2012). MRSA remains an important pathogen in nosocomial infections, such as pneumonia (Shorr et al., 2015) and bacteremia (Goudarzi, 2017). *S. aureus* causes also non-invasive and invasive infections, such as endocarditis and intravascular infections, osteomyelitis, vertebral discitis, epidural abscess, septic arthritis, pyomyositis, mastitis, necrotizing fasciitis, orbital infections, endophthalmitis, parotitis, staphylococcal toxinoses, urogenital infections, and central nervous system infections (David and Daum, 2017). On the other hand, *S. aureus* is a pathogen related to food contamination and represents a serious problem for both the food industry and healthcare systems (Pobiega et al., 2019b). In the food industry, foodborne pathogens possess the ability to escape from routine sterilization (such as high temperature and pressure, mechanical flushing) by forming a biofilm (Miao et al., 2019). In addition, foodborne illnesses caused by *S. aureus* generally involve toxicity associated with enterotoxins causing staphylococcal gastroenteritis. The high stability of staphylococcal enterotoxins and their resistance to heat, acidity, and most proteolytic enzymes such as pepsin and trypsin assure that these toxins remain hermetically active in the gastrointestinal tract (Mourenza et al., 2021).

1.3. Therapeutic properties of propolis

The standardization of propolis is necessary to be considered as an alternative therapeutic agent. The chemical constituents responsible for the beneficial biological activities of propolis are flavonoids and other phenolics such as cinnamic acids and their esters. Several articles confirmed the significant correlation between the total phenolic content and

antimicrobial activity against different bacteria, fungi, *Helicobacter pylori*, protozoa, viruses and parasites, and antioxidant activity (Pasupuleti et al., 2017). The beneficial effects of propolis have been used for centuries as an external antiseptic and internal remedy for multiple human ailments; to treat tuberculosis, inflammatory diseases, duodenal ulcers (Barros et al., 2008), and gastric disturbances, to relieve various types of dermatitis, reduce fever, and its use continues till today in home remedies and personal products. The main biological properties of propolis are antibacterial, antiviral, antifungal, anthelmintic, antiulcer, antioxidant, antiradiation, hepatoprotective, antitumor, antimutagenic, anti-angiogenic, cyto/chemo-preventive, anti-inflammatory, wound healing, immunomodulating, anti-diabetic, cardioprotective, local anesthetic, regenerative (cartilaginous and bone tissue, dental pulp), cicatrizing, it is also used in cosmetic products, and as an additive and food preservative (Bezerra et al., 2020; Desamero et al., 2019; Dobrowolski et al., 1991; El-Guendouz et al., 2018; Nani et al., 2018; Rufatto et al., 2017; Silva et al., 2019). Recently, clinical studies revealed that propolis solution could improve clinical coronavirus 2019 (COVID-19) symptoms and decrease viral clearance time (Dilokthornsakul et al., 2022). Propolis has been demonstrated to be safe and non-toxic for human use. However, some cases of allergic reactions such as contact dermatitis have been reported by beekeepers (Siheri et al., 2017). The studies employed different experimental approaches such as disc diffusion and macro- and micro-dilution methods to investigate the antimicrobial activity. The dilution method is used to determine the minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC). The biofilm formation and biofilm eradication assays applied for the investigation of the antivirulence effect of propolis, by measuring the minimal biofilm inhibition concentration (MBIC) and the minimal biofilm eradication concentration (MBEC) (Savka et al., 2015). The interaction properties between propolis extract and antibiotics on *S. aureus* and other microorganisms have been described by previous studies (Grecka et al., 2019; Grecka and Szweda, 2021; RegueiraNeto et al., 2017; Sadrzadeh-Afshar et al., 2020; Wojtyczka et al., 2013a).

1.3.1. Antifungal activity

The researchers have endorsed the importance of using natural products such as propolis to treat fungal infections caused by *Candida* species. Although the antimicrobial activity of propolis has been investigated over recent years as an alternative for conventional therapeutic strategies, the antifungal activity of propolis is still underestimated. Therefore, propolis needs

more evaluation to determine its therapeutic role. An ethanolic extract of Turkish propolis showed the highest antifungal activity against 76 *Candida* isolates (*C. albicans, C. parapsilosis*, *C. tropicalis*, and *C. glabrata*) that were isolated from the blood cultures of intensive care unit patients (Mutlu Sariguzel et al., 2016). Siqueira and the co-workers have investigated the fungistatic and fungicidal activities of alcoholic extract of Brazilian red propolis (BRP) against 19 *Candida* species (including; 12 *C. albicans*, 5 *C. tropicalis*, and 2 *C. glabrata*), which were isolated from chronic periodontitis cases. All *C.* species were sensitive to BRP, although 42% of *C. albicans* isolates were resistant to fluconazole (Siqueira et al., 2015). Ethanolic extract of BRP showed fungistatic activity on 12 *C.* strains (*C. albicans, C. tropicalis*, and *C. neoformans*) with MIC value equal to 256 μg/mL (Neves et al., 2016). The antifungal activity of aqueous and organic extracts (water, 95% ethanol, 70% ethanol, methanol, and dichloromethane) of French propolis was determined against *C. albicans*, *C. glabrata*, and *Aspergillus fumigatus*. The antifungal and antibiofilm capacity of propolis extract has been demonstrated against clinical isolates *Fusarium solani*, *F. oxysporum*, and *F. subglutinans*, which are a common species that cause onychomycosis (Galletti et al., 2017a). Ethanolic extract of Iranian propolis exhibited high antifungal activity against fluconazole-resistant *C. albicans* that were isolated from nails, the oral cavity, and vaginal cavity. The subinhibitory concentrations of propolis significantly reduced germ tube formation (Haghdoost et al., 2016a). Bezerra et al. found that the green propolis ethanolic extract showed significant antifungal activity, using disk diffusion assay, against *Candida* species. Propolis is considered a good oral antiseptic to prevent caries (Djais et al., 2019), and it has potential use in modifying the adhesive properties of *C. albicans*, thus preventing the pathogen's ability to form biofilms (Feldman et al., 2014). Additionally, propolis extracts could prevent yeast cells from forming biofilms while showing very low cytotoxicity in human cells (Capoci et al., 2015).

1.3.2. Antibacterial activity

Antimicrobial preventative therapies have shown the ability to avert NIs in the short term but result in the disruption of the microbiota, leading to future antibiotic-resistant infections (Kollef et al., 2021). Antimicrobial resistance represents one of the most important challenges in NIs. Resistance occurs due to selective pressures from regular antibiotic use, causing the evolution of existing bacteria. The common resistant bacteria found in the ICU are MRSA (Sikora and Zahra, 2022). The antibacterial potential of propolis varies considerably from one bacterial strain to another, and depends on the propolis sample used. Literature

suggested that the alcohol fractions of propolis possess significant antibacterial activity against Gram-positive as compared to Gram-negative bacteria. The *in vitro* antibacterial activity results from the synergy between propolis compounds (Ramos and Miranda, 2007). The polyphenols and aromatic compounds are accountable for the antibacterial properties of different types of propolis. Several scientific studies have shown that propolis and its derivatives have antibacterial activity against *E. coli*, *S. aureus*, *Streptococcus* spp., *Salmonella* Typhi, *Enterococcus* spp., *Bacillus* spp., and *P. aeruginosa* (Anjum et al., 2019; Przybyłek and Karpiński, 2019; Rufatto et al., 2017). An antibacterial study of Mediterranean propolis samples was carried out by the disc diffusion method against Gram-positive and Gram-negative bacteria and oral pathogens. It is noteworthy that the diterpene content was directly proportional to antimicrobial activity against all tested bacteria. Moreover, the samples showed particularly strong activity on Gram-positive bacteria (*S. aureus, S. epidermidis,* and *Streptococcus mutans*) (Graikou et al., 2016). Further studies on propolis samples collected from Mediterranean areas confirmed the effectiveness of propolis extracts on *S. epidermidis*, *S. aureus*, *a*nd MRSA (El-Guendouz et al., 2018). Korean, German, and Irish propolis have exhibited mild to moderate antibacterial activities (Graikou et al., 2016). Ethanol fractions of Polish propolis have shown significant antibacterial activity against *S. aureus* (Grecka et al., 2019). On the other hand, propolis is one of the natural antimicrobial substances that can be used efficiently to replace chemical preservatives in extending the life of raw materials and food products (Pobiega et al., 2019b).

1.4. Mechanisms of action of propolis

Propolis and some of its derivatives are responsible for either killing bacterial cells directly by interacting with them through different mechanisms, or by modifying the immune response of host cells (Almuhayawi, 2020). It is evident from the literature that several possible mechanisms might account for the lower antibacterial activity of propolis against Gram-negative bacteria. One possible reason could be the synthesis of a wide variety of hydrolytic enzymes by Gram-negative microorganisms (Grecka et al., 2019). These hydrolytic enzymes may interfere with the active components of propolis and result in the development of resistance (Bryan et al., 2015). Several underlying mechanisms have been proposed by different research groups regarding the antimicrobial activity of propolis, including the inhibition of cell division, nucleic acid synthesis, protein synthesis, impeding cytoplasmic membrane function, altering membrane permeability, reducing the ability to form biofilms, bacteriolysis, inhibiting the

energy generation pathway, and reducing bacterial resistance towards certain conventional antibiotics (Przybyłek and Karpiński, 2019). A study by Aru et al. found that Turkish propolis extract caused an apoptotic effect on cancer cell lines, and promoted cell cycle arrest by activating the expression of cell cycle p21 proteins. Using MTS assay, the same propolis samples showed moderate anti-proliferative activity on cancer cell lines (Aru et al., 2019). Propolis-derived showed a significant decrease in HRV RNA replication into human epithelial adenocarcinoma cervix (HeLa) cell cultures. Kaempferol and *p*-coumaric acid may interfere with expression of intercellular adhesion molecules (Kwon et al., 2019). It was detected that Russian propolis causes cell lysis and bacterial cell membrane damage within mature biofilms (Ambi et al., 2017). It was found that the antiviral activity of propolis is the result of a synergistic interaction between its compounds (Dilokthornsakul et al., 2022). *In silico* studies have shown that propolis compounds (eg; caffeic acid, chrysin, galangin, myricetin, rutin, hesperetin, pinocembrin, luteolin, quercetin, kaempferol, *p*-coumaric acid, and genistein) have positive binding affinities to coronavirus (SARS-CoV-2) proteins, thus interfering with virus entry and viral RNA replication (Dilokthornsakul et al., 2022; Zulhendri et al., 2022). Nevertheless, very little is currently known about the molecular mechanisms associated with the biological effects of propolis (Boisard et al., 2020), and the mechanisms underpinning its activity against microorganisms are still not clear. However, for a long time, it has been considered that the activities of propolis compounds against microorganisms are more related to the synergistic effect of polyphenols than to individual effects (Koo et al., 2000; Martins et al., 2002).

2. Aims of the study

The application of propolis in the pharmaceutical industry requires an understanding of its chemical composition and biological properties. Poplar-type propolis is the most studied and best-known type in terms of its content of biologically active components (Ristivojevic et al., 2015). The chemical composition of propolis is quite complex and variable for different reasons. *Candida albicans* and *Staphylococcus aureus* are considered as an important opportunistic pathogens responsible for various community-onset and hospital-acquired infections, due to the pathogenic capacity such as biofilm formation, fungal germ tube formation, and resistance to classical antibiotics. Propolis is known to have antibacterial and antifungal activities. However, Propolis's mechanism of action is supposed to be very complex and not well studied.

This study aimed to characterize the Hungarian propolis and to investigate the antimicrobial activity, as well as to detect the mode of action belonging to its cytotoxicity. To achieve these objectives, the following experiments were performed:

- The chemical characterization of propolis samples from different geographical regions in Hungary.
- Antibacterial, antifungal, and antibiofilm activities of ethanolic extract of propolis (EEP) were investigated on *C. albicans* and *S. aureus*, as well as the effect on *C. albicans* germ tube formation.
- The mode of action of propolis was evaluated on *C. albicans* cells, in order to provide new information about the action of this complex substance as an antifungal agent. Therefore, the bioabsorption of EEP, the probable cell wall and cell membrane damage generating effects were analysed. Furthermore, DNA fragmentation and nuclei damage were determined.

3. Materials and Methods

3.1. Microorganism and culture conditions

In the present study, two strains of *C. albicans* were used; *C. albicans* ATCC 44829 used in all experiments, and *C. albicans* SZMC 1424 used for examining the antbiofilm and germ tube formation. Two Hungarian clinical isolates of MRSA (SA H23 and SA H24) were obtained from the Department of Medical Microbiology, University of Pécs, Hungary. The reference strains methicillin-susceptible *S. aureus* ATCC 29213 (MSSA) and methicillin-resistant *S. aureus* ATCC 700699 (MRSA) were used as negative and positive controls in the susceptibility test, respectively. The fungal cells were precultured in the YPD medium (2% (w/v) glucose, 1% (w/v) peptone, and 0.5% (w/v) yeast extract, pH 5.6) and YPD agar (2% (w/v) agar, 2% (w/v) glucose, 1% (w/v) peptone, and 0.5% (w/v) yeast extract, pH 4.5) overnight. RPMI-1640 (1% (w/v) RPMI-1640, 3% (w/v) MOPS, and 2% (w/v) glucose), and MM medium (1% (w/v) glucose, 0.5% (w/v) $(NH_4)_2SO_4$, 0.04% (w/v) KH_2PO_4, and 0.05% (w/v) $MgSO_4$) were used for the inoculation, all the media supplemented with 25 µg/mL adenine for the strain *C. albicans* ATCC 44829 and adjusted to pH 7.2 ± 0.2. A mid-log-phase culture was incubated in an orbital shaker 150 rpm at 30°C for 18 h. The bacterial strains were cultured in tryptic soy broth (TSB) and tryptic soy agar (BD™, Heidelberg, Germany) with 2% (w/v) NaCl at 37°C for 24 h. The cell culture was washed in PBS buffer (0.8% (w/v) NaCl, 0.02% (w/v) KCl, 0.18% (w/v) Na_2HPO_4 2-hydrate, and 0.24% (w/v) KH_2PO_4).

3.2. Raw material of propolis and the extractions method

Raw propolis samples were collected from 6 regions of Hungary (Figure 3); Pécs (1), Szombathely (2), Szolnok (3), Csikóstőttős (4), Héhalom (5), and Somogybabod (6). The debris of bees or plants and excess wax were removed from the propolis samples. The samples were ground to a fine powder (Figure 4), then 100 g of propolis were extracted in 450 mL 80 % (v/v) ethanol in a water bath at 70°C for 30 min. The ethanolic extracts were sterilized through a 0.22 µm pore size filter (Millipore, Burlington, MA, USA) to obtain 222.2 mg/mL a stock concentration ethanolic extract of propolis (EEP) (Figure 5). It was stored at 4°C in dark (Alencar et al., 2007).

Figure 3: Raw propolis samples collected from six different geographical regions of Hungary, Pécs (1), Szombathely (2), Szolnok (3), Csikóstőttős (4), Héhalom (5), and Somogybabod (6).

Figure 4: The texture and colour of raw propolis samples from six regions in Hungary: Pécs (1), Szombathely (2), Szolnok (3), Csikóstőttős (4), Héhalom (5), and Somogybabod (6).

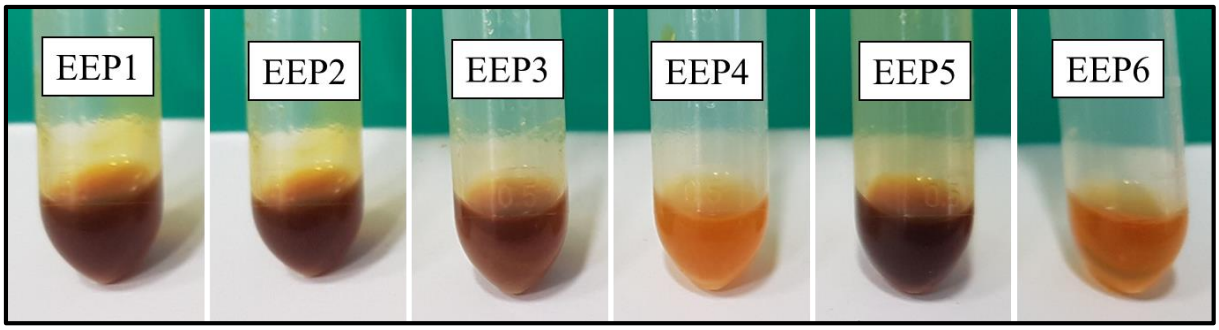

Figure 5: Ethanolic extracts of propolis (EEP) of six samples; Pécs (1), Szombathely (2), Szolnok (3), Csikóstőttős (4), Héhalom (5), and Somogybabod (6).

3.3. Chemical characterization of propolis extracts

3.3.1. Determination of the total phenolic content (TPC)

The TPC in the propolis extracts was evaluated indirectly by relating the reducing capacity of propolis and gallic acid standard compound using the Folin-Ciocalteu method (Moreira et al., 2008). Briefly, 500 µL of 200 µg/mL EEP was mixed with 500 µL of Folin-Ciocalteu reagent (10% v/v) and 500 µL of Na_2CO_3 (2% w/v), and incubated in dark at room temperature for 1 h. The absorbance of the reaction mixture was determined at 700 nm against the blank (the reagent mixture without EEP) using a Hitachi U-2910 spectrophotometer (Tokyo, Japan). Gallic acid standard solutions (0.01-0.5 mM) were used for constructing the calibration curve ($y= 85.344x - 0.0053$; $R^2= 0.9995$). The TPC was expressed as milligram (mg) of gallic acid equivalents (GAE) per gram (g) of propolis dry weight (DW).

3.3.2. Determination of total flavonoids content (TFC)

The flavonoids were determined by the aluminium chloride colorimetric method, as reported by (Dias et al., 2012). Briefly, 125 µL of 1 mg/mL EEP was mixed with 625 µL of distilled water and 37 µL of 5% $NaNO_2$ solution. After 5 min, 75 µL of 10% $AlCl_3$ solution was added, and subsequently, 250 µL of 1 M NaOH and 137 µL of distilled water were added after 6 min to the mixture and well vortexed. The intensity of the pink colour of the reaction mixture was measured at 510 nm against the blank (the same mixture without EEP) using a Hitachi U-2910 spectrophotometer (Tokyo, Japan). Catechin standard solutions (0.022-1.5 mM) were used for constructing the calibration curve ($y= 0.6814x + 0.0061$, $R^2= 0.9997$). The TFC was expressed as milligram of catechin equivalents (CE) per gram of propolis dry weight (DW).

3.3.3. Spectrophotometric analysis of UV-visible spectra

To distinguish the sub-type of poplar-type propolis according to the total flavonoid content and scavenging activity (Mărghitaş et al., 2013). The ethanolic extracts of propolis were diluted to 22 µg/mL in ethanol, and the mixtures were scanned to obtain the absorption spectra at wavelengths between 200 and 400 nm by UV-Vis spectrophotometer (Hitachi, U-2910, Tokyo, Japan), calibrated for the pure ethanol solvent. The recording speed and the sampling interval were 100 nm/min and 0.5 nm, respectively. Deconvolution of the absorption spectra was performed with OriginPro 2016 software.

3.3.4. Gas chromatography–mass spectrometry (GC-MS)

The trimethylsilyl (TMS) ether derivatives of ethanolic extracts of propolis were subjected to GC-MS analysis. Briefly, about 2.2 mg of freeze-dried propolis extract was mixed with 50 µL of dry pyridine (Merck, Budapest, Hungary) and 75 µL bis (trimethylsilyl) trifluoroacetamide (MSTFA) (Merck, Budapest, Hungary), heated at 80°C for 20 min. Then, derivatized of the extract was injected into a GC-MS QP2020 (Shimadzu, Duisburg, Germany) equipped with an Agilent DB-5 (30 m × 0.25 mm ID, 1.00 µm film) (Agilent, Santa Clara, CA, USA). Helium was utilized as a carrier gas using a linear velocity of 40 cm/s at the split ratio of 1:20. The injection temperature was set at 280°C. The oven program which started with an initial temperature of 100°C, was heated up to 320°C at a rate of 5°C/min. The EI ion source temperature, the ionization voltage, and the solvent cut time were 230°C, 70 eV, and 4.0 min, respectively. All spectra were recorded in scan mode with 0.3 s event time from 4.5 to 60 min in the mass range of 45–600 *m/z*. The detected compounds were identified by analysing both chromatograms and mass spectra using the Hexane solution of C7-C33 *N*-alkanes (Restek, Bellefonte, PA, USA) and comparing the results with those listed in the computer searches on commercial libraries.

3.4. Antimicrobial effect of EEP

3.4.1. Antifungal susceptibility testing on *C. albicans*

The susceptibility of *C. albicans* to EEP was determined according to the Clinical and Laboratory Standards Institute (CLSI) M11-A8 standard broth microdilution method (CLSI, 2012). Briefly, two-fold serial dilutions of EEP (6.25-400 µg/mL) were mixed in a 1:1 ratio

with fungus suspensions that were adjusted to the final concentration of 2.5 x 10^3 cells/mL in 96-well cell culture plates (Costar 3595, Corning, Kennebunk, ME, USA), and incubated for 48 h at 35°C. The solvent concentration was kept constant (1%) in the system. The growth proportional absorbance of the suspensions was measured at 595 nm with a plate reader (Thermo Multiskan EX, Berlin, Germany). The minimum inhibitory concentrations (MIC) values were determined as the lowest concentration at which 90% growth inhibition occurred.

3.4.2. Survival rate determination and adaptation assay

The adaptation assay aimed to determine the effect on survival rates of *C. albicans* cells in case of pre-treatment with a subinhibitory concentration of EEP during the subsequent high exposure to propolis extracts. Fungal culture was grown overnight at 30°C in YPD broth. The cells were harvested by centrifugation at 3000 rpm for 5 min (Hettich, Rotina 420R, Auro-Science, Budapest, Hungary) and diluted to10^7 cells/mL. Pre-treatment was performed with 50 μg/mL EEP at 30°C and 150 rpm for 1 h in an orbital shaker. One part of this cell suspension (referred to as a pre-treated control) was further diluted and plated onto YPD agar plates. The remaining aliquot was split in half and exposed to 200 and 400 μg/mL EEP. After incubation for 48 h at 30°C, the number of colony-forming units was estimated, and the percentage of survival was recorded. To test the adaptation hypothesis, and to determine the minimal fungicidal concentration (MFC) an identical experimental setup was carried out without the pre-treatment procedure.

3.4.3. Antibacterial susceptibility testing of *S. aureus*

S. aureus strains were tested for their susceptibility to EEP1 sample, oxacillin (Sigma-Aldrich, Darmstadt, Germany), cefoxitin (Sigma-Aldrich, Darmstadt, Germany), and vancomycin (Sigma-Aldrich, Darmstadt, Germany) using standard broth microdilution method (CLSI, 2012). Briefly, the density of the bacterial cells was adjusted to a final concentration of 10^5 CFU/mL in Muller Hinton broth (MHB) (Biolab, Budapest, Hungary). The cell suspension was mixed in 1:1 ratio with two-fold serial dilutions of EEP1 (12.5–100 μg/mL), oxacillin (0.125–8 μg/mL), cefoxitin, and vancomycin (0.25–16 μg/mL), severally into 96-well cell culture microtiter plates (Costar 3599, Corning, Kennebunk, USA). The concentration of 80% (v/v) ethanol (solvent of propolis) was kept constant (1%) in each well. The culture was incubated at 35°C for 20–24 h. The absorbance of the growth was measured at 595 nm using a Thermo Multiskan EX plate reader (Berlin, Germany). The minimum inhibitory concentration

(MIC) value was determined as the lowest concentration at which 90% growth inhibition occurred.

3.4.4. Chequerboard broth microdilution method

The broth microdilution chequerboard method was used to study the possible synergistic effect between EEP1 with selected antibiotics (oxacillin, cefoxitin, and vancomycin). Briefly,100 µL of bacterial suspension adjusted to 10^8 CFU/mL was distributed into a 96-well microtiter plate (Costar 3599, Corning, Kennebunk, USA) containing 50 µL of two-fold serial dilutions of EEP1 (3.13–400 µg/mL) and 50 µL of selected antibiotics (0.03–16 µg/mL). The plate was incubated at 35°C for 20–24 h. The absorbance of the growth was measured at 595 nm using a Thermo Multiskan EX microtiter plate reader (Berlin, Germany). A calculation matrix was created to convert the absorbance to percentages of the growth. The type of interaction between the EEP1 and the selected antibiotics was defined by the calculation of the fractional inhibitory concentration index (FICI). The FICI was computed according to the following equations:

FICI = (MIC value of the selected antibiotic in combination/MIC value of the selected antibiotic alone) + (MIC value of EEP1 in combination/MIC value of EEP1 alone).

The combination effect of antibiotics with EEP1 was considered, as synergistic when FICI ≤ 0.5, as additive when 0.5 < FICI < 1, indifferent when 1 ≤ FICI < 4, and antagonistic when FICI > 4 (AL-Ani et al., 2018).

3.5. Effect of propolis extract on virulence factors

3.5.1. Effect of propolis extract on biofilm formation of *C. albicans*

The effect of EEP1 and EEP2 on biofilm formation and eradication was investigated in 96-well flat-bottom microtiter plates (Sarstedt REF833934500, Numbrecht, Germany) on the two strains of *C.albicans* (ATCC 44829 and SZMC 1424). Briefly, equal volumes of an inoculum of 2.5 x 10^3 cell/mL were added to equal volumes of a series of two-fold dilutions of EEP to get final concentrations ranging from 3.125 to 200 µg/mL. The plates were incubated for 48 h at 35°C to develop biofilms. The biofilms were washed with PBS, then fixed for 20 min with formalin. Fixed biofilm was stained with 0.13% crystal violet solution for 20 min. SDS solution (0.5% (w/v) SDS, 50% (v/v) PBS and 50% (v/v) ethanol) was applied to dissolve the crystal violet bonded to the biofilm. The absorbance of the dye which is proportional to the

thickness of the biofilm was measured by a microtiter plate reader (Thermo Multiskan EX, Berlin, Germany) at 595 nm.

3.5.2. Effect of propolis extract on biofilm formation of *S. aureus*

For testing the effect of EEP1 on the biofilm of *S. aureus*, the cell number of an exponential-phase culture was adjusted to 10^3 cells/mL into TSB supplemented with 0.25% (w/v) glucose. The cell suspension was treated with two-fold serial dilutions of EEP1 (12.5–200 μg/mL) in the final volume of 200 μL (1:1, v/v) using 96-well microtiter plates (Sarstedt, REF 833934500, Numbrecht, Germany), and incubated at 37°C for 24 h. The cells were washed three times with 200 μL sterile PBS (pH 7.2), then the plates were left to dry at room temperature. The biofilm was fixed with 100 μL of 99% (v/v) methanol for a 15 min incubation. For the quantification of biofilm biomass, the dried biofilm was stained with 200 μL of 0.13% (w/v) crystal violet for 15 min. The unbound dye was removed by washing three times with 200 μL of PBS. The crystal violet dye was eluted with 200 μL of 33% (v/v) acetic acid glacial to solubilize the biofilm-bound dye by incubating for 15 min. The absorbance of biofilm biomass was measured at 595 nm using a Thermo Multiskan EX plate reader (Berlin, Germany). The absorbance of an inoculated well without propolis treatment served as a positive control and the absorbance of an uninoculated well served as a negative control. The minimum biofilm inhibitory concentration (MBIC) was defined as the lowest concentration that inhibited at least 90% biofilm formation. Then the cut-off value (ODc) was established; ODc = average OD of negative control + (3 × standard deviation (SD) of negative control); OD = average OD of a strain subtracted from ODc. For the interpretation of the results, strains were divided into the following categories: $OD \leq ODc$ = not biofilm-former, $ODc < OD \leq 2 \times ODc$ = weak biofilm-former, $2 \times ODc < OD \leq 4 \times ODc$ = moderate biofilm-former, $4 \times ODc < OD$ = strong biofilm-former (Miao et al., 2019; Stepanović et al., 2007).

3.5.3. Effect of propolis extract on the eradication of mature biofilm of *C. albicans* and *S. aureus*

The evaluation of biofilm eradication of strong biofilm former strain *C. albicans* SZMC 1424 was carried out as earlier described for fungi biofilm formation assay (See Section 3.5.1.), except inoculum were allowed to grow initially for 24 h aiming biofilm formation prior to the addition of the EEP1 or EEP2 treatments (Freires et al., 2016).

The ability of EEP1 to eradicate the 24 h-old biofilm of *S. aureus* was determined as previously mentioned in the biofilm formation and quantification assay (See Section 3.5.2.). The cell suspension was incubated for 24 h at 37°C without EEP. Then, the supernatants were removed and the wells were treated with EEP1 (12.5–200 µg/mL) for 16 h at 37°C. The planktonic cells were discarded and only the tightly attached biofilm was stained with crystal violet, resazurin, and propidium iodide (PI) to quantify the biofilm biomass, metabolic activity, and cellular death, respectively. The crystal violet assay for the quantification of biofilm biomass was mentioned in Section 3.6. To quantify the metabolic activity of the cells present within the mature biofilm, the wells were labelled with 1 µM resazurin solution (200 µL) in dark for 30 min. The metabolic activity is proportional to the rate of resazurin reduction that was determined by measuring the fluorescence at ($\lambda_{Ex/Em}$= 560/590 nm). The dead cells were determined by treating the mature biofilm with 200 µL of 20 µM PI in the dark for 15 min. The PI is an intercalating fluorescent agent, binding of PI to DNA causes a redshift of the excitation maximum to 540 nm and the emission maximum to 640 nm. The fluorescence measurements were determined using a PerkinElmer EnSpire multimode plate reader (Auro-Science Consulting Ltd., Budapest, Hungary). The fluorescence values for resazurin and PI were converted to percentages. Then, the percentage was calculated by supposing the positive control as 100% metabolically active cells (fluorescence of the cells in the presence of EEP1/fluorescence of the cells in the absence of EEP1 × 100) and 0% dead cells ([fluorescence of the cells in the presence of EEP1/fluorescence of the cells in the absence of EEP1 × 100] − 100). Furthermore, the minimum biofilm eradication concentration ($MBEC_{50}$) was computed as the lowest concentration that eradicates at least 50% of biofilm (Miao et al., 2019).

3.5.4. Effect of propolis extract on germ tube formation (GTF)

To determine the effect of propolis extracts on the germ tube formation of the two *C. albicans* strains (ATCC 44829 and SZMC 1424), exponential growth phase cultures were washed and 10^7 cells/mL cells were suspended in 1 mL of horse serum supplemented with 50 µg/mL adenine (Sigma-Aldrich, Darmstadt, Germany). The cells were treated with 50 and 200 µg/mL of EEP. Untreated cells were applied as the negative control. The cultures were incubated for 30, 60, 120, and 180 min at 37°C in an orbital shaker at 150 rpm (Kim et al., 2007). The GTF was quantified under 400× magnification of a light microscope (Nikon Eclipse 80i, Tokyo, Japan).

3.6. Mechanism of action of EEP on *C. albicans*

3.6.1. Cellular uptake of EEP

The decrease in the concentration of propolis in cell suspension was measured at different time points. The suspensions of 10^7 cells/mL exponentially growing cultures of *C. albicans* ATCC44829 in MM media were incubated in an orbital shaker at 30°C at 150 rpm in the presence of 200 μg/mL EEP1. After 5, 10, 15, 30, 60, 120, 180 and 240 min samples were taken and the cells were removed by centrifugation at 3000 rpm, for 5 min. The absorbance of the supernatants was measured at the absorption maximum of propolis extract at 295 nm with a spectrophotometer (Hitachi U-2910, Tokyo, Japan). The absorbance of two-fold serial dilutions (3.125-200 μg/mL) of EEP1 at the same wavelength was measured to obtain a calibration curve.

3.6.2. Effect of propolis on cell wall

To determine the cell wall damaging effect of EEP1 on *C. albicans* ATCC 44829, two-fold serial dilutions of EEP1 (12.5-400 μg/mL) were applied in the presence and absence of 0.8 M D-sorbitol anhydrous (Sigma-Aldrich, Darmstadt, Germany) an osmotic protector, based on the CLSI M11-A8 standard broth microdilution method (Ferreira et al., 2014).

3.6.3. Effect of propolis on cell membrane

To detect the effect of EEP1 on membrane permeability, the exponentially growing *C. albicans* ATCC44829 cells were cultured in YPD medium until the late logarithmic phase. The cells were washed and resuspended in Sorensen's buffer (pH 6.5) and the cell number was adjusted to 10^8 cells/mL. Cells were treated with 100, 200, and 400 μg/mL EEP1, untreated cells were applied as the negative control. After 0, 1, 2, 4, 6 hours treatment the cells were harvested by centrifugation (3000 rpm, 5 min) and the absorbance of the supernatants was measured at 260 nm with a spectrophotometer (Hitachi U-2910, Tokyo, Japan) (Gucwa et al., 2018; Horváth et al., 2010).

For the membrane protection assay, the susceptibility of *C. albicans* ATCC 44829 to EEP1 (200, 400, and 600 μg/mL) was determined following microdilution technique as earlier described in the CLSI guidelines M11-A8 (CLSI, 2012) in the presence of 50, 100, and 200 μg/mL ergosterol (Sigma-Aldrich, Darmstadt, Germany). The concentrations 0.5, 1.0, and 1.5

μg/mL amphotericin B (AmB) (Sigma-Aldrich, Darmstadt, Germany) were used as positive control (Ferreira et al., 2014).

3.6.4. DNA fragmentation and nuclei damage

In order to detect genomic DNA (gDNA) fragmentation of *C. albicans* ATCC44829, 10^7 cells/mL were treated with 50 and 200 μg/mL EEP1 for 1 h at 35°C in MM medium. Untreated cells were applied as negative control, the treatment with 5 mM H_2O_2 was applied as the positive control (Park and Lee, 2010). The DNA was extracted according to the modified method of Suman et al. (2012). Briefly, the cells were harvested and resuspended in TE buffer (10 mM Tris base, pH 8.0, 1 mM EDTA, 0.5% (v/v) SDS) then disrupted in liquid nitrogen. The phenol-chloroform method was applied for the DNA purification and precipitation. The DNA was eluted in TE buffer (pH 8.0). The Agilent 4200 TapeStation system (Agilent Technologies, Santa Clara, CA, US) and the Agilent Genomic DNA ScreenTape assay was used to determine the fragmented DNA content, according to the manufacturer's instructions.

The same samples that were used for DNA fragmentation were applied for the detection of nuclei damage. After the treatment, the cells were washed and incubated with 10 μg/mL DAPI (4′,6-diamidino-2-phenylindole) (Sigma-Aldrich, Darmstadt, Germany) for 10 min in dark. For the quantitative assessment of damaged nuclei at least 300 stained cells per sample were computed under fluorescence microscope (Nikon Eclipse 80i, Tokyo, Japan) at ($\lambda_{ex/em}$= 358/461 nm).

3.7. Statistical analysis

All experiments were carried out in triplicates, the data were presented as mean ± standard deviation (SD). Data were analysed by analysis of variance (ANOVA) for multiple comparisons between the groups, followed by Dunn's post hoc test. Analysis results and graphics were made using OriginPro 2016 and Past 3.1 software. Differences between samples were considered significant when $p < 0.05$ (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$).

4. Results

4.1. Chemical characterization of propolis samples

4.1.1. Determination of the total phenolics and flavonoids contents

In this study, colorimetric assays using the Folin-Ciocalteu, and aluminium chloride reagents were used for the determination of phenolic and flavonoid contents, respectively in the Hungarian propolis samples. The TPC and TFC of the EEP samples were measured using the gallic acid and catechin standards, respectively (Table 2). The TPC values were in the range of 10.4-71.1 mg GAE/g, and TFC values were in the range of 33.8-273.2 mg CE/g. The EEP1 sample represented the highest concentration of phenolic and flavonoids and showed 6.8 to 8.1 times higher contents than those of the EEP6 sample, which showed the lowest values of TPC and TFC among the studied samples. Since all the samples were collected and extracted by the same method, the diversity of the vegetation between the regions is likely the reason for the significant difference in the phenolic and flavonoids contents.

Table 2: Expression of total phenolic contents (TPC) and total flavonoids contents (TFC) in milligrams of gallic acid equivalents per gram of propolis (mg GAE/g), and milligrams of catechin equivalent per gram of propolis (mg CE/g), respectively.

Propolis samples	TPC (mg GAE/g) ± SD	TFC (mg CE/g) ± SD
EEP1	71.1 ± 4.3 ^{a}	273.2 ± 10.2 ^{a}
EEP2	55.8 ± 2.0 ^{b}	172.8 ± 11.5 ^{b}
EEP3	47.9 ± 0.2 ^{c}	164.1 ± 2.7 ^{b,c}
EEP4	44.0 ± 0.5 ^{c}	142.5 ± 4.2 ^{c,d}
EEP5	34.6 ± 1.0 ^{d}	147.3 ± 12.9 ^{c,d}
EEP6	10.4 ± 1.4 ^{e}	33.8 ± 3.0 ^{e}

EEP1-EEP6: Ethanolic extracts of propolis samples 1-6. $^{a–e}$ Different letters indicate significant differences between the regions within the same column ($p < 0.05$). Values represent mean ± SD (n= 3).

4.1.2. Spectrophotometric analysis of UV-visible spectra

The samples collected from six different regions showed 3 peaks at the same wavelengths, λ_1= 271 nm± 8.5, λ_2= 293 nm± 0.5, and λ_3= 325 nm± 2.3 (Appendix, Table 3). All propolis samples have the same pattern with a maximum absorbance at λ_{max}= 293 nm. EEP1 exhibited the highest absorption compared to the other EEP spectrums. However, the EEP6 showed significantly lower absorption (Figure 6). Therefore, the results may indicate a similarity in the quality of the chemical compounds that constitute all of propolis samples but in different quantities.

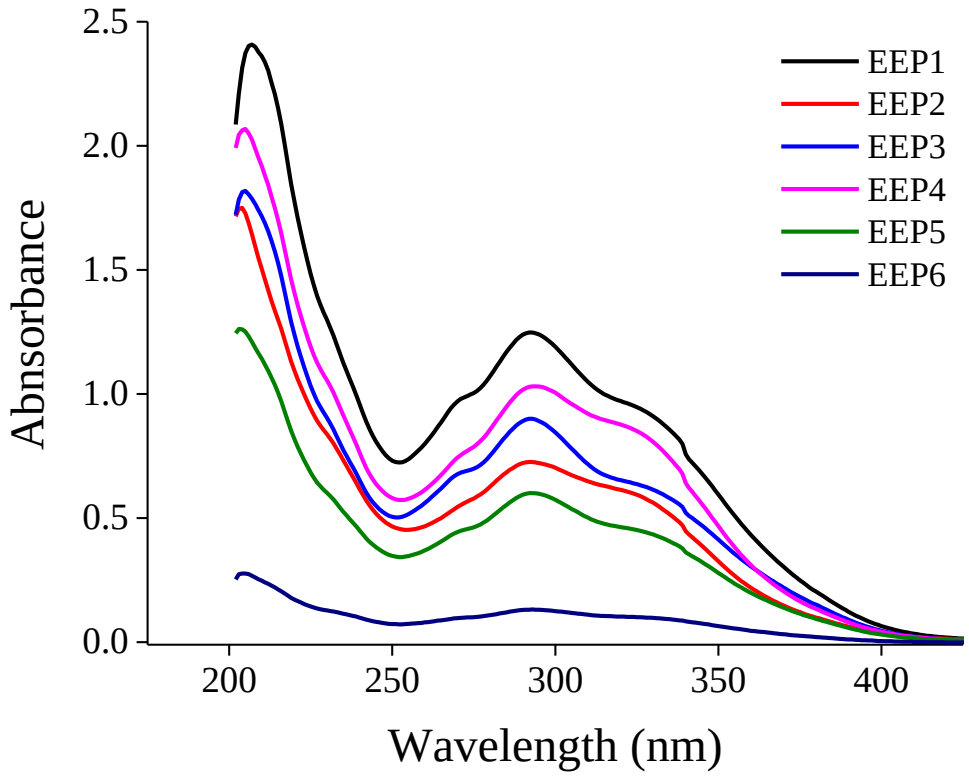

Figure 6: Absorption UV-Vis spectra of propolis extracts. All samples have the same pattern. Most of EEP samples showed strong absorption in the region between 250 and 350 nm, with a very intense peak at 293 nm and shoulders at 271 and 325 nm.

4.1.3. Chemical constituents of propolis extracts

The biological activities of EEP samples are highly dependent on their chemical composition. In the current study, the chemical compositions of Hungarian propolis ethanolic extracts were analysed using GC-MS. The components were identified according to the retention indices, the MS data were compared with databases.

Table 3: Retention time (RT), retention indices (RI), and the relative concentrations (percentage of the area under the peak) of the major chemical components (above 0.5% presence) in EEP samples.

Compounds	RT	RI	EEP1	EEP2	EEP3	EEP4	EEP5	EEP6
Methoxyethanol	4.5	746	0.15	0.11	0.26	0.24	0.28	0.73
Glycerol	16.3	1249	0.35	0.33	0.99	0.19	0.84	1.14
Benzoic acid	16.4	1253	3.49	6.76	5.04	2.54	3.20	1.49
Butanedioic acid	22.0	1466	0.09	0.99	0.67	0.31	0.68	0.47
Vanillin	24.0	1548	0.25	0.75	0.23	0.42	0.13	0.07
Furanacetaldehyde	28.0	1720	0.08	0.10	0.08	0.24	0.05	1.44
Glucose	28.8	1755	0.03	0.05	0.04	0.03	0.09	0.56
Fructofuranose	29.3	1777	1.31	2.73	3.43	3.31	3.55	16.46
Cinnamic acid	30.5	1832	1.92	2.07	1.61	1.30	1.99	0.53
Glucopyranose	31.3	1870	0.48	1.40	1.63	2.88	1.58	6.43
Propenoic acid	31.8	1893	0.33	0.60	0.28	0.36	0.40	0.08
Eudesmol	31.9	1900	1.94	3.38	1.22	1.05	1.67	0.40
Coumaric acid	32.8	1943	1.70	4.38	1.43	3.19	1.64	0.67
Dimethoxycinnamic acid	34.5	2029	2.04	2.31	1.63	1.10	2.13	0.91
Palmitic Acid	34.7	2039	0.37	0.25	0.36	0.29	0.49	1.21
Isoferulic acid	35.3	2074	1.44	3.26	1.10	1.01	1.64	0.48
Cinnamate	36.4	2129	6.69	8.26	6.94	8.94	5.98	3.36
***p*-coumarate**	37.1	2170	0.87	1.03	0.66	0.72	0.89	0.27
Caffeic acid	37.5	2187	5.61	3.87	3.47	4.63	4.55	2.19
Farnesol	37.8	2205	0.54	0.26	0.87	0.43	0.53	0.12
Octadecynoic acid	42.3	2472	0.56	1.02	0.40	0.49	0.50	0.11
Benzetriol	43.6	2557	1.12	2.33	2.32	1.76	1.82	0.74
Sucrose	44.3	2603	0.38	0.79	0.96	5.48	0.71	10.53
Trehalose	46.3	2698	0.09	0.12	0.17	0.15	0.20	1.36
Ethyl gallate	46.6	2712	8.93	7.94	5.56	7.73	6.58	5.71
Genistein	46.8	2723	23.30	17.36	24.44	18.77	21.86	13.83
Chrysin	47.4	2751	26.02	18.90	24.86	25.45	26.64	19.01
Lignoceric acid	48.4	2802	0.73	0.81	1.01	0.62	0.67	0.91
Hexadecane-1,2-diol	49.9	2875	0.53	0.18	0.47	0.11	0.26	0.45
Isorhamnetin	58.2	2823	3.34	1.57	2.76	1.75	1.93	1.40

The chemical composition amounts are presented by the relative concentration (Table 3), which is proportional to the area under the peak for each component obtained in the chromatogram. A total of 122 individual compounds and derivatives were identified in all EEP samples, while the EEP1 and EEP2 samples comprised 114 different compounds, the EEP3, EEP4, EEP5, and EEP6 samples consisted of 110, 101, 116, and 85, respectively. However,

thirty compounds that showed more than 0.5% of the area under the peak were highlighted (Table 3), these compounds covered between 93-96% of the total amount of chemicals. The identified components were classified into different groups according to their chemical characteristics such as alcohols, terpenes, aromatic acids, cinnamic acids, fatty acids, flavonoids, phenols, and polysaccharides (Figure 7). The compounds that showed high amounts belong to the category of flavonoids and isoflavonoids with a total relative concentration of 52.9% to 34.5%. The identified flavonoids were represented mainly in chrysin and genistein with concentrations ranging from 18.9 to 26% for chrysin and from 13.8 to 23% for genistein. The second-largest quantity was for the category of phenols (including cinnamic acids and cinnamate ester) in all of EEP1, EEP2, EEP3, EEP4, and EEP5 samples. However, the monosaccharaides and the sugars were present in considerable amounts (38%) in the sample EEP6 (Figure 7).

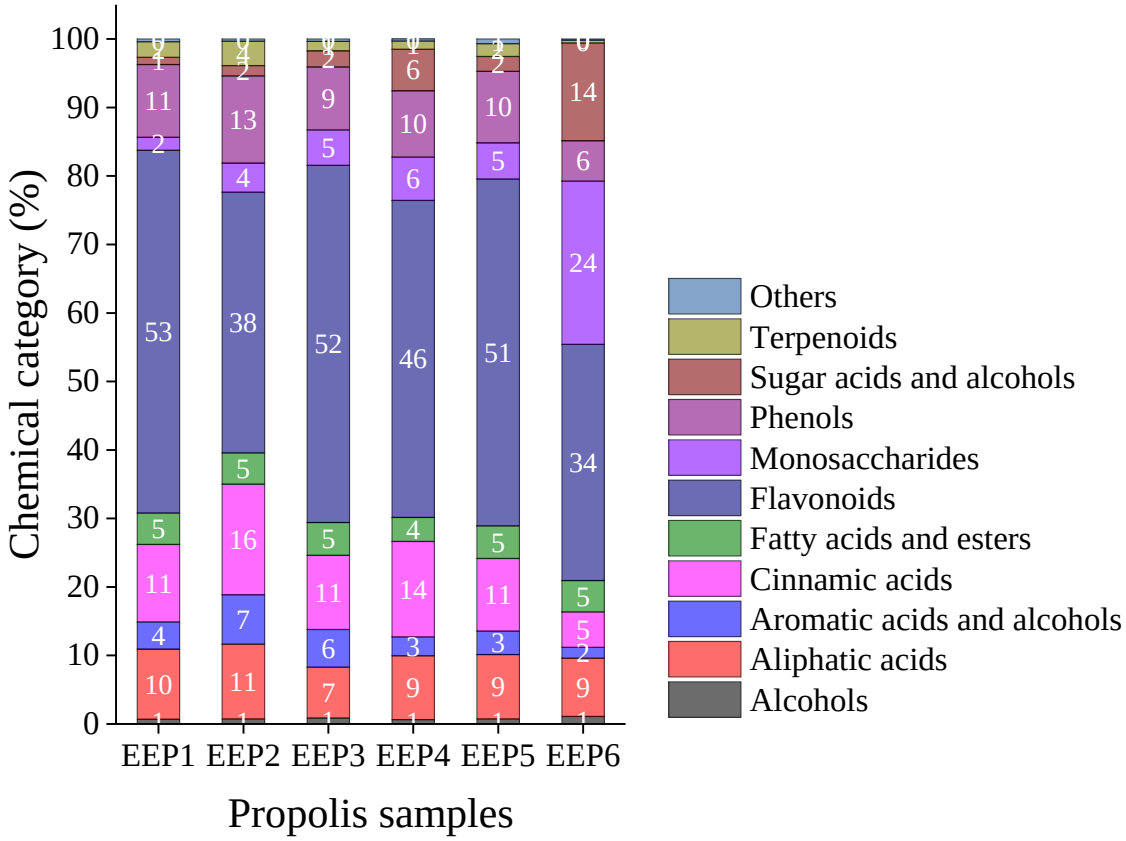

Figure 7: Chemical composition of six ethanolic extracts of propolis (EEP) using GC-MS analysis. The columns show the relative concentrations (%) of chemicals grouped into 11 categories for each propolis sample.

4.2. Antimicrobial effect of propolis

4.2.1. Antifungal activity

The cytotoxicity of EEP was characterized by determining the susceptibility of the *C. albicans* cells after 48 h incubation with the treatment using the microdilution method. All the extracts showed concentration-dependent susceptibility. All the extracts were potent on *C. albicans* cells, the MIC values were in the range of 100-200 µg/mL, except the sample of EEP6 that was significantly weaker compared to the other samples (Figure 8).

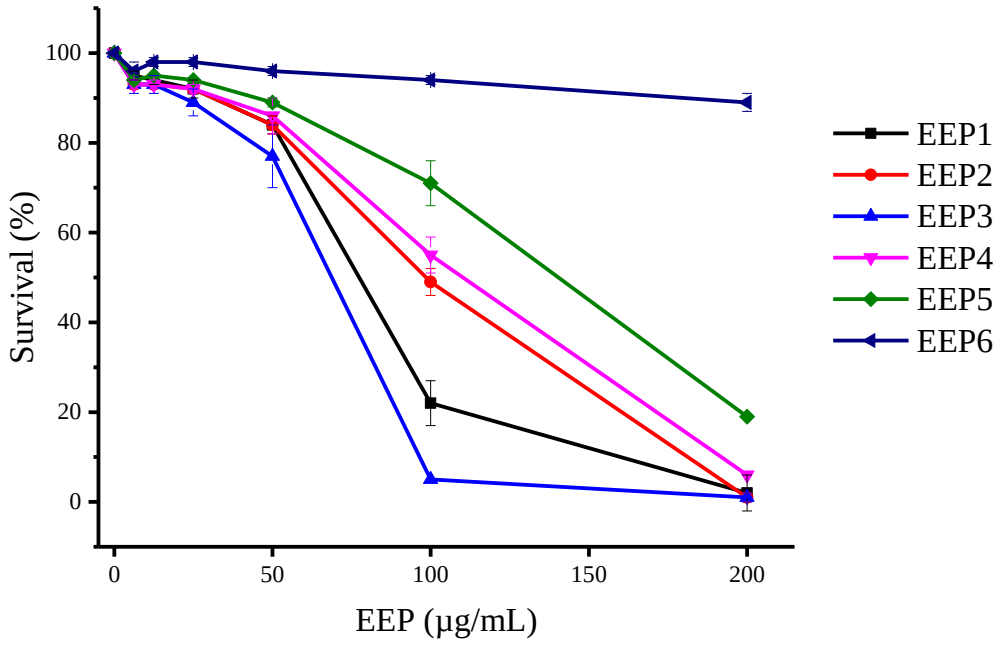

Figure 8: The survival rate of *C. albicans* ATCC 44829 on exposure to 6.25-400 µg/mL of ethanolic extracts of propolis (EEP) after 48 h incubation at 35°C, using the broth microdilution method. The absorbance was measured at 595 nm with a 96-well plate reader spectrophotometer.

4.2.2. Ethanolic extracts of propolis induced cytotoxicity and adaptation

In time-dependent inhibition assay, the colony-forming units (CFU) of *C. albicans* ATCC 44829 cells were examined after exposure of the cells to MIC and 2 × MIC of EEP1 for 30 and 60 min. The CFU values were decreased by 78% and 98% after 30 and 60 min treatment at 200 µg/mL, respectively (Figure 9A). Therefore, the MFC value of EEP1 was equal to 200

µg/mL. Nevertheless, the pretreatment of cells with a subinhibitory concentration (50 µg/mL) of EEP1 for 1 h caused a considerable increase in the survival rate after the re-treatment of cells with the same concentrations of EEP1 for another 1 h. The colony-forming ability increased by 17% and 11% at 200 and 400 µg/mL EEP1, respectively. An adaptive response to EEP1 was demonstrated at the cell level, although the increase in cell growth was statistically significant ($p < 0.05$) only with 2 × MIC after 30 min of incubation (Figure 9B).

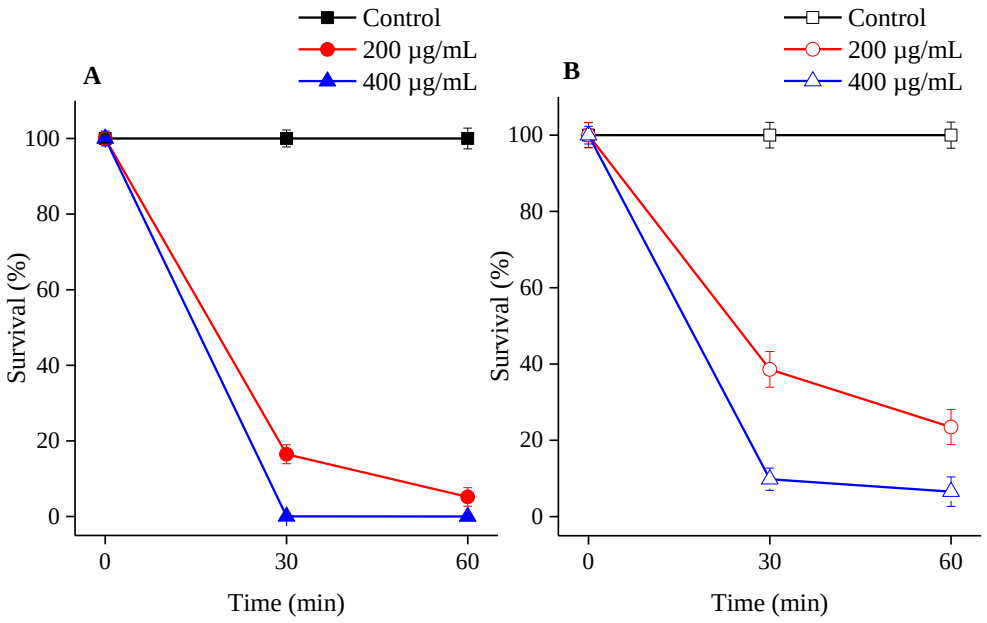

Figure 9: The survival rates of *C. albicans* ATCC 44829 in the presence of 200 and 400 µg/mL of EEP1 for 30 and 60 min (A), and the survival rates of pre-treated cells with 50 µg/mL EEP1 for 60 min (B). The cells were cultured on an agar plate for 48 h at 30°C, the determined number of colony-forming units was converted to the percentage of survival.

4.2.3. Antibacterial activity

An antibiotic susceptibility test was performed against the *S. aureus* strains using oxacillin, cefoxitin, and vancomycin. The two MRSA clinical isolates (SA H23 and SA H24) were found resistant to oxacillin, and cefoxitin but sensitive to vancomycin at 1 µg/mL (Figure 10). The reference *S. aureus* ATCC 29213 (MSSA) strain was susceptible to the above antibiotics and showed MIC at 0.25 µg/mL, 4 µg/mL, and 1 µg/mL, for oxacillin, cefoxitin, and vancomycin, respectively. The reference *S. aureus* ATCC 700699 (MRSA) strain was found resistant to oxacillin, and cefoxitin. However, this strain showed intermediate resistance to vancomycin with MIC equal to 8 µg/mL. In addition, the anti-staphylococcal activity of the

EEP1 sample was tested. All the strains were very sensitive to EEP1 with a MIC values at 50 µg/mL.

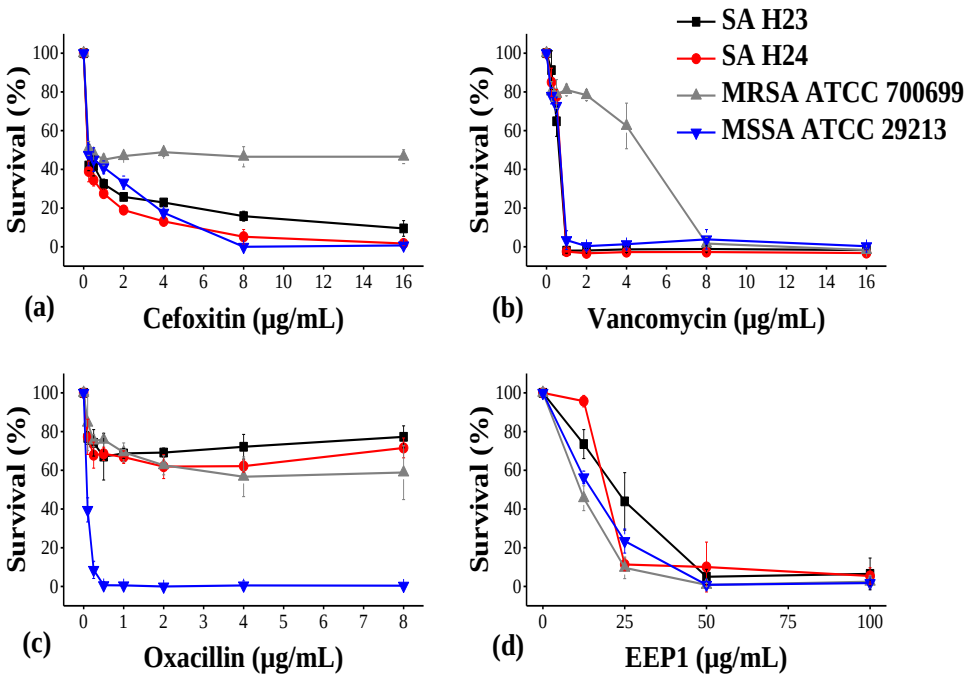

Figure 10: Inhibitory effect of antibiotic drugs: (a) cefoxitin (0.25–16 µg/mL); (b) vancomycin (0.25–16 µg/mL); (c) oxacillin (0.125–8 µg/mL); and (d) EEP1 (12.5–100 µg/mL) on *S. aureus*; clinical isolates (SA H23 and SA H24), and reference strains methicillin-susceptible *S. aureus* ATCC 29213 (MSSA) and methicillin-resistant *S. aureus* ATCC 700699 (MRSA). According to the broth microdilution method, the culture was incubated at 35°C for 20–24 h. The absorbance of the growth was measured at 595 nm using a plate reader.

4.2.4. Killing effect of EEP in combination with antibacterial drugs

MRSA, SA H23 and SA H24 strains were resistant to oxacillin, and neither MIC_{50} nor MIC_{80} were observed at 10^5 nor 10^8 CFU/mL. MIC_{50-80} of oxacillin for MSSA was measured at 0.125-0.25 µg/mL at 10^5 CFU/mL and 1-4 µg/mL at 10^8 CFU/mL. The results of EEP1 showed that MIC_{80} equals 25 µg/mL in all the strains at 10^5 CFU/mL, while it was increased significantly (50 µg/mL to more than 200 µg/mL) at 10^8 CFU/mL. As indicated in Table 4, the FICI values of chequerboard microdilution result of two-drug combinations between EEP1 and antibiotics (oxacillin, cefoxitin, and vancomycin) demonstrated synergistic combinations with all the antibiotics against MSSA, while the resistant strains were shown synergistic effect only

with the vancomycin. However, The MICs of all the antibiotics showed a significant reduction in case of interaction with propolis on all the strains, except the interaction of oxacillin and EEP on the strain SA H23 which was indifferent. However, these concentration combinations did not show complete inhibition due to the high cell number (Table 4).

Table 4: The MIC of EEP and antibiotics alone and in combination on *S. aureus* (at inoculum of 10^8 CFU/mL); clinical isolates (SA H23 and SA H24), and reference strains methicillin-susceptible *S. aureus* ATCC 29213 (MSSA) and methicillin-resistant *S. aureus* ATCC 700699 (MRSA), and the type of interaction according to fractional inhibitory concentration index (FICI), (N) not calculated.

S. aureus strains	**Drugs**	**MIC (µg/mL)**		**FICI (type of interaction)**
		Alone	**In combination**	
SA H23	EEP1	200	< 200	**N**
	Oxacillin	< 4	< 4	
	EEP1	200	200	**N**
	Cefoxitin	< 8	0.13	
	EEP1	200	3.13	**0.03 (synergistic)**
	Vancomycin	2	0.03	
SA H24	EEP1	200	200	**N**
	Oxacillin	< 4	0.06	
	EEP1	200	200	**N**
	Cefoxitin	< 8	0.13	
	EEP1	200	3.13	**0.03 (synergistic)**
	Vancomycin	2	0.03	
MSSA	EEP1	100	25	**0.27 (synergistic)**
	Oxacillin	4	0.06	
	EEP1	100	3.13	**0.05 (synergistic)**
	Cefoxitin	2	0.03	
	EEP1	100	3.13	**0.05 (synergistic)**
	Vancomycin	2	0.03	
MRSA	EEP1	400	400	**N**
	Oxacillin	< 4	0.06	
	EEP1	400	200	**N**
	Cefoxitin	< 8	0.13	
	EEP1	400	6.25	**0.05 (synergistic)**
	Vancomycin	4	0.13	

4.3. Effect of propolis extract on fungal and bacterial virulence factors

4.3.1. Biofilm formation of *C. albicans*

The crystal violet analysis method was performed to evaluate the activity of EEP1 and EEP2 on the biofilm of *C. albicans* ATCC 44829 and SZMC 1424. The two strains showed different degrees of biofilm formation. Although *C. albicans* ATCC 44829 was a weak biofilm former, its biofilm formation was significantly blocked at concentrations of 12.5 µg/mL EEP1. *C. albicans* SZMC 1424 showed strong biofilm formation ability. However, it was inhibited significantly by 92% at 3.125 µg/mL EEP1 ($p < 0.001$).

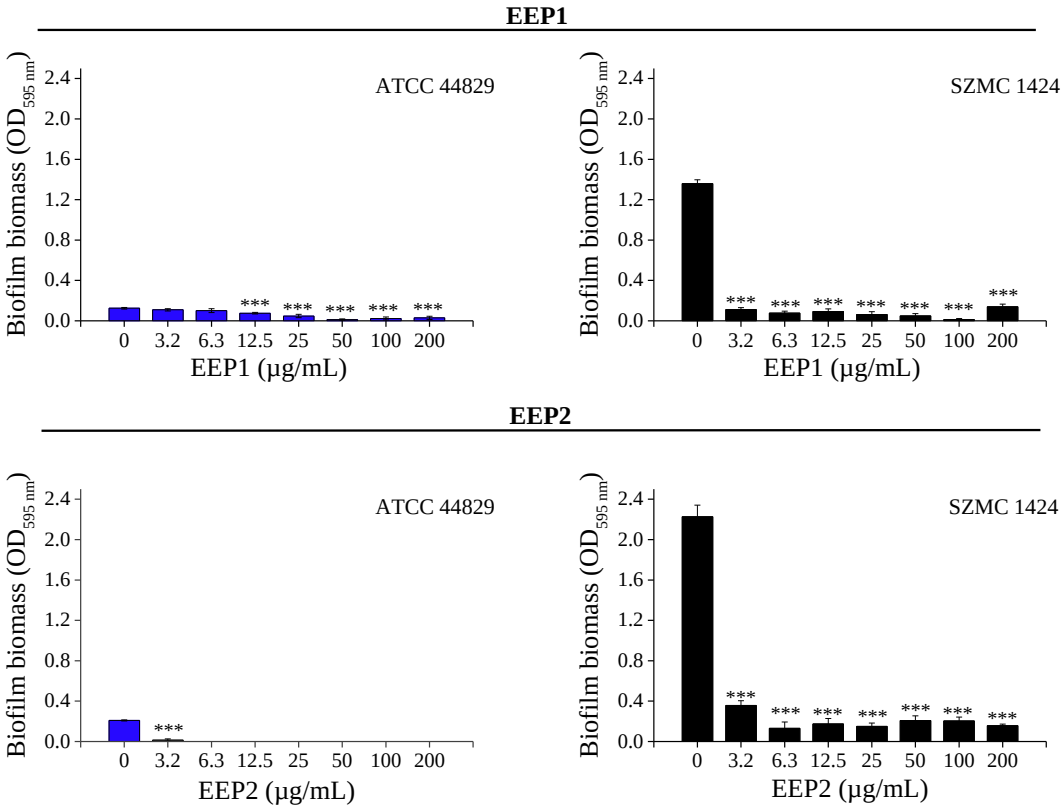

Figure 11: The biofilm formation of *C. albicans* strains (ATCC 44829 and SZMC 1424) in the presence of 3.125-200 µg/mL of propolis extracts (EEP1 and EEP2) and the absence of propolis extracts (control) after 48 h incubation at 37°C, using crystal violet assay, the absorbance of biofilm biomass was measured by 96-well plate reader spectrophotometer.

In the case of the EEP2 sample, the biofilm of *C. albicans* ATCC 44829 was completely inhibited above 3.125 µg/mL. However, the biofilm biomass of the strong biofilm former isolate (*C. albicans* SZMC 1424) was significantly reduced by 84% in the presence of 3.125

μg/mL EEP2. Although propolis is effective in biofilm inhibition, the complete prevention of strong biofilm former by EEP2 was not observed even at high concentrations (Figure 11).

4.3.2. The eradication of *C. albicans* mature biofilm

It is hypothesized that the eradication of mature biofilms is more difficult than inhibiting planktonic cells. The challenge of EEP1 and EEP2 to destroy the mature biofilms was examined on the *C. albicans* SZMC 1424 (strong biofilm former strain). The two propolis extracts showed different biofilm degradation abilities. In the case of EEP1, the biofilm thickness was effectively reduced in the range of 6.25-50 μg/mL EEP1. On the other hand, the 100 and 200 μg/mL EEP1 did not degrade the biofilm biomass. However, the treatment of the same strain with EEP2 did not reduce the biofilm biomass but on the contrary significantly promoted it by 28-43% in the range of 50-200 μg/mL compared to the positive control (Figure 12).

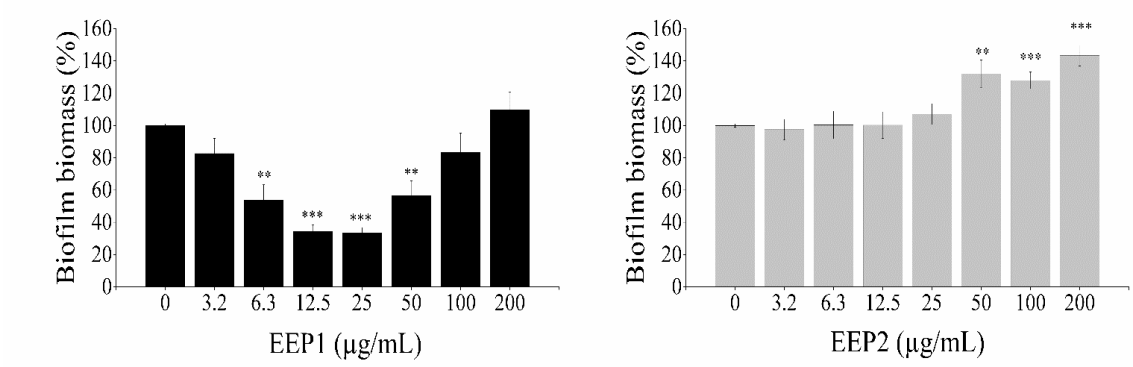

Figure 12: Effect of propolis extracts (EEP1 and EEP2) at the concentrations of 3.125-200 μg/mL on preformed biofilms of *C. albicans* SZMC 1424. The cells were cultivated for 24 h at 37°C to form biofilms, then the mature biofilms were treated with propolis extracts for an additional 24 hours. Using the crystal violet assay, the absorbance of biofilm biomass was measured by 96-well plate reader spectrophotometer.

4.3.3. Effect of propolis extract on germ tube formation (GTF)

Germ tube formation is one of the fungal mechanisms that enhance the adherence of the pathogen to the host. Therefore it could be an important therapeutic target for antifungal agent research. Germ tube formation assay is a diagnostic test in which the fungal cells are suspended in horse serum to induce germination at 37°C, and observed by microscope at defined time points. The examination was performed to investigate the germ tube formation ability of *C. albicans* strains (ATCC 44829 and SZMC 1424) in the presence of EEP1 and EEP2 at the subinhibitory concentration (50 μg/mL) and MIC (200 μg/mL). After 30 min of

treatment, no germination appeared on the cells. Compared to the positive control after 1 h exposure, the GTF of the *C. albicans* ATCC 44829 was significantly suppressed by 50% and 77% in the presence of 50 and 200 µg/mL of EEP1, respectively. However, after 3 h treatment, the reduction in the GTF was decreased by 30% and 51%. The strain *C. albicans* SZMC 1424 showed slightly higher resistance to the EEP1 compared to *C. albicans* ATCC 44829, since a significant reduction of its germination was only shown after 1 h and 3 h with 200 µg/mL. Additionally, there was no significant effect observed after 2 h of treatment either of the propolis extract.

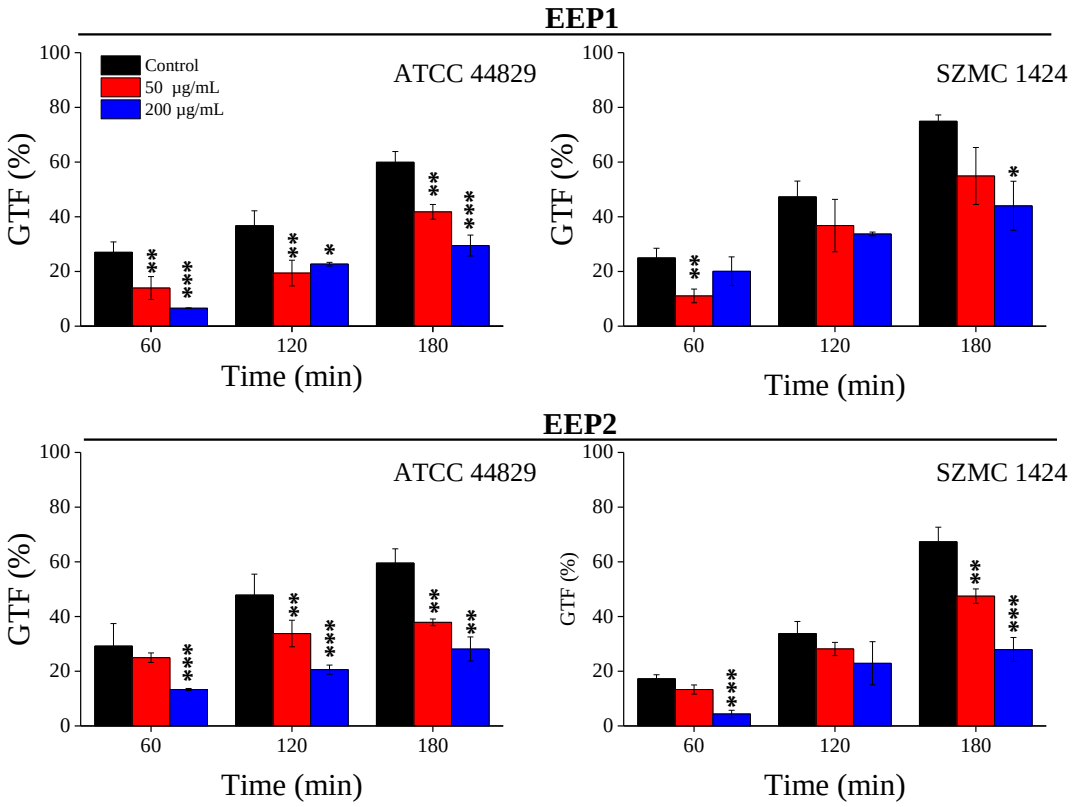

Figure 13: The effect of 50 and 200 µg/mL of propolis extracts (EEP1 and EEP2) on germ tube formation of *C. albicans* ATCC 44829 and *C. albicans* SZMC 1424. The cells were suspended in horse serum and incubated in an orbital incubator at 37°C for 60, 120, and 180 min, the germination was calculated by a light microscope.

The exposure of both strains to EEP2 showed a similar effect. The number of *C. albicans* ATCC 44829 germ tubes was significantly reduced by 54%, 57%, and 53% over the time at 200 µg/mL. However, the treatment of *C. albicans* SZMC 1424 with EEP2 for 3 h was more effective compared to EEP1 treatments (Figure 13). The MIC of both propolis extracts suppressed the germination effectively after 3 h, but it did not exceed the inhibition of 58%.

4.3.4. Biofilm formation of *S. aureus*

The crystal violet staining was applied to investigate the effect of EEP1 on *S. aureus* biofilm formation. The result was interpreted according to the ODc that was calculated to separate the growth of the biofilm at different concentrations of EEP1 into 4 categories: strong, moderate, weak, and no biofilm formation (Figure 14). All the tested *S. aureus* strains were strong biofilm formers in the absence of EEP1. However, the biofilm formation was significantly inhibited in the presence of 100–200 µg/mL EEP1. The MBIC values of EEP1 were 50 µg/mL for ATCC 700699, 100 µg/mL for the two MRSA clinical isolates (SA H23 and SA H24), and 200 µg/mL for ATCC 29213. Interestingly, MRSA biofilm was the most sensitive to propolis treatment.

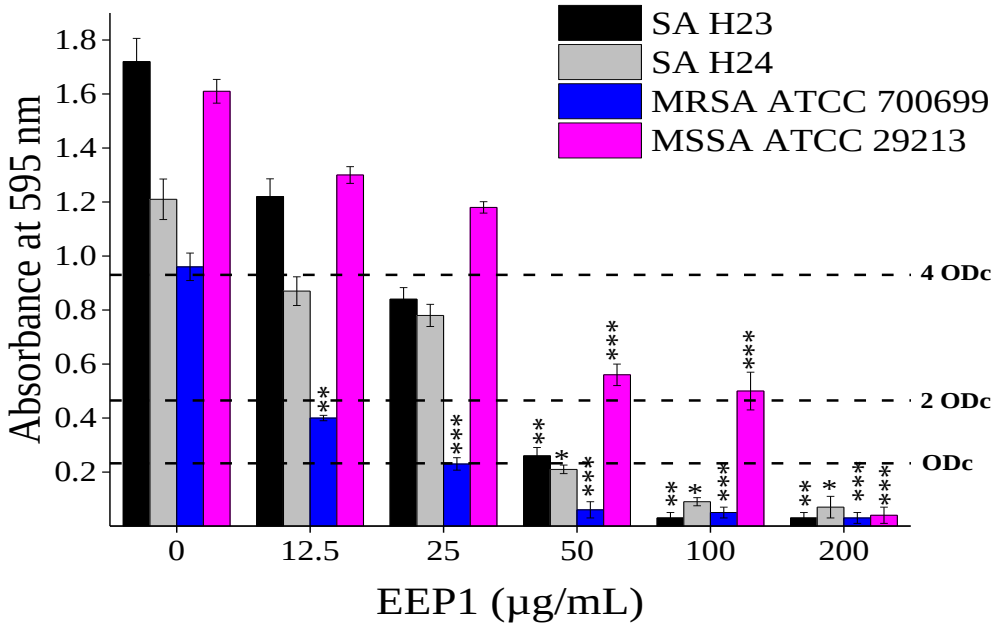

Figure 14: The effect of EEP1 (12.5–200 µg/mL) on the prevention of biofilm formation of *S. aureus* SA H23 and SA H24 clinical isolates, *S. aureus* ATCC 29213 (MSSA), and *S.aureus* ATCC 700699 (MRSA) strains after 24 h incubation at 37°C. (ODc) the cut-off value of the optical density. (OD ≤ ODc) means no biofilm, (ODc < OD ≤ 2 ODc) means weak biofilm, (2 ODc < OD ≤ 4 ODc) means moderate biofilm, (4 ODc < OD) means strong biofilm. Asterisks indicate statistically significant differences between each treatment of EEP1 and in absence of EEP1 (* p <0.05, * p < 0.01, *** p < 0.001).

4.3.5. The eradication of *S. aureus* mature biofilm

In this assay the cultures of *S. aureus* were grown for 24 h at 37°C, then the formed biofilm was treated with various EEP1 concentrations for 16 h, and the biofilm eradication was detected by crystal violet colorimetric assay. The EEP1 significantly enhanced the biofilm degradation in each strain and showed $MBEC_{50}$ values of 15, 18, 48, and 52 µg/mL against ATCC 700699, SA H23, SA H24, and ATCC 29213, respectively.

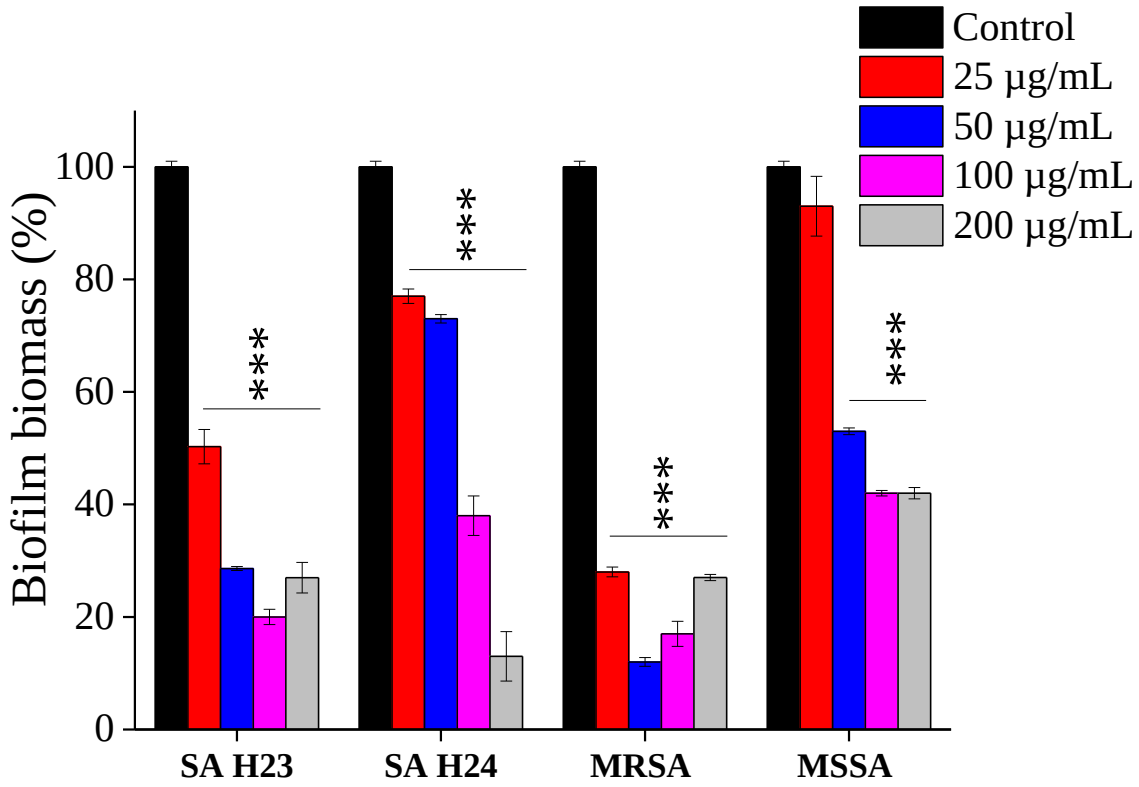

Figure 15: The effect of EEP1 on mature biofilm biomass of *S. aureus* SA H23, SA H24 clinical isolates, *S. aureus* ATCC 29213 (MSSA), and *S. aureus* ATCC 700699 (MRSA). The 24 h-old biofilm was treated with EEP1 (25–200 µg/mL) for 16 h at 37°C. Using the crystal violet assay, the absorbance of biofilm biomass was measured by a 96-well plate reader spectrophotometer.

The biofilm of MSSA and SA H24 strains showed more resistance to the EEP1, however, the thickness of biofilms was degraded at 200 µg/mL of EEP1 by 47% and 87%, respectively. The most sensitive biofilm was observed in the case of ATCC 700699 and SA H23 strains, where the degradations of the 24h-mature biofilms were 88% and 71%, respectively after treatment with 50 µg/mL of EEP1. However, unexpected growth of the biofilm biomass of the same strains was observed in the presence of high EEP1 concentration (200 µg/mL) (Figure 15).

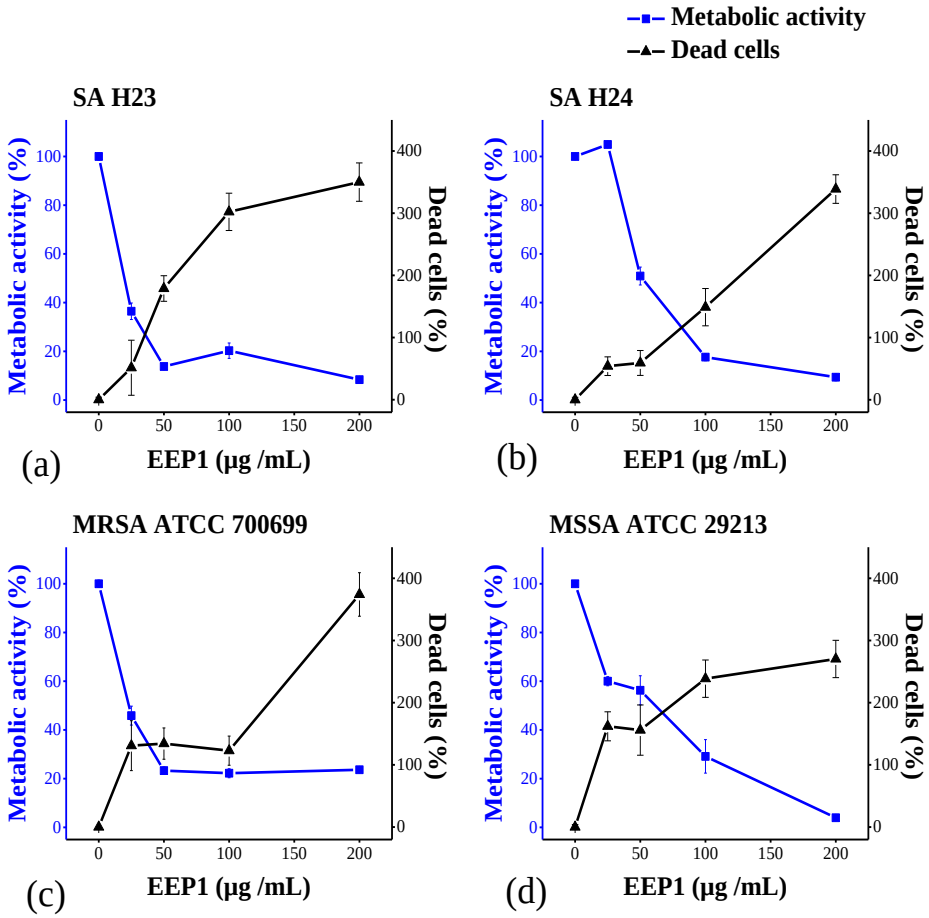

Figure 16: The effect of EEP1 (25–200 µg/mL) on 24 h-old biofilms formed by: (a) clinical isolates of *S. aureus* SA H23, (b) SA H24, (c) *S. aureus* ATCC 700699 (MRSA), and (d) *S. aureus* ATCC 29213 (MSSA). The mature biofilms were treated with EEP1 for 16 h at 37°C. Using the double fluorescent staining, the metabolic activity of the cells (blue lines) and dead cells (black lines) were detected within mature biofilms, in proportion to resazurin and propidium iodide dyes, respectively.

The biofilm formation of some bacteria is one of the important microbial defence strategies against antibiotics. In this study, double fluorescent staining with resazurin and PI were applied on 24 h-old biofilms to detect the simultaneous effect of propolis on the reductive metabolic activity and the cell viability in the mature biofilm of *S. aureus*. The PI binds specifically to the DNA through the penetration into the cells only with disrupted membranes. This study has clearly shown the concentration-dependent cytotoxic effect of EEP1 on cells within the structure of biofilm. EEP1 significantly decreased the cellular metabolic activity of the four *S. aureus* strains within the biofilm up to 90% at 200 µg/mL (4 MIC value). At the

concentration of 50 µg/mL (MIC value) of EEP1, MSSA and SA H24 showed higher metabolic activities than that of MRSA and SA H23 (Figure 16), which is in parallel with the resistance presented and the higher thickness of 24 h-old biofilm biomass (Figure 15), indicating a protective effect. The significant decrease in cellular metabolic activity was proportional to the increase in dead cells (Figure 16).

4.4. Mechanism of action of propolis

4.4.1. Propolis biosorption kinetics

Due to the observed relatively fast fungicidal effect of EEP1, the biosorption kinetics of MIC (200 µg/mL EEP1) was determined on *C. albicans* ATCC 44829 cell suspension. The initial concentration of EEP1 was reduced by 54% after 5 min of incubation.

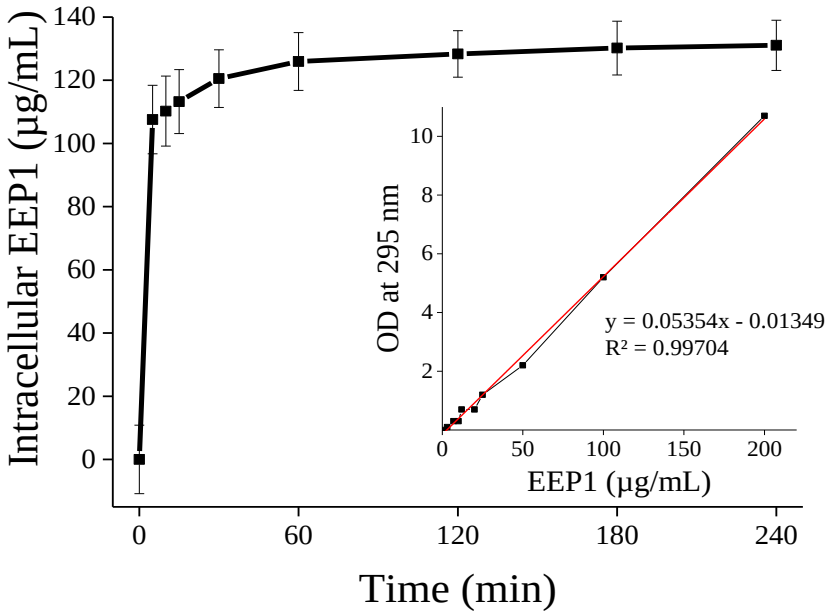

Figure 17: The uptake of propolis extract (EEP1) by *C. albicans* ATCC 44829. The cells were incubated in an orbital shaker at 30°C at 150 rpm in the presence of 200 µg/mL EEP1, the extracellular concentration of the EEP1 was measured hourly for 4 h photometrically at 295 nm. The calibration curve (included in the graph) was determined under the same conditions.

The decrease of EEP1 in the extracellular medium reached a maximum of 66% after 60 min, indicating a rapid bioabsorption and bioaccumulation process. The EEP1 uptake was saturated after 1 h of incubation whereas the cells were able to uptake approximately 131 µg/mL EEP1 by the end of 4 h of incubation (Figure 17).

4.4.2. Effect of propolis on cell wall and cell membrane

To test the hypothesis that propolis may target the fungal cell wall, the microdilution method was used to determine the susceptibilities of *C. albicans* ATCC 44829 in the presence and absence of sorbitol as an osmotic stabilizer agent. The MIC values of EEP1 were 200 µg/mL in both cases, which indicated that the EEP1 did not affect the yeast cell wall biosynthesis. Paradoxically, the growth of the cells was significantly reduced in the presence of sorbitol at 100 µg/mL EEP1, which may indicate the synergistic interaction of propolis with sorbitol on the cell viability over that concentration (Figure 18).

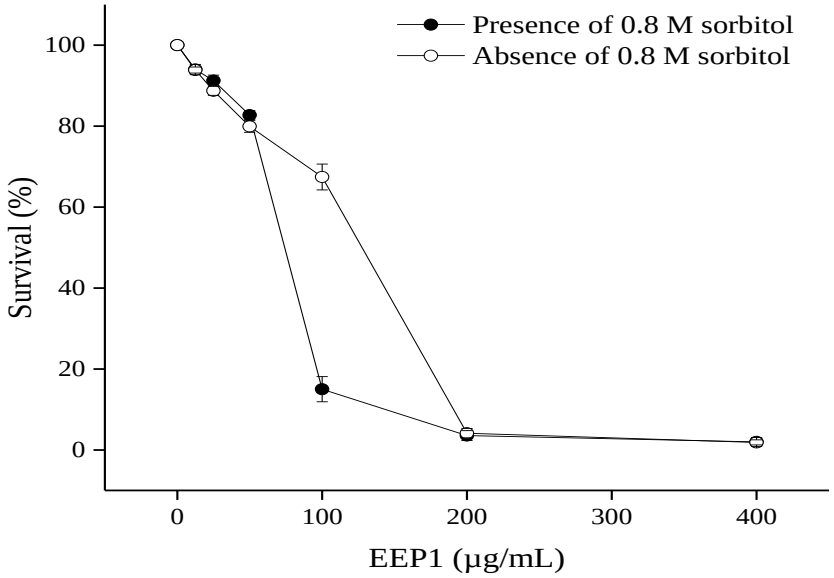

Figure 18: Effect of propolis extract (12.5-400 µg/mL of EEP1) on the cell wall integrity of *C. albicans* ATCC 44829, in the presence and absence of 0.8 M sorbitol. After the incubation of the cells for 48 h at 35°C, the absorbance was maseared using 96-well plate reader.

The time-dependent leakage of intracellular substances (nucleotides, nucleosides, and free bases, etc.) that absorb light at 260 nm was determined, to detect the action of propolis on the cellular membrane permeability of *C. albicans* ATCC 44829. After 1 h treatment, the measurements showed statistically significant loss of intracellular substances in the presence of 400 µg/mL EEP1. While a significant increment in the leakage by 4.2 and 10.8 times was observed with 200 and 400 µg/mL EEP1 after 2 h, respectively, compared to the untreated cells ($p < 0.05$). The treatment for 6 h caused 1.7, 2.9, and 4.8 time increase of the absorbance in the presence of 100, 200, and 400 µg/mL of EEP1, respectively. The ethanolic extract of propolis

caused concentration and time-dependent loss of the plasma membrane barrier function of *C. albicans* cells, which might play a considerable role in the anti-candiadal effect (Figure 19A).

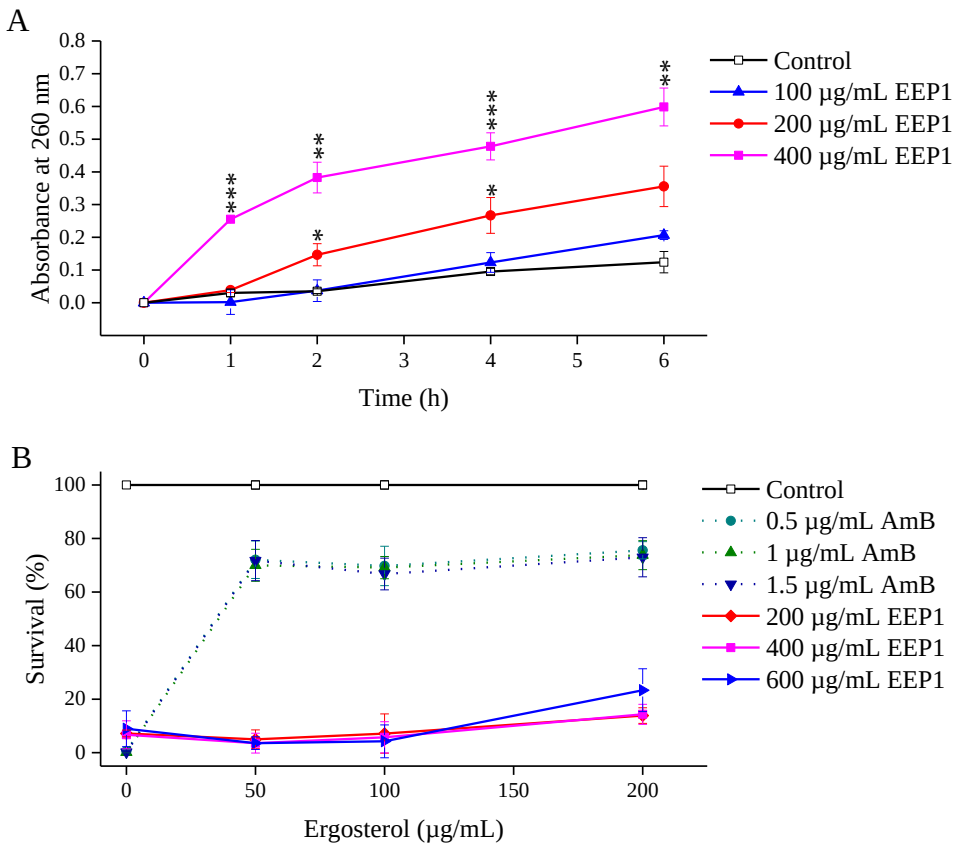

Figure 19: The effect of propolis extract on the cell membrane of *C. albicans* ATCC 44829. (A) The leakage of intracellular substances absorbing at 260 nm was measured with 100, 200, and 400 µg/mL EEP1every hour for 6 h spectrophotometrically. (B) Effect of ergosterol on the survival rate in the presence of 200, 400, and 600 µg/mL of EEP1, and in the presence of 0.5, 1.0, and 1.5 µg/mL AmB (positive control).

Considering the possible fungal cell membrane interference of EEP1, the susceptibility tests were applied in the presence of various concentrations of ergosterol to investigate the ergosterol binding capacity of EEP1 on *C. albicans* ATCC 44829. A compound that has an affinity to ergosterol rapidly forms complexes with the free molecules, thus preventing interactions with the fungal membrane ergosterol, thereby the survival rate of the cells increases. However, in the presence of 200 µg/mL ergosterol the survival rate revealed 13.86, 14.33, and 23.35% with 200, 400, and 600 µg/mL EEP1 (Figure 19B). Thus, the slightly increasing tendency of survival suggested slight interaction between the EEP1 and ergosterol in the yeast cellular membrane, compared to the positive control of AmB.

4.4.3. DNA fragmentation and nuclei damage

The examination aimed to establish whether the propolis is capable to affect the integrity of *C. albicans* gDNA. Consequently, the DNA extracted from the cells treated with EEP1 was analysed with the 2400 TapeStation platform. Visualization of the DNA extracts using the gel-electrophoresis and electropherogram (Figure 20A and B) allows a rapid assessment of the gDNA quantity and size. The DNA extracted from the untreated cells made a clear single band at 15000 bp which indicated intact gDNA. Treatment for 1 h with 5 mM H_2O_2, 50 µg/mL, and 200 µg/mL of EEP1 exhibited an intense smear through the range of 250-3000 bp and demonstrated higher DNA degradation compared to the negative control. The generated concentration of fragmented DNA was approximately saturated upon 50 µg/ml EEP1 treatment compared to the positive control. Hence, DNA fragmentation could be the principal reason for EEP1-induced cell death. These results suggested the possibility of propolis-induced alterations in nucleus morphology, which were investigated with DAPI staining assay under fluorescent microscope evaluating at least 300 cells in each sample. Propolis-treated cells showed an increase in cytoplasmic volume. In addition, cells treated with 200 µg/mL EEP1 showed green light emission, which could be the interaction of propolis with cell components (Figure 21).

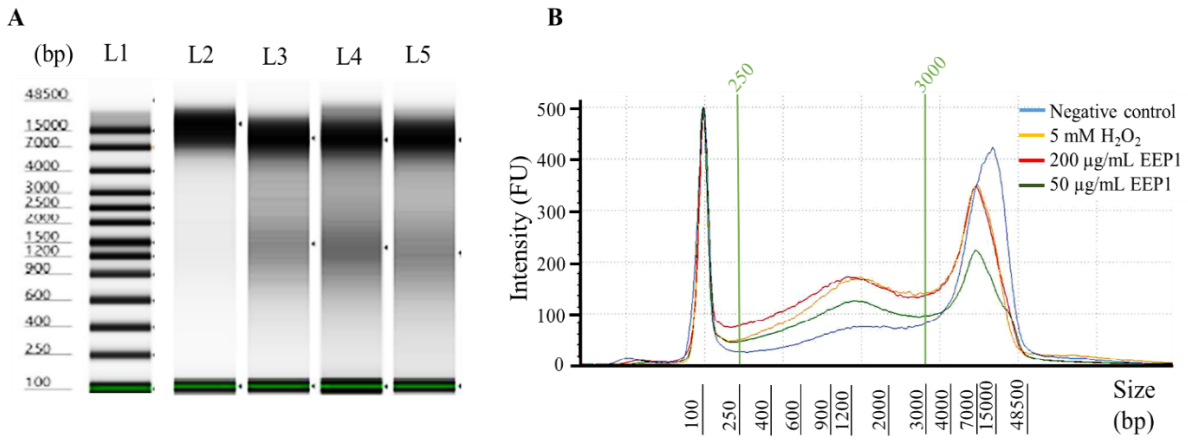

Figure 20: The quality assessment of genomic DNA extracted from *C. albicans* ATCC 44829 after a 1 h exposure to propolis extract (EEP1) was performed using the Agilent 4200 TapeStation system and the Agilent Genomic DNA ScreenTape assay. (A) The gel image; (L1) molecular weight markers, (L2) negative control, (L3) positive control (5 mM H_2O_2), (L4) 50 µg/mL EEP1, (L5) 200 µg/mL EEP1. (B) An overlay of the electropherograms.

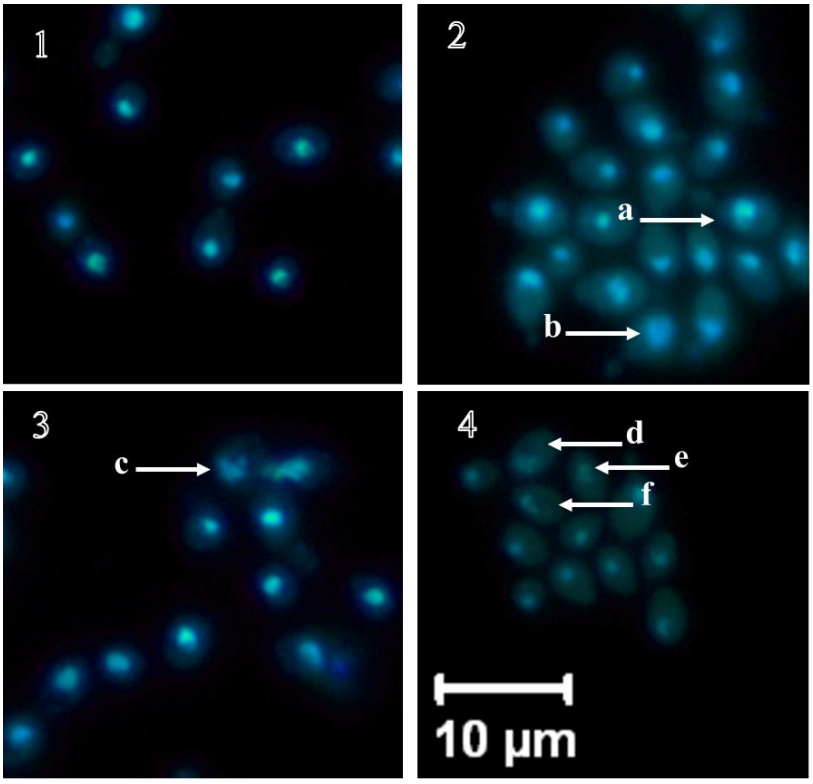

Figure 21: Show damage to the nucleus of *C. albicans* ATCC 44829 1 h after the exposure to propolis extract (EEP1) by DAPI staining; (1) Negative control, (2) Positive control (5 mM H_2O_2), (3) 50 µg/mL EEP1, (4) 200 µg/mL EEP1. The untreated cells showed homogeneous nuclei in shape and density. Arrows point to changes in the nucleus; (a) chromatin condensation, (b) enlarged nucleus, (c) irregular shape of the nuclear membrane, (d-f) shrunken and fragmented nuclei, and micronucleus. The cells were visualized by a fluorescence microscope.

Table 5: Summarizing the percentage DNA fragmentation of *C. albicans* ATCC 44829 after 1-hour exposure to 5 mM H_2O2 and propolis extract (50 µg/mL and 200 µg/mL EEP1) in the 250–3000 bp region using the Agilent Genomic DNA ScreenTape assay, the percentage of damaged nuclei by DAPI staining.

Treatments	Fragmented DNA (%)	DAPI staining (%)
Negative control	28.78	1.86
5 mM H_2O_2	49.68	1.91
50 µg/mL EEP1	52.32	2.07
200 µg/mL EEP1	52.94	6.61

The 50 µg/mL EEP1 caused a 1,1-time increment, while 200 µg/mL EEP1 showed a 3.6 times increase of nuclei damage compared to the negative control (Table 5). This result

supports the damage of gDNA of *C. albicans* cells that were detected with gel-electrophoresis DNA fragmentation assay, although displaying a different pattern of concentration dependence.

5. Discussion

Natural products are well known as a promising source for the discovery of major new pharmaceuticals (Sforcin and Bankova, 2011). The application of propolis extracts in the pharmaceutical industry requires an understanding of their chemical composition and biological properties (Ristivojević et al., 2015). The antimicrobial activity of propolis extracts would give new opportunities to fight against fungal and bacterial infections, but the responsible mechanism of action is still not completely clear. Therefore, the chemical composition of ethanolic extract of Hungarian propolis was determined, and the biological action was investigated on *C. albicans and S. aureus* species.

5.1. Chemical characterization

The phenolic and flavonoid contents in propolis resin in propolis resin differ from one sample to another (López and Sawaya, 2012). In Hungary, the main sources of collected propolis are originated from the bud secretion of poplar (*Populus* spp.) and birch (*Betula* spp.), which is a source of resin (Przybyłek and Karpiński, 2019). Propolis is a substance having different colours from dark-brown, red, green, and yellow due to the change of the chemical composition (Table 1), especially flavonoid and phenolic compounds (López and Sawaya, 2012). It was found a significant correlation between the phenolic concentration, the antioxidant activity, and the colour of propolis samples from Chile and Spain (Revilla et al., 2017). Therefore, the concentration of phenolic or flavonoids in propolis could be proportional to the colour intensity of the extracts. The visual comparison of our samples shows that the extracts with high TPC and TFC have dark colours (except the EEP4), while the EEP6 has a light yellow colour (Figure 5). In contrast, the raw propolis of the samples 6 have black colour and crumbly texture (less sticky) compared to the other samples (Figure 4). The information about the characterization of Hungarian propolis is almost non-existent. Our propolis samples showed a phenolic content about 4 times lower than other samples collected from different regions of Hungary (Molnár et al., 2017). However, the TFC was significantly high compared to other European and Brazilian propolis (Table 6).

Table 6: The determination of the total phenolics (TPC) and flavonoids contents (TFC) using the Folin-Ciocalteu and the aluminium chloride colorimetric methods, respectively, in propolis samples from the temperate and tropical regions.

Propolis sample	Solvent	TPC (mg/g)	TFC (mg/g)	Reference
Bolivia	Methanol	43-176 (mg GAE/g)	5.5-57.1 (mg CE/g)	(Nina et al., 2016)
Brazil	Ethanol	1.3-3.9 (mg GAE/g)	0.14-0.15 (mg QE/g)	(Torres et al., 2018)
		249-482 (mg GAE/g)	30-187 (mg QE/g)	(Devequi-Nunes et al., 2018)
Chile	Methanol	115-208 (mg GAE/g)	17-140 (mg CE/g)	(Nina et al., 2015)
Croatia	Methanol	10-220 (mg GAE/g)	5-50 (mg CE/g)	(Ivana Tlak et al., 2017)
Czech Republic	Ethanol	129.8 (mg CAE/g)	2 (mg QE/g)	(AL-Ani et al., 2018)
France	Ethanol, methanol, and water	239-292 (mg GAE/g)	20.5-80 (mg QE/g)	(Boisard et al., 2014)
Germany	Ethanol	46.5 (mg CAE/g)	1.9 (mg QE/g)	(AL-Ani et al., 2018)
Hungary	Ethanol	104.6-286.9 (mg GAE/g)	-	(Molnár et al., 2017)
Ireland	Ethanol	52.8 (mg CAE/g)	2.9 (mg QE/g)	(AL-Ani et al., 2018)
Korea	Ethanol	49-239 (mg GAE/g)	20-50 (mg QE/g)	(Wang et al., 2016)
Poland	Ethanol	14.6-150.8 (mg GAE/g)	-	(Wieczy et al., 2017).
		76-105.3 (mg CAE/g)	11-15.7 (mg QE/g)	(Pobiega et al., 2019a).
Romania	Ethanol	190-243 (mg GAE/g)	90-139 (mg CE/g)	(Gatea et al., 2015).
		180-343 (mg GAE/g)	24-144 (mg QE/g)	(Gatea et al., 2016).
Turkey	Ethanol	27.5-199.7 (mg GAE/g)	30.7-302.9 (mg QE/g)	(Gezgin et al., 2019; Ozdal et al., 2019)
		314 (mg GAE/g)	523 (mg QE/g)	(Ozdal et al., 2018)
Venezuela	Ethanol	19.1-107 (mg GAE/g)	2.6-8.5 (mg QE/g)	(Mohtar et al., 2020)

(GAE) Gallic acid equivalent, (CAE) caffeic acid equivalent, (CE) catechin equivalent, (QE) quercetin equivalent. (-) Not available.

The researchers unanimously agreed that the chemical composition of propolis is influenced by the vegetation (Mohtar et al., 2020), it also varies from hive to hive even in the same region, depending on the honeybee species (Asem et al., 2020), and solvent of extraction (AL-Ani et al., 2018; Narimane et al., 2017; Wieczy et al., 2017). In addition, the mild climate conditions could be the reason for the propolis production with large TPC and TFC (Gezgin et al., 2019; Ozdal et al., 2019). Normally, the antimicrobial activity of propolis is proportional to the TPC and TFC (Torres et al., 2018).

To characterize the type of the Hungarian propolis samples, the UV-Vis spectra were recorded and analysed. UV-Vis spectrometry is a quick method that can be used to classify the propolis extracts according to their main chemical composition. The UV-Vis pattern of propolis extracts is the result of the contribution of spectral properties of the main phenolic compounds identified in propolis. According to Marghitas and co-workers (2013), three types of propolis may be distinguished using simple spectrophotometric registration of UV-Vis spectra of different propolis extracts (Mărghitaş et al., 2013). The spectra having absorption maximum λ_{max}= 295 nm show the highest total flavonoid content and radical scavenging activity. Our propolis samples were presented with a maximum absorption (Appendix, Table 3) consistent with the absorption of the phenolic compounds of Portuguese propolis, such as the derivatives and the isomers of caffeic acid, ferulic acid, and pinobanksin (Falcão et al., 2013a). The UV-Vis absorption spectra of Serbian, Italian, French, and German propolis samples are characterized by a broad absorption band in the near UV region, with a strong peak at 270, 290, and 320 nm, thank the high ratio of phenolic compounds and it was classified as orange-type of propolis. These findings are in agreement with the results obtained in this study. Moreover, our samples characterized by a high content of chrysin and genistein. However, other characteristic components of orange-type propolis such as galangin, apigenin, kaempherol, quercetin, and pinocembrin were not identified in our samples (Fabris et al., 2013; Mărghitaş et al., 2013; Ristivojević et al., 2017, 2015). Thus, the Hungarian propolis likely belonged to the orange subtype of poplar-type propolis according to the UV-Vis analysis. However, the GC-MS analysis may reveal a new type of propolis. As it is known, propolis is a mixture of substances collected from various kinds of buds of resinous plants. The propolis originating from temperate climatic regions mainly consisting of the bud exudates of *Populus* species, which is called poplar-type propolis (Isidorov et al., 2014; Rojczyk et al., 2020). According to the study of NFCSO in 2014, the poplar trees in Hungary formed 8% of the total forest area (21%). As an expected result, the investigated samples showed a chemicals-profile of propolis similar to the chemicals profile of bud exudates of *Populus* species. Poplar buds and resins are

very rich in bioactive compounds like flavonoids and phenolic compounds (Tyśkiewicz, 2019), among the identified flavonoids that occurred at the highest concentration, are chrysin and pinocembrin, also caffeic acid, ferulic acid, *p*-hydroxybenzoic acid, and *p*-coumaric acid (Bankova et al., 2002; Isidorov and Vinogorova, 2003). The concentrations of phenols and flavonoids in our samples were higher than other poplar-type propolis collected from various Euroasia origin. Genistein which accounts for 34.4-13.9% in Hungarian samples, was not common in many of the propolis previously studied (Dezmirean et al., 2017; Isidorov et al., 2014; Isidorov and Vinogorova, 2003; Kartal et al., 2002; Molnár et al., 2017; Ristivojević et al., 2015; Vardar-Ünlü et al., 2008). Genistein is an angiogenesis inhibitor and a phytoestrogen, mainly found in *Glycine max L.* and *Trifolium* species. It has received wide attention due to the numerous biological activities such as binding to estrogen receptors, inhibition of tyrosine kinases, and reducing inflammation (Vanden Braber et al., 2018). It is also known as an effective antioxidant, antiproliferative and anti-skin cancer (Spagnuolo et al., 2015; Tuli et al., 2019), and antimicrobial agent (Choi et al., 2018). Chrysin is a flavone extracted from various plants, such as the *Passiflora coerulea*, it is found in honey and propolis (Mani and Natesan, 2018). The anticancer activity of propolis is related mainly to chrysin, besides other polyphenols (such as caffeic acid, *p*-coumaric acid, ferulic acid) that affect the DNA biosynthesis in cancer cells (Celińska-Janowicz et al., 2018). Some studies reported that chrysin has antimicrobial properties because it could destroy the integrity of the microbial cell wall and cell membrane. Also, it played a crucial role in protecting plants against microbial invasion (Liu et al., 2014; Suresh Babu et al., 2006; Vardar-Ünlü et al., 2008). Propolis also contains farnesol, vanillin, hydrocarbons, and vitamins B6, which have antioxidant properties (Kurek-Górecka et al., 2013). Farnesol is mainly secreted by *C. albicans* and *C. dubliniensis*. It is participating in the control of morphogenesis in *C. albicans*. It is well known that farnesol blocks the transformation of yeast cells to hyphae in *C. albicans*, prevents cell adhesion, and promotes detachment of biofilms from some surfaces. The farnesol molecule represented up to 0.87% in the Hungarian propolis samples. In addition, farnesol treatment may induce apoptosis and disordered mitochondria due to the presence of reactive oxygen species, the exposure to the farnesol leading to necrosis due to the distraction of the cell membrane (Fernandes et al., 2016; Shirtliff et al., 2009). All samples analysed in this study showed high amounts of bioactive compounds in different proportions. However, EEP6 showed a significantly lower concentration in terms of flavonoids and phenols and higher in terms of the saccharides. The biological activities of propolis extracts are highly dependent on its chemical composition.

5.2. Antimicrobial and anti-virulence activities

5.2.1. Antifungal activity

To the best of our knowledge, the antifungal activity of Hungarian propolis has not previously been evaluated (Papp et al., 2021). There has been only limited research on the antifungal and antivirulence activities of propolis (Boisard et al., 2015b; Gucwa et al., 2018). The present study discussed the cytotoxic activity of ethanolic extracts of propolis on *C. albicans* strains. The MIC values of EEP fall in the range of those found in previous studies. According to Simões et al. (2009), phytochemicals that produce MIC in the range of 100-1000 μg/mL in the *in vitro* susceptibility tests are routinely classified as antimicrobials. This proposal is in agreement with those previously obtained for Iranian propolis ethanolic extracts against *C. albicans* isolates that the MIC values varied from 120.2 to 970.6 μg/mL (Haghdoost et al., 2016). While other studies suggest that the crude extract of any natural product that displays MIC lower than 500 μg/mL is a promising substance (Duarte et al., 2007; Tiveron et al., 2016). Therefore, different Brazilian red propolis showed a weaker MIC (in the range of 250-1000 μg/mL) (López et al., 2015) than the tested Hungarian propolis extracts. The organic extracts showed significant antifungal activity against *C. albicans* and *C. glabrata*. The ethanolic extract showed considerable activity against *C. albicans* with a MIC equal to 31.25 μg/mL. This study may be concluded that organic solvents extracts of French poplar-type propolis are associated with a potent antifungal activity correlated with high flavonoid contents (Boisard et al., 2015a). The time and concentration-dependent fungicidal effect of EEP1 has been demonstrated with the determination of survival rates of *C. albicans* by counting the CFU after 30 min and 60 min. It has been shown that the observed MIC resulted in more than 90% decrement in the number of CFUs after 1 h treatment (The MFC was equal to 200 μg/mL). The MFC of our samples was in the range compared to the fungicidal activity of Brazilian red propolis extract that was observed (64-512 μg/mL) against *C. albicans* strains (Freires et al., 2016; Siqueira et al., 2015), and it was stronger than the Iranian propolis that showed MFC in the range of 480.8 to 3900.4 μg/mL (Haghdoost et al., 2016). The 30 min timeframe which was needed for the 78% of CFU decrement assumed a rapid biosorption of propolis extract by the cells. The results revealed that in the first 5 min more than half of the original amount of the EEP1 was adsorbed and/or bioaccumulated by the cells. However, pretreatment of the cells with a subinhibitory concentration of propolis proved the existence of an adaptive response to propolis extract at a cell level, which was reflected in the increased effective fungicidal concentration. According to

these results, it can be supposed that low-concentration therapy and subsequent adaptation can enhance the survival as well as the virulence of *C. albicans*.

5.2.2. The antibiofilm and antigermination activities of propolis against *C. albicans*

C. albicans biofilm is composed of yeast and a network of filamentous cells embedded in an extracellular matrix that confer resistance to treatment and may act as a persistent source of infection (Veiga et al., 2018). Extracellular polymeric matrix causes difficulty in penetration of antimicrobial agents and thus contributes to the virulence of microorganisms (Tyagi et al., 2013). The blockage of the virulence factors could be an important therapeutic target for antifungal agent development (Hmoteh et al., 2018). The adhesion of the cells relies on several cell wall proteins called adhesins. These proteins promote attachment to other epithelial and microbial cells or abiotic surfaces by binding to a specific amino acid or sugar residues (Djais et al., 2019). Therefore, propolis can be one of the agents that can modify the surface characteristics of *C. albicans* and may alter its adherence capacity, thereby preventing biofilm formation (Feldman et al., 2014). On the other hand, propolis extracts can prevent yeast cells from forming biofilms with low cytotoxicity in human cells (Galletti et al., 2017b; Ranfaing et al., 2018; Veiga et al., 2018). Another study investigated the effect of Brazilian propolis extract against 29 clinical isolates of *C. albicans* isolated from vaginal specimens. The EEP showed strong antibiofilm activity against all the isolates (Capoci et al., 2015). Capoci and co-workers (2015) suggested that propolis can be potentially used to treat candidiasis. Our data confirmed that propolis could be a promising agent to combat *Candida* infections by preventing the development of biofilms. The Hungarian propolis samples showed significantly low MBIC values at 3.125 µg/mL. It was found that 0.1% of Spanish propolis extract significantly reduces the biofilm formed by *C. glabrata* in a dose-dependent manner (Fernández-Calderón et al., 2021). The infections associated with biofilms are extremely challenging to treat as the adherent fungi, which have already developed thick old-biofilm, are protected against the host immune system response and antifungal attack (Bryan et al., 2015). While the increased thickness of preformed biofilm indicates higher virulence of the strain. This confirms that *Candida* cells in the planktonic form are more affected by propolis extract and therefore the biofilm-stage is an important characteristic in promoting the persistence of *Candida* in the host and increases treatment tolerance. The extracellular matrix is one of the barriers facing the drug for penetrating and destroying the biofilm (Capoci et al., 2015). EEP1 and EEP2 differentially

affected the eradication of the old-biofilm of *C. albicans* SZMC 1424. Interestingly, the EEP1 degraded the biofilm biomass in the range of 6.25-50 µg/mL while the EEP2 strengthened and contributed to the increase of the biofilm thickness in the range of 50-200 µg/mL. Wojtyczka and co-workers found that the bacterial biofilm formation ability was inhibited at concentrations ranging from 0.39 to 1.56 mg/mL of EEP. However, an increment in the proliferation was observed after 12 and 24 h of incubation in the presence of EEP concentrations ranging from 0.025 to 0.39 mg/mL and from 6.25 to 12.5 mg/mL. The phenomenon of accelerating the formation of biofilms at low and high concentrations of EEP could be due to the presence of nutrients in EEP that act as a growth stimulator. These results were consistent with our findings, and indicate that the antimicrobial activities of EEP were significantly affected by the incubation time and EEP concentration (Wojtyczka et al., 2013b). In addition, the effect of EEP2 was tested on other strong biofilm former strains of *C. albicans* SZMC 1425 and SZMC 1426 (Appendix, Figure 2), it showed an effect similar to that of EEP1 on SZMC 1424. We concluded that 100-200 µg/mL of EEP were not efficient on biofilm eradication of *C. albicans* strains. The possible mechanisms of biofilm resistance and amplification to propolis include limited penetration through the extracellular matrix at a high concentration of propolis or switching the regulation of the gene expressions. Hyphal development is important for the formation of substantial biofilm biomass (Djais et al., 2019). The increment in OD revealed by crystal violet staining, which is proportional to the biofilm biomass was justified by the increase of yeast filamentation and possibly occurred as a response of *C. albicans* to the exposure of propolis extract triggered environmental stress (Calderone and Fonzi, 2001; Capoci et al., 2015). *C. albicans* produce germ tubes and somatic hyphae. These are harmful virulence factors that facilitate the invasion of host tissue and the dissemination of the pathogen. Furthermore, it is associated with an increased synthesis of proteins and ribonucleic acids. Germ tube development has been documented to be a crucial functional mechanism that enhances adherence of the pathogen to the host cell surfaces. (Haghdoost et al., 2016; Hmoteh et al., 2018). Nevertheless, the variation in germ tube inhibition was not observed between the isolates after the treatment with the same extract neither between extracts after the treatment on the same strain (Appendix, Figure 3). Our results were similar to a previous study that showed a significant reduction in the germination of *C. albicans* isolates by 36.7% to 22% in the presence of 1/2 MIC and 1/4 MIC of EEP, respectively. The MIC values of EEP in this study varied from 120.2 to 970.6 µg/mL (Haghdoost et al., 2016). In addition, Corrêa and co-workers indicate that the propolis extract interferes with germination and efficiently inhibits the filamentation of *C. albicans* at a concentration of 1675 µg/mL of TPC for up to 6 h (Corrêa et al., 2020). This

bioactivity could be related to the interference of propolis and especially phenols with cell wall synthesis, or directly affecting DNA replication.

5.2.3. Antibacterial activity

As a result of the high rate of infection with drug-resistant bacteria over the past few decades, efforts have been intensified not only to discover new antibiotics but also to find new strategies to fight the infection (Miklasińska-Majdanik et al., 2018). It was reported that MRSA acquired the *mec*A gene that is present within the Staphylococcal Chromosomal Cassette *mec* (SCC*mec*) and reduced the binding affinity of *β*-lactam antibiotics (methicillin, oxacillin, cefoxitin, etc.) on the peptidoglycan layers of *S. aureus* (Chovanová et al., 2016). Slightly different, vancomycin is a conventional glycopeptide that inhibits the late stage of cell wall biosynthesis in *S. aureus* and other Gram-positive microorganisms, by binding to the C-terminal (the D-Alanyl-D-Alanine) residue of the peptidoglycan (Howden et al., 2010). Previous studies reported that the antibacterial activity of EEP varies depending on many factors; such as the type of propolis, the extraction method, and the method of testing on bacterial susceptibility (Bueno-Silva et al., 2017b; Regueira Neto et al., 2017). To the best of our knowledge, only one study was evaluated the antimicrobial activity of the Hungarian propolis. The ethanolic extracts of propolis collected from different regions in Hungary were selected to examine the antibacterial activity on different bacterial strains including *S. aureus* using an agar well diffusion assay. All studied bacteria showed bactericidal effects at 200 μg/mL with a diameter of 12 to 22.5 mm from the inhibitory zone, the antimicrobial activity of EEP was independent of the bacterial species (Molnár et al., 2017). The Brazilian propolis showed a very broad range of MIC values from 31.2 μg/mL to >1024 μg/mL against *S. aureus* strains (Bueno-Silva et al., 2017b; Regueira Neto et al., 2017). Numerous studies confirmed the high antimicrobial potential of propolis using *in vitro* and *in vivo* assays against some important pathogens. The antimicrobial activity of Croatian propolis samples was revealed against *S. aureus*, most of the samples did not show activity against Gram-negative bacteria (*E. coli*). In addition, the MIC values were slightly elevated, ranging from 0.391 to 12.5 mg/mL on Gram-positive bacteria. The antimicrobial activity of EEP was evaluated against *S. aureus* and MRSA, using the microdilution method. MIC values were determined in the range of 1 to 6 mg/mL, in comparison with our EEP results, it is considered to be significantly less effective (Torres et al., 2018). It was confirmed the safety of most samples of propolis when the human gingival fibroblasts (HGFs) were incubated with 10 μg/mL and 100 μg/mL EEP *in vitro*. However, some

samples at concentrations of 500 µg/mL and 1000 µg/mL induced a cytotoxic effect resulting in decreased mitochondrial activity of HGFs. (Wieczy et al., 2017). The high anti-staphylococcal activity of EEP was observed for samples collected from Taiwan, Turkey, Oman, and Ireland, with MIC values at 3.75, 8, 42, and 80 µg/mL, respectively (AL-Ani et al., 2018; Lu et al., 2005; Popova et al., 2013; Uzel et al., 2005). Nevertheless, our study demonstrated a very potent antibacterial activity of Hungarian samples against *S. aureus* including oxacillin and cefoxitin resistant strains as well as vancomycin-intermediate MRSA compared to other efficient propolis samples, collected from Turkey (Gezgin et al., 2019), Australia (Wang et al., 2021), Iran (Kashi et al., 2011), Romania (Vică et al., 2021), and Croatia (Ivana Tlak et al., 2017).

The use of combination therapies between two pre-existing drugs is a promising alternative therapy, whereby the effectiveness of the treatment is enhanced at the reduced concentration of the two drugs (Lai et al., 2017; Wojtyczka et al., 2013a). The drug's association with phenolic compounds could enhance the activity of common antibiotics against a range of resistant pathogens (Miklasińska-Majdanik et al., 2018). In this study, the anti-staphylococcal activity of propolis was investigated alone and in combination with oxacillin, cefoxitin, and vancomycin at higher inoculum size. It was used 10^8 CFU/mL to simulate an organism density that is often associated with many infections. Staphylococcal infection often results in a high bacterial density (10^8 to 10^{10} cells/g of tissue) (LaPlante and Rybak, 2004). The MIC may vary according to the size of the inoculum used especially with some β-lactam antibiotics (Mizunaga et al., 2005). The increases in the bacterial inoculum from 10^5 to 10^8 CFU/mL raised the MIC of EEP1 from two to eight-fold (Figure 10 and Table 4). The study of Grecka and co-workers in 2019 mentioned synergistic interaction between EEP and antibiotics (amikacin, kanamycin, gentamicin, tetracycline, and fusidic acid), which are acting on the inhibition of protein synthesis (Grecka et al., 2019). The interaction with cefoxitin also had a positive effect but was not significant compared to the absence of EEP (Wojtyczka et al., 2013a). It was also reported that the Polish propolis sample had shown additive interaction with oxacillin (Grecka et al., 2019). The present study showed that the combination of EEP with vancomycin might boost the activity to reduce the cell wall synthesis. Such similar findings on synergistic effects between EEP and antibiotics acting on cell wall biosynthesis were reported (AL-Ani et al., 2018; Krol et al., 1993). The study of Surek et al. evaluated the interaction of Brazilian propolis with antibacterial agents using the broth microdilution chequerboard tests. EEP samples showed a promising synergistic effect with gentamicin against MRSA at 62.5 µg/mL of EEP and 0.83 µg/mL of gentamicin after 18 h. None of the extracts showed synergism with oxacillin

and vancomycin against MRSA (Surek et al., 2021). Most of the studies conclude a synergistic interaction between EEP and the drugs that interfere with protein synthesis on the cells. Furthermore, *β*-lactams and vancomycin antibiotics could positively act with propolis on the cell wall of *S. aureus* strains. It was reported that the type of the EEP and antibiotics interactions depends on one strain to another, due to the presence or absence and the type of SCC*mec* carried by the cell (Fernandes Júnior et al., 2005; Grecka et al., 2019; Reichmann and Pinho, 2017; Wojtyczka et al., 2013a). Such a similar finding was observed in our present study, in which SA H23 and SA H24 strains carried SCC*mec* type IVa and II types, respectively (Naorem et al., 2020). While the reference strain ATCC 29213 has no SCC*mec*, and ATCC 700699 harboured SCC*mec* type IVa, this might result in the variation of the interaction of EEP with antibiotics.

5.2.4. The antibiofilm activity of propolis against *S. aureus*

Biofilm formation is an important *S. aureus* virulence factor, characterized by the attachment of multi-layered cells to abiotic and biotic surfaces (Yoshii et al., 2017). Biofilm formation is influenced by a variety of conditions such as environment, availability of nutrients, and above all the presence of the regulatory genes and their expression (Piechota et al., 2018). In this study, no difference in terms of biofilm production ability was observed between MSSA and MRSA strains in the absence of EEP1. It was demonstrated that there was a relationship between phenotypic biofilm formation and the presence of *ica*A and *ica*D genes (Ghasemian et al., 2016). However, it was reported that not all *ica*-positive isolates produce strong biofilm (Naorem et al., 2020). On the other hand, the biofilm of the MSSA was the most resistant, which confirms that the biofilms are produced by distinct mechanisms in MRSA and MSSA. Generally, the biofilm of MSSA is formed in an *ica*-dependent manner (PIA-dependent) by PIA that is encoded by *ica*ADBC gene, whereas the biofilm of MRSA is formed in an *ica*-independent manner (PIA-independent) by surface proteins (Miao et al., 2019). Possibly the EEP1 inhibits biofilm formation in MSSA *via* a mechanism that differs from that responsible for the resistant strains (Ghasemian et al., 2016; Piechota et al., 2018; Yoshii et al., 2017). The Hungarian propolis effectively prevented the biofilm formation of *S. aureus* strains in the range of 50 and 200 μg/mL of EEP1, while a previous study showed inhibition of *S. epidermidis* biofilm with EEP in the range of 0.78 to 1.56 mg/mL after 24 h incubation (Wojtyczka et al., 2013b). Another study found that EEP has the ability to impair *Proteus mirabilis* biofilm in the range of 25-100 mg/mL (Kwiecińska-Piróg et al., 2018). In line with our results, ethanolic

extract of Italian propolis has shown the ability to reduce no more than 65% of the biofilm biomass of *P. aeruginosa* at 100 µg/mL after 24 h treatment. On the other hand, the viability of sessile bacteria was diminished by 42% at the same concentration (de Marco et al., 2017).

According to the World Health Organization (WHO), MRSA is not necessarily more dangerous than MSSA. However, MRSA has a higher mortality rate, as it is related to bacteremia infection more than MSSA. But MSSA can also be mortal in the healthcare field, especially for infants. It is considered that biofilms contribute to more than 80% of all infections in humans. The formation of biofilms by MRSA and MSSA strains is an important virulence factor affecting its persistence in both the environment and the host organism since bacterial cells in biofilms show increased resistance against conventional antimicrobial treatments and host immune factors (Piechota et al., 2018). It is cumbersome to remove mature biofilms and reduce the growth of dormant bacteria inside, due to reduced metabolic activity and reduced cell divisions and the difficulty of drug penetration into the biofilm (Yoshii et al., 2017). In the same context, the tested propolis sample was able to eradicate the mature biofilm with the MIC value, but was not able to eliminate it completely even at 4 MIC. It has been well characterized that bacteria in biofilms can tolerate up to 10-1000 times higher concentrations of antibiotics than planktonic bacteria (Sharma et al., 2019). Our result showed unexpected growth of the biofilm biomass was observed in the presence of high EEP1 concentration, such a similar result was reported in the biofilm of *S. epidermidis* and further suggested that the efficiency of propolis can be reduced over the time, and after 24 h the propolis stimulates biofilm formation and added that the high concentration of EEP could be used as a nutrient by bacteria for its proliferation (Wojtyczka et al., 2013b). Another study found that treatment of MRSA biofilm with 1/2 MIC, MIC (900 µg/mL), and 2 MIC of EEP, caused a significant decrease of the cellular activity in biofilm using the XTT reduction assay. Moreover, a significant decrease in biomass of MRSA biofilms was detected after treatment with 1/2 MIC, MIC, and 2 MIC EEP by crystal violet assay. Thus, EEP not only inhibited the planktonic cell growth but also affected the adhesion on a solid surface (Wang et al., 2021). Furthermore, *S. aureus* biofilm was effectively eliminated from the prosthetic materials with 10% Brazilian green propolis alcohol solution after immersion for 5 minutes (de Azevedo et al., 2021). However, accurate determination of the appropriate concentration is very important. Noteworthy, considerable antibiofilm activity was demonstrated by propolis on matured biofilm of *S. aureus* with MBEC values ranging from 2-4 µg/mL (Hazem et al., 2017).

The exposure times of propolis treatment to biofilm were different from one study to another. Nonetheless, *S. aureus* biofilms were completely inactivated with 2 µg/mL EEP after

40 h long treatment, indicating that the activity is dependent on treatment times (Ambi et al., 2017). Our result was in good agreement with the 90% elimination of the living *S. epidermidis* cells from the biofilm structure at 4 MIC EEP (Grecka et al., 2020). Similarly, another group investigated the ethanolic extracts of Brazilian propolis on matured biofilms of *S. aureus*, the result showed a reduction of 93% of the viability of the cells at 125 μg/mL. However, the total biofilm biomass eradication was insignificant (de Oliveira Dembogurski et al., 2018). The outcomes of previous research revealed a high efficiency of EEP in the eradication of MSSA biofilms incubated for 24 h at 37°C, with MBEC in the range of 64-128 μg/mL. It was concluded that the antibiofilm activity of propolis is the most clinically beneficial aspect (Grecka et al., 2019). In addition, the antibiofilm activity of Russian propolis has been reported using the MTT assay, which showed a 50% decreased viability of *S. aureus* in the mature biofilms, and demonstrated severe cell wall damage as a possible means of cell lysis (Bryan et al., 2015).

5.3. Mechanisms of action

The fungal cell wall is the first barrier responsible for growth, adaptation, and permeability regulation of fungal pathogens during infection (Gucwa et al., 2018). Corrêa and colleagues found that Brazilian propolis damages the integrity of *C. albicans'* cell wall and cell membrane, and causes leakage of intracellular organelles. The study hypothesizes that the antifungal efficacy of propolis is due to the capacity of polyphenols to form a complex with soluble proteins by disrupting the synthesis of chitin, which leads to cell wall disruption (Corrêa et al., 2020). In the same context, it is remarkable that propolis has been found to have the ability to cause severe cell wall damage to cells within the biofilm matrix, showing that the mechanism of action of propolis is structural rather than functional (Bryan et al., 2015). According to the rapid decrement of the viability of *C. albicans* and the rapid biosorption process, we assumed that propolis may act on the cell wall and the cell membrane. However, the antifungal activity of EEP1 did affected with the presence of sorbitol, indicating that the cell wall was not the target for our propolis sample, which is in line with the previous finding of Polish propolis (Gucwa et al., 2018). Contrariwise, the presence of sorbitol slightly enhanced the cytotoxicity of the propolis extract, probably by the penetration into the cell and causing hyperosmotic intracellular milieu.

The membrane permeability of the cells was observed after 2 h incubation by the release of the substances absorbing light at 260 nm, although significant fungicidal effect was shown

within 30 min treatment. The shifted leakage of the cellular substances may indicate the indirect effect of propolis on cellular membrane damage. Thus, the action of propolis in association with ergosterol was investigated. The results indicated that the effect of propolis analysed by the efflux method was not related to the direct binding to ergosterol. Notwithstanding, this finding was not consistent with the observations of other studies (Chang et al., 2018; Corrêa et al., 2020; Eskandarinia et al., 2020). The ergosterol and membrane depolarization assays suggest that the cell membrane might be a potential target for Polish propolis (Gucwa et al., 2018). Various compounds like phenolics and flavonoids are responsible for their antifungal activity by affecting the permeability of the cytoplasmic membrane, which leads to the total leakage of the cellular constituents, leading to complete cell death. Moreover, it has been found that phenolic compounds can disrupt the cell membrane of *C. albicans*, such as caffeic acid derivatives and curcumin (Iadnut et al., 2019).

The exposer to fungicidal and subinhibitory doses of EEP1 caused twice higher DNA damage as compared to untreated cells, accompanied by morphological damage to the cell nucleus and nuclear fragmentation. It was suggested that propolis might act by inhibiting DNA replication and indirectly block cell division (Ota et al., 2001). In addition, previous studies indicated that propolis extract exerted an apoptotic effect and promoted cell cycle arrest on cancer cell lines (Aru et al., 2019). However, the DNA ladder pattern was not found in the EEP1-treated fungal cells, which is found in many apoptotic systems as the result of internucleosomal DNA cleavage. Apoptotic cell death in certain yeast cells has also been shown to occur in the absence of a DNA ladder (Mousavi, 2004). The mechanisms by which *C. albicans* undergoes apoptosis are still unclear (de Castro et al., 2013).

The Hungarian propolis caused leakage of cellular metabolites after 2 h by the disruption of the cell membrane. In addition, the effect on DNA integrity in the presence of a subinhibitory dose of EEP1 after 1 h of treatment may indicate that the primary target of propolis extract is the nucleus and cell membrane as a secondary mechanism of action.

6. Conclusion

Propolis is one of the excellent natural antimicrobial agents. Hungarian propolis samples showed diversity and richness in terms of phenolic and flavonoid contents. It was characterized by the presence of genistein and chrysin in high amounts. The propolis extracts have strong antimicrobial effects on the planktonic cells of *C. albicans* and *S. aureus*. On the other hand, the concentration and treatment time have a critical role in inhibiting bacterial and fungal virulence, because it was found that propolis can protect and stimulate the mature biofilm with high concentrations. However, Propolis is a promising agent to inhibit the biofilm formation in the early phase of *Candida* infections, as well as it was able to penetrate and eradicate the matured biofilm of *S. aureus*, and decrease the viability of the cells. The interactions of EEP with antibiotics to combat the emergence of multidrug-resistant bacterial infections need further investigation for the *in vivo* application. The gDNA and the fungal cell membrane are the most probable targets of the Hungarian propolis. Hence, this study supports the use of propolis as a novel natural substance to fight against candidiasis and MRSA infections.

7. Future perspectives

The application of propolis in the pharmaceutical and food industries requires an understanding of the chemical composition and its mode of action on pathogens. The variation in botanical origin is an important factor that can determine the ideal biological properties for each sample. Therefore, future research need to standardizes and characterize the different types of propolis from all over the world. However, the activity of components of propolis can be based on a single action or synergistic interaction of some chemicals. Antibiotic resistance is a global health challenge, raising the need to search for alternative methods of controlling MDR pathogens. Future researchers should focus on the biological activity of each component of propolis alone and in combination to be able to determine the exact chemical and ratios of chemicals that is contributing to the therapeutic activity of propolis. Also, it is necessary to condense the effort to deeply study the effect of propolis *in vivo*. Lastly, it is important to carry out advanced techniques to find the mechanism of action of propolis extracts on the microbial cells side by side with the cells of a host organism.

8. Summary in English

Propolis is a complex substance of exudates that honeybees collect from various plant sources, it is widely used in folk medicine. The main objectives of this study were to characterize the chemical composition of the ethanolic extracts of Hungarian propolis (EEP), evaluate the antifungal, antibacterial, and antibiofilm effects on *Candida albicans*, methicillin-resistant and sensitive *Staphylococcus aureus* (MRSA and MSSA), as well as to understand the possible mode of action on fungal cells. Total phenolic and flavonoid contents of EEP were measured using colorimetric assays. The chemical composition was investigated by GC-MS. The antimicrobial effect was determined by measuring the minimum inhibitory concentrations (MIC) using the broth microdilution method. In addition, the virulence activity was evaluated according to the biofilm prevention and eradication by crystal violet assay, as well as the examination of germ tube formation. The mechanisms of action involved in the cytotoxic effect were examined by sorbitol, ergosterol, and 260 nm efflux assays. On the other hand, Interaction between EEP1 and antibiotics was assessed by the chequerboard broth microdilution method. Hungarian propolis contains considerable amounts of phenolics and flavonoids. The chemical analysis identified the presence of compounds that belonged to different groups, chrysin and genistein were the most dominant compounds in all the extracts. The EEP effectively inhibited the growth of the planktonic cells of *C. albicans* and *S. aureus* at 200 and 50 μg/mL, respectively. The propolis treatments prevented the formation of fungal and bacterial biofilm as well as the formation of germ tubes. Moreover, the biomass of mature biofilms formed by *S. aureus* was eradicated, in parallel with the decrease in the metabolic activity of the cells. However, the MIC value was unable to degrade the *C. albicans* mature biofilms, but rather promoted it. On the other hand, the propolis extract caused concentration and time-dependent damage to the cell's viability and the permeability of the cellular membrane. Nevertheless, the cells showed very quick uptake of propolis that caused DNA fragmentation and nucleus damage. Synergistic interactions have been determined after the co-exposition of EEP and vancomycin on *S.aureus*. Hence, the simultaneous application of EEP and vancomycin could enhance its effect against MRSA infection. The Hungarian propolis demonstrated strong antifungal and antibacterial activities, due to the richness of bioactive components. Propolis extract approves the efficiency of this natural substance, and the ability to use it as an alternative agent. However, the treatment duration and concentration should be well known.

9. Összefoglalás (Summary in Hungarian)

A propolisz a népi gyógyászatban széles körben használt összetett váladékanyag. A tanulmány fő célja egyrészt a magyarországi propolisz etanolos kivonatok (EEP) kémiai összetételének jellemzése, valamint a *Candida albicans*-ra és meticillin-rezisztens és érzékeny *Staphylococcus aureus*-ra gyakorolt antimikrobiális és antibiofilm hatásának felmérése volt. További célként tűztük ki az EEP-nek a *C. albicans* sejtekre gyakorolt hatásmódjának megismerését. Az EEP összfenol és -flavonoid tartalmát kolorimetriás módszerekkel, a kémiai összetételt GC-MS-sel határoztuk meg. Az EEP antimikrobiális hatását a minimális gátló koncentrációk (MIC) meghatározásával, mikrohígításos módszerrel végeztük. A biofilm képzésre és a biofilm degradációra gyakorolt hatását kristályibolya-módszerrel vizsgáltuk, valamint a *C. albicans* esetében a csíratömlő képzésre gyakorolt hatását fénymikroszkóppal határoztuk meg. A citotoxicitásban szerepet játszó feltételezett hatásmechanizmusokat, amelyek a *C. albicans* sejtmembránjára és sejtfalára irányultak, szorbit-esszével, ergoszterin-esszével és a 260 nm-en abszorbeáló anyagok sejtbőli kiáramlásának mérésével, a genotoxikus hatást pedig DNS-fragmentációval detektáltuk. Az EEP felvételének kinetikáját fotometriás módszerrel, különböző antibiotikumokkal vizsgált kölcsönhatását a checkerboard-titrálás módszerével értékeltük. Az EEP-k kémiai elemzése különböző csoportokba tartozó vegyületek jelenlétét azonosította. A legdominánsabb vegyület a krizin és a genistein volt mindegyik kivonatban. *C. albicans* esetében 200 µg/mL, míg *S. aureusnál* 50 µg/mL EEP hatékonyan gátolta a planktonikus sejtek növekedését. A propolisz kezelések hatékonyan megakadályozták a biofilmek képződését, valamint gátolták a *C. albicans* csíratömlők keletkezését. Ezen túlmenően a *S. aureus* által alkotott érett biofilmek biomasszája lecsökkent, ezzel párhuzamosan csökkent a sejtek metabolikus aktivitása is. A MIC-val történő kezelés azonban nem tudta lebontani a *C. albicans* érett biofilmjét, inkább elősegítette a biofilm további fejlődését. A hatásmechanizmus vizsgálatok tekintetében indirekt módon vizsgálva az EEP koncentráció- és időfüggő módon befolyásolta a 260 nm-en abszorbeáló intracelluláris anyagok kiáramlását. Azonban a gomba sejtmembránjában az ergoszterinhez való direkt kötődést vagy a sejtfal károsodásával kapcsolatos direkt hatást nem észleltünk. Ennek ellenére a sejtek gyorsan felvették az EEP-t, amely DNS-fragmentációt és sejtmagkárosodást okozott. Szinergikus kölcsönhatásokat mutattunk ki az EEP és vancomycin együttes használatát követően *S. aureus*-on. A magyarországi propolisz erős antimikrobiális hatást fejt ki, és intenzíven gátolja a biofilmek és csíratömlők képződését. Kutatási eredményeink igazolják ennek a természetes anyagnak a hatékonyságát és alternatív szerként való felhasználását.

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12. List of publications and conferences

12.1. Article related to this thesis

Papp, Z., **Bouchelaghem, S.**, Szekeres, A., Meszéna, R., Gyöngyi, Z., Papp, G., **2021**. The scent of antifungal propolis. Sensors 21, 2334. https://doi.org/10.3390/s21072334, (Q2, IF: 3.576)

Bouchelaghem, S., 2021. Propolis characterization and antimicrobial activities against *Staphylococcus aureus* and *Candida albicans*: A review. Saudi Journal of Biological Sciences 29, 4. https://doi.org/10.1016/j.sjbs.2021.11.063, (Q1, IF: 4.219)

Bouchelaghem, S., Das, S., Naorem, R.S., Czuni, L., Papp, G., Kocsis, M., **2022**. Evaluation of total phenolic and flavonoid contents, antibacterial and antibiofilm activities of Hungarian propolis ethanolic extract against *Staphylococcus aureus*. Molecules 27, 574. https://doi.org/10.3390/molecules27020574, (Q1, IF: 4.412)

12.2. Article not related to this thesis

Khallef, M., Benouareth, D.E., Konuk, M., Liman, R., **Bouchelaghem, S.**, Hazzem, S., Kerdouci, K., **2019**. The effect of silver nanoparticles on the mutagenic and the genotoxic properties of the urban wastewater liquid sludges. Environ Sci Pollut Res 26, 18403–18410. https://doi.org/10.1007/s11356-019-05225-8 (Q2, IF: 3.172)

12.3. Conference presentations related to this thesis

Sarra Bouchelaghem ; Sourav Das ; Lilla Czuni ; Zoltán Gazdag ; Csaba Fekete ; Tamás Kőszegi ; Romen Naorem ; Gábor Papp. Antimicrobial activities of Hungarian propolis alone and in combination with anitbiotics and its antibiofilm activity on Staphylococcus aureus. XVI. János Szentágothai Multidisciplinary Conference and Student Competition – Abstracts. Pécs, Hungary: János Szentágothai Scholastic Honorary Society, Faculty of Sciences, University of Pécs (2019) pp. 16-16. , 1 p. Publication: 30648441 Published Core Chapter in Book (Abstract) Scientific

Sarra Bouchelaghem ; Hedvig Dalma Kurnász ; Réka Meszéna ; Lilla Czuni ; Zoltán Gazdag ; Csaba Fekete ; Matthias Kun-Paul ; Gábor Papp. Dose dependent virulence and cytotoxic effect of Hungarian propolis on *Candida* albicans. XVI. János Szentágothai Multidisciplinary Conference and Student Competition – Abstracts. Pécs, Hungary: János Szentágothai

Scholastic Honorary Society, Faculty of Sciences, University of Pécs (2019) pp. 246-246. , 1 p. Publication: 30648501 Published Core Chapter in Book (Abstract) Scientific

Réka Meszéna ; Hedvig Dalma Kurnász ; **Sarra Bouchelaghem** ; Lilla Czuni ; Zoltán Gazdag ; Csaba Fekete ; Gábor Papp. The effect of Hungarian propolis samples on proliferation and biofilm formation on *Candida* strains. XVI. János Szentágothai Multidisciplinary Conference and Student Competition – Abstracts. Pécs, Hungary: János Szentágothai Scholastic Honorary Society, Faculty of Sciences, University of Pécs (2019) pp. 249-250. , 2 p. Publication: 30648486 Published Core Chapter in Book (Abstract) Scientific

12.4. Conference presentations not related to this thesis

Kurnász Hedvig Dalma ; Meszena Réka ; Czuni Lilla ; **Bouchelaghem Sarra** ; Rich Zoltán ; Urban Peter ; Fekete Csaba ; Papp Gábor. Investigation of the antifungal and voriconazole interaction effects of Hungarian propolis samples. Mycological publications-clusiana 56 : 1 pp. 114-115. , 2 p. (2017). Publication: 3331498 Published Core Journal Article (Abstract)

13.Appendix

I. Materials and methods

a. The microorganisms

Table 1: The microorganisms used in this study. (*pvl*): Panton-Valentine leukocidin gene, (*mecA*): gene found in methicillin-resistant *S. aureus* (MRSA), (*erg*): ergosterol biosynthesis genes, (*ade*): adenine gene, (ND) no data.

Name	Laboratory code	Standard reference	Genotype
Candida albicans	001	ATCC 44829	*erg*$^{+}$, *ade*$^{-}$
Candida albicans	SZMC 1424	Clinical isolate	ND
Candida albicans	SZMC 1425	Clinical isolate	ND
Candida albicans	SZMC 1426	Clinical isolate	ND
Staphylococcus aureus	MRSA	ATCC 700699	*pvl*$^{-}$ SCC*mec* type II
Staphylococcus aureus	MSSA	ATCC 29213	*mec*A^{-}
Staphylococcus aureus	SA H24	Clinical isolate	ND
Staphylococcus aureus	SA H23	Clinical isolate	ND

b. The media used for cells culture

1) YPD + adenine medium

Add 1 L of distilled water to 20 g dextrose, 10 g peptone, 5 g yeast extract, and 25 mg adenine (and 20 g of agar for YPD plates). Sterilize by autoclaving for 20 min at 100°C. Store the YPD medium at 4°C.

2) RPMI-1640

Add 1 L of distilled water to 18 g glucose, 10.5 g RPMI-1640, 25 mg adenine, 4 mg thiamine, and 2 mg biotin. Adjust the pH to 7.2 using HCl or NaOH. Then, Sterilize with 0.2 µm filter into sterile bottles. Store the medium at 4°C.

3) TSB + 0.25% Glucose

Add 800 mL of distilled water to 30 g tryptic soy agar (BD™, Heidelberg, Germany), and 2.5 g glucose (and 20 g of agar for TSB plates). Then, complete the volume until 1 L with distilled water. Sterilize by autoclaving for 20 min at 100°C. Store the medium at 4°C.

4) MM media

Add 1 L of distilled water to 10 g glucose, 5 g $(NH_4)2SO_4$, 0.4 g KH_2PO_4, 0.5 g $MgSO_4$, 25 mg adenine, 4 mg thiamine, and 2 mg biotin. Adjust the solution to pH ≈ 7.4, then sterilize the solution by autoclaving for 20 min at 100°C. Store the medium at 4°C.

5) Muller Hinton broth (MH) + 2% NaCl

Add 800 mL of distilled water to 20 g NaCl, 17.5 g acid hydrolysate of casein, 3 g beef extract, and 1.5 g starch. Then, complete the volume until 1 L with distilled water. Sterilize by autoclaving for 20 min at 100°C. Store the medium at 4°C.

6) Crystal violet 0.13%

Add in glass bottle, 940.7 mL of PBS, 54.05 mL formalin 37%, 5.2 mL ethanol 99%, and 1.3 g crystal violet. Pass the solution through a paper filter and store at room temperature in dark.

7) PBS puffer

Prepare 800 mL of distilled water in a glass beaker, and add 8 g of NaCl, 0.2 g KCl, 1.8 g of Na_2HPO_4; $2H_2O$, 0.24 g of KH_2PO_4. Adjust the solution to pH= 7.4, then complete the volume until 1 L with distilled water. Sterilize by autoclaving for 20 min at 100°C. Store the puffer at room temperature.

c. Testing reagents and chemicals

The main chemicals that were used in the study are: 4′,6-Diamidino-2-Phenylindole (DAPI) (Sigma), Acetic acid glacial (Sigma), Adenine sulfate >98% (Fluka Chemie AG), Aluminium chloride 99% (Alfa Aesar), Amphotericin B (AmB) (Sigma), Catechin (Sigma), Cefoxitin sodium salt 89% (Sigma), D-sorbitol anhydrous (Sigma), Ergosterol ≥95% (Sigma), Ethanol absolute (Sigma), Ethylenediaminetetraacetic acid (EDTA) (Sigma), Folin-Ciocalteu's reagent (VWR chemicals), Gallic acid (Sigma), Horse serum (Sigma), Hydrogen peroxide solution 30% (Sigma), Lauryl sulfate sodium salt (SDS) (Calbiochem), Methanol 99% (Sigma), MOPS 99.5% (Sigma), Oxacillin sodium salt 95% (Sigma), Phenol-chloroform (Sigma), Propidium iodide (Sigma), Resazurin (Sigma), RPMI-1640 Medium (Sigma), Trisaminomethane (Tris) (Sigma), Vancomycin (Sigma).

d. Propolis samples

Table 2: The ethanolic extracts of propolis (EEP) samples and the regions of the raw propolis.

Sample	Extract	Origin	GPS coordinates
1	**EEP1**	Pécs	Elevation: 133 m; Latitude: 46°4'21.85"N; Longitude: 18°13'56.16"E
2	**EEP2**	Szombathely	Elevation: 215 m; Latitude: 47°13'50.47"N; Longitude: 16°37'18.64"E
3	**EEP3**	Szolnok	Elevation: 85 m; Latitude: 47°9'43.69"N; Longitude: 20°10'56.9"E
4	**EEP4**	Csikóstőttős	Elevation: 137 m; Latitude: 46°20'19.67"N; Longitude: 18°9'32.79"E
5	**EEP5**	Héhalom	Elevation: 134 m; Latitude: 47°46'43.43"N; Longitude: 19°35'17.35"E]
6	**EEP6**	Somogybabod	Elevation: 156 m; Latitude: 46°40'10.7"N; Longitude: 17°46'36.57"E

e. Instruments used

The instruments used in this study are: Sanyo orbital incubator (Auro-Science Consulting Kft., Budapest, Hungary), incubator (Thermo Scientific Heraeus Function Line B12, Langenselbold, Germany), Hettich centrifuge (Rotina 420R, Auro-Science, Budapest, Hungary), spectrophotometer (Hitachi U-2910, Tokyo, Japan), GC-MS QP2020 (Shimadzu,

Duisburg, Germany), plate reader (Thermo Multiskan EX, Berlin, Germany), PerkinElmer EnSpire multimode plate reader (Auro-Science Consulting Ltd., Budapest, Hungary), light microscope (Nikon Eclipse 80i, Tokyo, Japan). The Agilent 4200 TapeStation system (Agilent Technologies, Santa Clara, CA, US), fluorescence microscope (Nikon Eclipse 80i, Tokyo, Japan).

II. Results

a. Spectrophotometric analysis of UV-visible spectra

Table 3: Absorption of UV-Vis spectra of propolis extracts. All samples collected from six different regions showed 3 peaks at the similar wavelengths.

Peaks (nm)	EEP1	EEP2	EEP3	EEP4	EEP5	EEP6	Average (SD)
Peak 1	267.0	266.5	265.5	**289.6**	268.5	267.2	**271 (8.5)°**
Peak 2	292.6	**291.6**	293.1	293.1	292.6	292.9	**293 (0.5)**
Peak 3	322.3	**326.6**	321.2	**327.8**	324.9	324.4	**325 (2.5)**

b. Biofilm formation of *C. albicans*

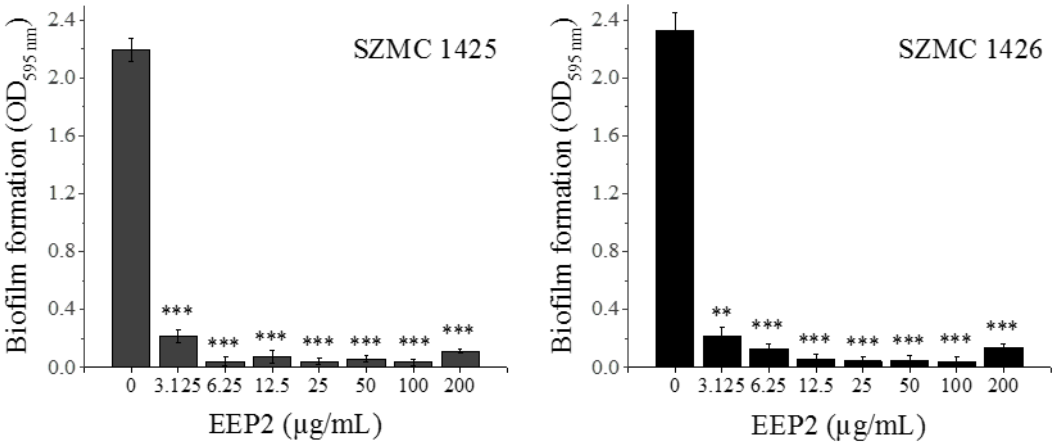

Figure 1: The biofilm formation of *C. albicans* strains (SZMC 1425 and SZMC 1426) in the presence of 3.125-200 µg/mL of propolis extract (EEP2) and the absence of propolis extract (control) after 48 h incubation at 37°C, using crystal violet assay.

c. Biofilm eradication of *C. albicans*

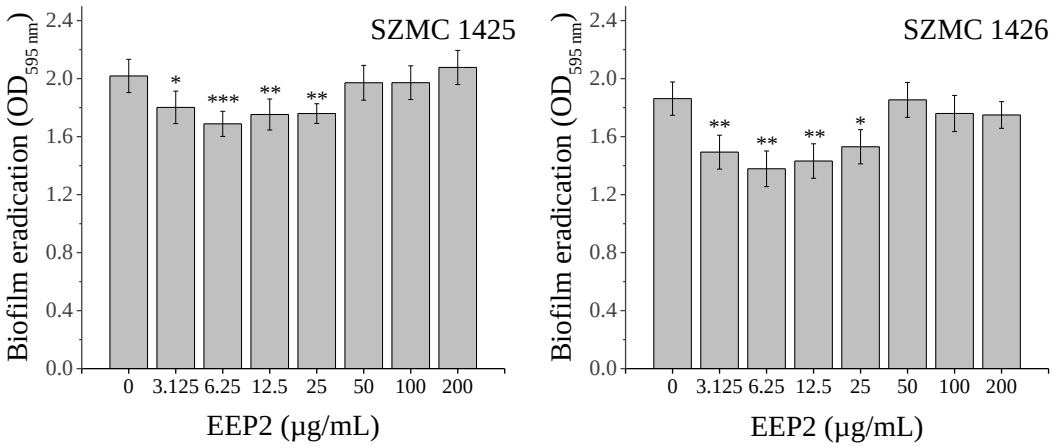

Figure 2: Effect of propolis extract (EEP2) at the concentrations of 3.125-200 µg/mL on preformed biofilms of *C. albicans* strains (SZMC 1425 and SZMC 1426). The cells were cultivated for 24 h at 37°C to form biofilms, then the mature biofilms were treated with propolis extracts for an additional 24 hours. Using the crystal violet assay, the absorbance of biofilm biomass was measured by spectrophotometer.

d. Germ tube formation (GTF)

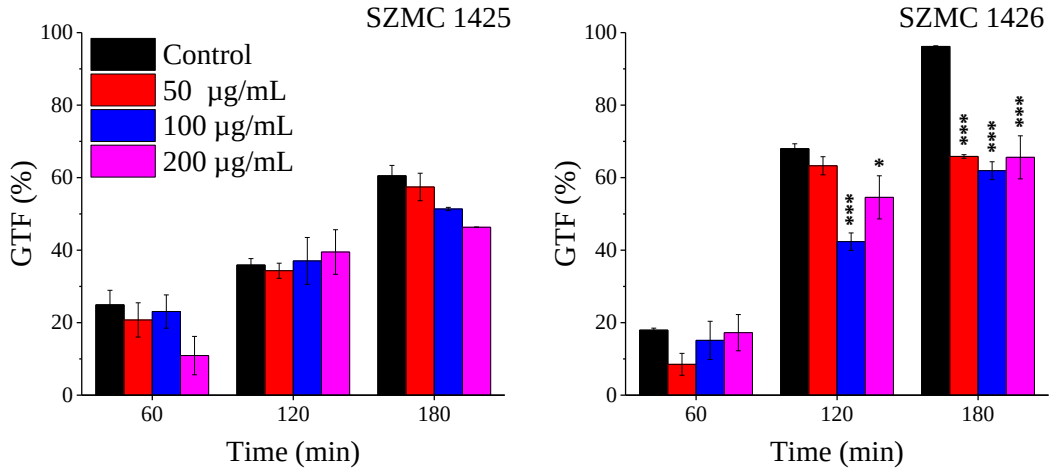

Figure 3: The effect of propolis extracts (50, 100 and 200 µg/mL of EEP2) on germ tube formation of *C. albicans* SZMC 1425 and SZMC 1426. After the incubation in an orbital incubator at 37°C for 60, 120, 180 min, the cells were counted using a light microscope.