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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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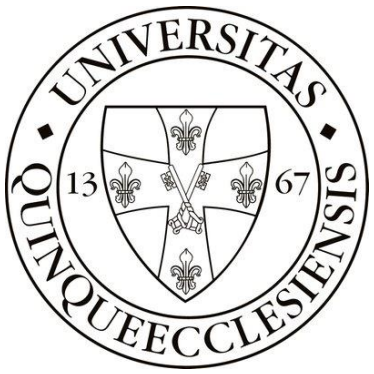

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1. Introduction

Staphylococcus aureus and *Candida albicans* are ubiquitous opportunistic pathogens that are widely distributed in our environment, food, skin and mucous membranes of healthy humans and animals (Lee et al., 2019; Todd and Peters, 2019). *S. aureus* and *C. albicans* are important nosocomial and community acquired human pathogens that can cause mild to severe illnesses (Todd and Peters, 2019). Emergence of antibiotic resistant staphylococcal and *candida* infections has resulted in significantly higher mortality and morbidity rates in both developed and developing countries (Koehler et al., 2019; Todd and Peters, 2019). Natural plant-based products and synthetic chemistry are two main fields to which scientific attention has shifted in the quest to develop new, economical, and potent antimicrobial agents in order to treat and prevent infectious diseases (Abreu et al., 2012; Anand et al., 2019). Propolis, commonly known as "bee glue" is a resinous and balsamic secretion of flowers, branches, shells, leaves, barks and buds of various plants. Honeybees transform this sticky substance by the aid of their salivary secretions and beeswax into propolis (Elnakady et al., 2017). Since ancient times, propolis has been used as a traditional folk medicine alone or in combination with other natural substances to treat wounds (Przybyłek and Karpiński, 2019; Wagh, 2013). Literature has demonstrated that propolis possesses several biological activities including antibacterial, antiviral, antifungal, anticancer, antioxidant, and antitumor activities (AL-Ani et al., 2018; Elnakady et al., 2017; Moreira et al., 2008; Silva-Carvalho et al., 2015). Some studies have shown that propolis exhibits synergistic effects and enhances the efficacy of conventional antibiotics when tested in combination (Stepanović et al., 2003). However, the standardization and the mechanism of action of propolis extracts remains a challenge (Silva-Carvalho et al., 2015; Toreti et al., 2013).

2. Aims

The application of propolis in the pharmaceutical industry requires an understanding of its chemical composition and biological properties. The chemical composition of propolis is quite complex and variable for different reasons. 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 analysis of chemical composition of propolis samples from different geographical regions in Hungary.
- Antibacterial, antifungal, and antibiofilm activities of ethanolic extracts 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 a new information about the action of this complex substance as an antifungal agent. Therefore, the bioabsorption of EEP, and 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 (ATCC 44829 used in all experiments), and (SZMC 1424 used for examining the biofilm 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-sensitive *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.

3.2. Raw material of propolis and the extractions method

Raw propolis samples were collected from 6 regions of Hungary; Pécs (1), Szombathely (2), Szolnok (3), Csikóstőttős (4), Héhalom (5), and Somogybabod (6). The samples were extracted in 80 % (v/v) ethanol at 70 °C for 30 min. The ethanolic extracts of propolis (EEP) were stored at 4 °C in dark (Alencar et al., 2007).

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). Gallic acid standard solutions were used for constructing the calibration

curve (y= 85.344x - 0.0053; R^2= 0.9995). The TPC was expressed as mg of gallic acid equivalents (GAE)/g of propolis dry weight (DW).

3.3.2. Determination of total flavonoids content (TFC)

The flavonoids were determined by the aluminum chloride colorimetric method, as reported by Dias et al., 2012. Catechin standard solutions 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 (CAE) per gram of propolis dry weight (DW).

3.3.3. Spectrophotometric analysis of UV-visible spectra

The propolis extracts were scanned to obtain the absorption spectra between 200 and 400 nm wavelengths (Mărghitaş et al., 2013).

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

The trimethylsilyl (TMS) ether derivatives of EEP were subjected to GC-MS analysis. The detected compounds were identified by analysing both chromatograms and mass spectra using the Hexane solution of C7-C33 *N*-alkanes, 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 CLSI M11-A8 standard broth microdilution method (CLSI, 2012). The minimum inhibitory concentrations (MIC) values were determined.

3.4.2. Fungicidal and adaptation essay

The fungicidal effect of EEP1 on *C. albicans* cells was determined before and after the pre-treatment of the cells with subinhibitory concentration of EEP. After incubation for 48 h at 30 °C, the number of colony-forming units (CFU) were estimated. The minimum fungicidal concentrations (MFC) values were determined.

3.4.3. Antibacterial susceptibility testing of *S. aureus*

S. aureus strains were tested for their susceptibility to EEP1 sample, oxacillin, cefoxitin, and vancomycin using broth microdilution method (CLSI, 2012). The MIC values were determined.

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) on *S. aureus* (CLSI, 2012). The fractional inhibitory concentration index (FICI) was calculated (AL-Ani et al., 2018).

3.5. Effect of propolis extract on virulence activity

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

The effect of EEP1 and EEP2 on biofilm formation was investigated in 96-well flat-bottom microtiter plates on the two strains of *C. albicans* (ATCC 44829 and SZMC 1424). The absorbance of crystal violet dye which is proportional to the thickness of the biofilm was measured by a microtiter plate reader at 595 nm.

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

The cell suspension was treated with EEP1 into 96-well microtiter plates. For the quantification, the absorbance of crystal violet fixed with biofilm biomass was measured at 595 nm. 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 (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 *C. albicans* 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 mature biofilm of *S. aureus* was determined as previously mentioned (See Section 3.5.2.), except inoculum were allowed to grow initially for 24 h aiming biofilm formation prior to the addition of the EEP1. 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. Furthermore, the minimum biofilm eradication concentration ($MBEC_{50}$) was computed as the lowest concentration that eradicate at least 50% of biofilm (Miao et al., 2019).

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

The fungal cultures were suspended in horse serum, treated with EEP, and incubated in an orbital shaker at 150 rpm (Li and Kim, 2014). The GTF was quantified under a light microscope.

3.6. Mechanism of action of EEP

3.6.1. Biosorption of EEP

The concentration of EEP1 decrease in *C. albicans* suspension was measured at 295 nm with a spectrophotometer.

3.6.2. Effect of propolis on cell wall

Two-fold serial dilutions of EEP1 was applied on *C. albicans* in the presence and absence of 0.8 M D-sorbitol (Ferreira et al., 2014).

3.6.3. Effect of propolis on cell membrane

The cells were treated with EEP1, then the supernatants was measured at 260 nm with a spectrophotometer (Gucwa et al., 2018; Horváth et al., 2010). For the membrane protection assay, the susceptibility of *C. albicans* was determined following microdilution technique (CLSI, 2012) in the presence of ergosterol. Amphotericin B (AmB) was used as a positive control (Ferreira et al., 2014).

3.6.4. DNA fragmentation and nuclei damage

The DNA of treated cells was extracted according to the modified method of Suman et al. (2012). Briefly, the cells were harvested and resuspended in TE buffer then disrupted in liquid nitrogen. The phenol-chloroform method was applied for the DNA purification and precipitation. The Agilent Genomic DNA ScreenTape assay was used to determine the fragmented DNA content. On the other hand, the cells were stained with DAPI (4′,6-diamidino-2-phenylindole) to quantify the damaged nuclei using fluorescence microscope.

3.7. Statistical analysis

All experiments were carried out in triplicates. Analysis variance (ANOVA) for multiple comparisons between the groups and graphics were made using OriginPro 2016 software. Differences between samples were considered significant when $p < 0.05$.

4. Results

4.1. Chemical characterization of propolis samples

4.1.1. Determination of the total phenolic and total flavonoid contents

The TPC values were found in the range of 10.4-71.1 mg GAE/g, and TFC values were found in the range of 33.8-273.2 mg CAE/g. The EEP1 sample represented the highest concentration of phenols and flavonoids and showed 6.8 to 8.1 times higher contents than that of the EEP6 sample. 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 TPC and TFC.

4.1.2. Spectrophotometric analysis of UV-visible spectra

The EEP samples showed 3 peaks at the same wavelengths (λ_1 = 269 nm± 0.37, λ_2 = 292 nm± 0.27, λ_3 = 322 nm± 1.46). All the propolis samples spectra of EEP having a maximum absorbance at λ_{max} = 292 nm. EEP1 exhibited the highest absorption compared to the others EEP spectra. However, The EEP6 showed significantly low absorption. This results is in agreement with the TPC and TFC determination. Therefore, the results may indicate a similarity in the quality of the chemical compounds that constitute all of propolis samples but in different quantities.

4.1.3. Chemical constituents of propolis extracts

The biological activities of EEP samples are highly dependent on its chemical composition. A total of 122 individuals and derivatives were identified in all EEP samples, while the EEP1 and EEP2 samples composed 114 different compounds, the EEP3, EEP4, EEP5, and EEP6 samples consisted of 110, 101, 116, and 85 compounds, respectively. However, thirty compounds covered 93-96% of the total amount of chemicals. The 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. The category of flavonoids and isoflavonoids showed the highest amounts in all samples with a total relative concentration ranged from 52.9% to 34.5%. The identified flavonoids were represented mainly in chrysin and genistein. The second largest quantity was for the category of phenols (cinnamic acids and cinnamate ester) in the strong samples. However, the monosaccharaides and the sugars showed a considerable amounts in the sample EEP6.

4.2. Antimicrobial effect of selected samples

4.2.1. Antifungal activity on *C. albicans*

All the extracts showed concentration-dependent susceptibility. The extracts were potent on *C. albicans* cells and showed MIC in the range of 100-200 μg/mL, except the sample of EEP6 that was significantly weaker compared to the other samples.

4.2.2. EEP induced cytotoxicity and adaptation

The colony-forming units (CFU) of *C. albicans* ATCC 44829 cells were examined after exposure of cells to MIC and 2 × MIC of EEP1 for 30 and 60 min. The CFU was decreased by 78% and 98% after 30 and 60 min at 200 μg/mL, respectively. Therefore, the MFC value of EEP1 was equal to 200 μg/mL. Nevertheless, the pretreatment of cells with a subinhibitory concentration of EEP1 for 1 h caused a considerable increase in the survival rate.

4.2.3. Antibacterial activity of propolis on *S. aureus*

The susceptibility of MSSA was confirmed and shows MIC at 0.25 μg/mL, 4 μg/mL, and 1 μg/mL, for oxacillin, cefoxitin and vancomycin, respectively. MRSA was intermediate to vancomycin and resistant to oxacillin and cefoxitin. The two clinical isolates were resistant to oxacillin and cefoxitin but sensitive to vancomycin. All the strains were very sensitive to EEP1 with MIC of 25 μg/mL.

4.2.4. Killing effect of EEP in combination with antibacterial drugs

The FICI result of the 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 MIC 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. Although, these concentration combinations did not show complete inhibition due to the high cell number.

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

4.3.1. Influence of EEP on biofilm formation of *C. albicans*

The two strains of *C. albicans* showed different degrees of biofilm formation. Although *C. albicans* ATCC 44829 was a weak biofilm former, its biofilm formation was significantly

blocked at 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 ($p < 0.001$). 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 significantly reduced by 84% in the presence of 3.125 μg/mL EEP2.

4.3.2. Effect of propolis extract on the eradication of *C. albicans* mature biofilm

The challenge of EEP1 and EEP2 to destroy the mature biofilms was examined on the *C. albicans* SZMC 1424 (strong biofilm former strain). Surprisingly, 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 of 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.

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. 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 EEP1. 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. Additionally, there was no significant effect observed after 2 h treatment neither with both of the propolis extracts. The exposure of the both strains to EEP2 showed a similar effect. The MIC of both EEP suppressed the germination effectively after 3 h, but it did not exceed the inhibition of 58%.

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

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 MRSA, 100 μg/mL for the two MRSA clinical isolates (SA H23 and SA H24), and 200 μg/mL for MSSA. Interestingly, MRSA was the most sensitive to propolis treatment.

4.3.5. Effect of propolis extract on the eradication of *S. aureus* mature biofilm

The EEP1 significantly enhanced the biofilm degradation in each strain and showed $MBEC_{50}$ values of 15, 18, 48, and 52 µg/mL against MRSA, SA H23, SA H24, and MSSA, respectively. The thickness of MSSA and SA H24 biofilms was degraded at 200 µg/mL of EEP1 by 47% and 87%, respectively. However, the most sensitive biofilm was observed in the case of MRSA and SA H23 strains at 50 µg/mL, where the degradations of the mature biofilms were 88% and 71%, respectively. 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 *S. aureus* strains within the biofilm up to 90% at 200 µg/mL. The significant decrease in cellular metabolic activity was proportional to the increase in dead cells.

4.4. Mechanism of action of EEP

4.4.1. Propolis biosorption kinetics

The initial concentration of EEP1 was reduced by 54% after 5 min of incubation. The decrease of EEP1 in the extracellular medium reached a maximum of 66% after 60 min, indicating a rapid biosorption and bioaccumulation process. The EEP1 uptake was saturated after 1 h of incubation whereas the cells were able to uptake approximately 131 µg/mL by the end of 4 h of incubation.

4.4.2. Effect of EEP1 on cell wall integrity, membrane permeability, and ergosterol binding

The MIC values of EEP1 were 200 µg/mL in presence and absence of sorbitol, 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. The time-dependent leakage of intracellular substances that absorb light at 260 nm was determined. 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-candidal effect.

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. In this study, no significant increase in survival rate was observed in the presence of ergosterol. Thus, the effect of EEP1 on *C. albicans* through binding with fungal ergosterol is negligible.

4.4.3. DNA fragmentation and nuclei damage

The DNA extracted from the untreated cells made a clear single band at 15000 bp which indicated intact gDNA. The treatment 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. Hence, DNA fragmentation could be the principal reason for EEP1-induced cell death. The DAPI staining assay showed a 3.6-fold increase of nuclei damage at 200 μg/mL EEP1 compared to the negative control.

5. Conclusion

The emergence of resistant strains has stimulated the discovery of new therapeutic agents. Propolis is one of the natural antimicrobial agents that can controls biofilm-associated infections. This study illustrated the richness and diversity of Hungarian EEP samples in terms of phenolic and flavonoid contents, such as chrysin, genistein, and cinnamate. The propolis extracts have strong antimicrobial effects on *C. albicans* and *S. aureus*. On the other hand, the concentration and the time of the treatments have a critical role in the inhibition of bacterial and fungal virulence. It 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*. The observations support that the genomic DNA and the fungal cell membrane are the most probable targets of the propolis components. However, the interactions of EEP with antibiotics to combat the emergence of multidrug-resistant bacterial infections need further investigation for the *in vivo* application. Hence, the mode of action of propolis remains problematic because of the significant variation of chemical compositions between samples. This study represents an innovative option to fight against candidiasis and MRSA infection. Further research should be extended to biotic surfaces.

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7. List of publications and conferences

7.1. 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. 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)

Cumulative impact factor (IF): 12.207

7.2. 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 sludge. Environ Sci Pollut Res 26, 18403–18410. https://doi.org/10.1007/s11356-019-05225-8
(Q2, IF: 3.172)

7.3. 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

7.4. 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)