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

Ph.D. thesis

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PÉCS, 2021.

I. Introduction

In recent years, the biological activities of natural products have received much attention because of the increasing interest in human health (Bernardini et al., 2018; Er, 2021; Gyawali and Ibrahim, 2014; Kokoska et al., 2019; Okińczyc et al., 2020). One of the most important natural products that are commonly used for their antimicrobial properties is propolis. It is produced by honeybees from resins and plant exudates. Plants protect themselves from pathogenic microbes primarily by producing secondary metabolites termed phytochemicals, many of which have been isolated and used in medicine for their antioxidant and antimicrobial properties (Berretta et al., 2020). Phytochemical compounds that are promising as antimicrobial agents in humans include all components of propolis (Tiku, 2018). These antimicrobial properties can be recognized by honeybees, which selectively collect these plant products and process them to make propolis (Berretta et al., 2020).

Propolis is used in folk and modern medicine for the prevention and treatment of colds, wounds and ulcers, rheumatism, sprains, heart disease, and diabetes (Huang et al., 2014). It has diverse biological properties such as anti-inflammatory (Wang et al., 2013), antimicrobial, antioxidant, antitumor (Bankova et al., 2000; Huang et al., 2014), antiulcer and anti-human immunodeficiency virus (HIV) activities (Ito et al., 2001). Its antioxidant and antimicrobial properties provide scope for use in food technology as a food preservative. One of the most important advantages is that, unlike the other preservatives, its residues may have a generally favorable effect on health (Bahtiti, 2013).

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

The Arabic coffee (*Coffea arabica* L.) is also another important natural product. It has been thought to be a good source of antioxidants for humans, because of the high total phenolic content and caffeine. The chemical profile of Arabic coffee includes phenolic compounds and their derivatives (such as chlorogenic acids), alkaloids (mainly caffeine), diterpenoid alcohols (such as cafestol and kahweol), carbohydrates, lipids, and volatile and heterocyclic compounds (Affonso et al., 2016; Magharbeh et al., 2020).
(Affonso et al., 2016; Magharbeh et al., 2020)

II. Aims of the study

1. In the first part of this study, we aimed to investigate the effect of propolis ethanolic extract (PEE) on the planktonic growth and biofilm-forming abilities of five commercial probiotics (Normaflore® oral suspension, Enterol®, BioGaia® ProTectis® Baby, Linex® Forte, and Protexin® Restore).
2. In the second part, we aimed to investigate the effect of caffeic acid phenethyl ester (CAPE) on planktonic growth, biofilm-forming ability, and cell death in different strains of *C. albicans*, *C. tropicalis*, *C. glabrata*, and *C. parapsilosis*. These *Candida* species were selected due to the high clinical importance as the main causative agents of candidiasis. The strains were selected to represent high biofilm-formers (*C. albicans* SZMC 1424, *C. tropicalis* SZMC 1366, *C. glabrata* SZMC 1374, and *C. parapsilosis* SZMC 8007) and non-biofilm formers (*C. albicans* ATCC 44829, *C. albicans* SZMC 1423, *C. tropicalis* SZMC 1512, *C. glabrata* SZMC 1378, and *C. parapsilosis* SZMC 8008).
3. In the third part of this study, we aimed to study the molecular interaction between CAPE and G- and F-actin. We also examined the ability of CAPE to cause a detectable functional change in the conformation of G- and F-actin.
4. In the last part of the study, we aimed to study the antioxidant properties of the methanolic extract of Arabic coffee beans (*Coffea arabica*) and its fractions.

III. Materials and methods

1. Effect of PEE on probiotics

1.1. Preparation of PEE

Poplar-type propolis sample was collected from a local beekeeper in Pécs/Hungary. It was extracted with ethanol 80% (v/v) in a water bath, at 70 °C, for 30 min and then filtered with a 0.22 μm vacuum filter to obtain its ethanolic extract (Alencar et al., 2007).

1.2. Antimicrobial susceptibility testing of probiotics planktonic cells against PEE

Determination of minimal inhibitory concentration (MIC) by the broth microdilution method was performed based on the recommended protocol of the National Committee for Clinical Laboratory Standard Institute (CLSI, 2012, 2007, 2002).

1.3. Probiotics biofilm-forming ability assay

In vitro biofilm formation by probiotics was assayed by the ability of cells to adhere to the wells of a 96-well tissue culture microplate. Biofilm quantification was done spectrophotometricaly based on crystal violet binding assay as described by Stepanović and co-workers (Stepanović et al., 2007).

1.4. Normaflore biofilm eradication assay

The effect of PEE on mature biofilms was verified as described by Nostro and co-workers (Nostro et al., 2007). Biofilm quantification was done spectrophotometricaly based on crystal violet binding assay as described by Stepanović and co-workers (Stepanović et al., 2007).

1.5. Normaflore autoaggregation assay

Autoaggregation ability was investigated as described by Jeon and co-workers (Jeon et al., 2017).

1.6. Normaflore swarming motility assay

Swarming motility was determined according to the size of the growth on semi-solid agar plates as described by O'May and co-workers (O'May et al., 2012).

2. Effect of CAPE on different *Candida* species

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

The broth microdilution method was performed to determine the minimal inhibitory concentration (MIC) based on the recommended protocol of the National Committee for Clinical Laboratory Standards Institute (CLSI, 2002).

2.2. *Candida* biofilm susceptibility assay and effect of CAPE on Candida biofilm-forming ability

In vitro biofilm formation by *Candida* was assayed by the ability of cells to adhere to the wells of a 96-well tissue culture microplate. Biofilm quantification was done spectrophotometricaly based on crystal violet binding assay as described by Stepanović and co-workers (Stepanović et al., 2007).

2.3. *Candida* biofilm eradication assay

The effect of CAPE on mature biofilms was verified as described by Nostro and co-workers (Nostro et al., 2007). Biofilm quantification was done spectrophotometricaly based on crystal violet binding assay as described by Stepanović and co-workers (Stepanović et al., 2007).

2.4. Biosorption of CAPE by *Candida* cells

Biosorption of CAPE was determined by measuring the remaining CAPE in the media after incubation for 2 h as described by Sun and co-workers with some modifications (Sun et al., 2015).

2.5. Cell death examination assay

Cells were treated with sub-lethal concentrations (MIC_{80}) of CAPE and incubated at 35 °C with shaking for 3 hours. Cell death examination was done using CF®488A Annexin V and Propidium iodide (PI) apoptosis kit according to the manufacturer's instructions to identify apoptosis and necrosis using flow cytometry.

2.6. Caspase inhibitor assay

Caspase inhibitor assay was performed as described by Yue and co-workers with some modifications (Yue et al., 2013).

2.7. Ultrastructural examination of *Candida* species by TEM

The apoptotic and necrotic markers were visualized using transmission electron microscope as described by Phillips and co-workers (Phillips et al., 2003).

3. Effect of CAPE on actin

3.1. Actin preparation

The skeletal α-actin was prepared and provided from acetone-dried muscle powder of rabbit skeletal muscle according to the method of Spudich and Watt (Spudich and Watt,

1971). Actin was labeled with N-(1-pyrene) Iodoacetamide based on the method of Criddle and co-workers (1985).

3.2. Anisotropy measurements

In steady-state anisotropy measurements, the anisotropy of unlabeled actin was monitored using the intrinsic fluorescence of tryptophan in the absence or presence of CAPE (Ujfalusi et al., 2012).

3.3. Actin polymerization assay

The polymerization assay was based on the fact that the fluorescence intensity of pyrene-bound actin increases by a factor of ~25 upon polymerization. The increment in the fluorescence intensity of pyrene-labeled actin was detected using fluorimeter (Bugyi et al., 2006).

3.4. Differential scanning calorimetry

The thermal denaturation of the actin was examined between 20 and 100°C using differential scanning calorimeter (Bugyi et al., 2006).

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

4.1. Plant material

The Arabic coffee beans were dried at 45 ºC overnight and afterward ground to powder.

4.2. Preparation of the plant extract and subsequent fractions

Ground beans were exhaustively defatted with n-hexane for 8 hr. The defatted beans were soaked again in 400 mL methanol for 8 hr. The total methanol extract was concentrated till dryness under vacuum. The methanol extract residue was then fractionated against three different solvents namely, ethyl acetate, acetone, and isopropanol using the Soxhlet apparatus.

4.3. Antioxidant activity assay

The antioxidant activity was determined using DPPH radical scavenging assay (Tepe et al., 2005).

IV. Results

1. Effect of PEE on free-living probiotic cells

PEE can reduce the planktonic growth of all tested probiotics. However, lower concentrations of PEE improved the viability of Linex, BioGaia, and Protexin microbes which are mostly containing anaerobic and/or microaerophilic bacteria. Similarly, the viability of those microbes was also improved when different concentrations of the antioxidant glutathione treatment was applied. Different probiotics have varied MIC_{80} values in the range of 100 – 800 µg/mL. BioGaia, which contains *L. reuteri* DSM 17938, has the highest MIC_{80} value (800 µg/mL), and this might be due to the antibiotic-producing properties of this strain.

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

It was found that Protexin and Enterol microbes were non-biofilm formers under the applied conditions. On the other hand, the microbes of BioGaia, Linex, and Normaflore can form weak biofilms. The weak biofilm-forming probiotics have various responses to the PEE treatments. The biofilm of BioGaia microbes was inhibited by the lowest concentration of PEE (12.5 µg/mL). However, the same concentration of PEE has a slight positive effect on the biofilm-forming ability of Linex microbes, but it still falls to the weak biofilm category. Interestingly, the biofilm of Normaflore microbes showed a unique property not only to tolerate higher concentrations of PEE but moreover, it has been enhanced and shifted from weak to strong biofilm at 12.5, 25 and, 50 µg/mL concentrations.

3. Effect of PEE on mature biofilms of Normaflore

Application of different concentrations of PEE revealed that the mass of the mature biofilm was improved up to 400 µg/mL. Whereas biofilm eradication was observed at 800 µg/mL. Interestingly, the mature biofilms of Normaflore microbes were shifted from weak to strong at low concentrations of PEE (12.5 and 25 µg/mL). Concerning planktonic cells which were found in the suspension above the biofilm, PEE revealed a dose-dependent inhibitory effect on their growth.

4. Effect of PEE on autoaggregation in Normaflore

PEE has a significant stimulatory effect on the autoaggregation ability of Normaflore microbes. After 24 hours of incubation, the autoaggregation rate at 12.5, 25, and 50 µg/mL of PEE was about 9, 14, and 21% higher than the negative control, respectively.

5. Effect of PEE on swarming motility in Normaflore

PEE has a significant inhibitory effect on the swarming motility of Normaflore microbes compared to the untreated group. The rate of swarming motility decreased about 12, 22, and 33% when treated with 12.5, 25, and 50 µg/mL of PEE, respectively.

6. Susceptibility of *Candida* planktonic cells to CAPE

It has been found that CAPE has a strain and dose-dependent effect. The MIC_{80} values were ranging from 12.5 to 100 µg/mL. The highest inhibitory effect was seen against *C. glabrata* SZMC 1378, *C. glabrata* SZMC 1374, and *C. parapsilosis* SZMC 8008 compared to the other strains. Whereas, the most resistant strain was *C. albicans* SZMC 1423.

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

CAPE has a species and dose-dependent inhibitory effect on the biofilm formation in the four strains. The minimum biofilm inhibitory concentrations (MBIC) were 50, 50, 50, and 100 µg/mL for *C. albicans* SZMC 1424, *C. glabrata* SZMC 1374, *C. parapsilosis* SZMC 8007, and *C. tropicalis* SZMC 1366, respectively.

8. Effect of CAPE on *Candida* biofilm eradication

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

9. Biosorption of CAPE by *Candida* cells

The biosorption of CAPE by different *Candida* strains occurs very quickly, and the maximum biosorption was achieved within the first 30 to 90 minutes. According to the amount of CAPE biosorbed, two groups can be recognized: the first group was able to biosorb 53-63 µg/mL of CAPE and it includes *C. albicans* SZMC 1424, *C. parapsilosis* SZMC 8007, and *C. parapsilosis* SZMC 8008; while the second group was able to biosorb 74-86 µg/mL and it includes *C. albicans* ATCC 44829, *C. albicans* SZMC 1423, *C. tropicalis* SZMC 1366, *C. tropicalis* SZMC 1512, *C. glabrata* SZMC 1374, and *C. glabrata* SZMC 1378.

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

CAPE can induce apoptosis in six of the tested strains at different levels. Among these strains, *C. albicans* SZMC 1423 and *C. albicans* ATCC 44829 revealed the highest percentage of early apoptotic cells (69.8 and 70.2%, respectively), whereas almost no apoptosis was seen in *C. glabrata* SZMC 1374, *C. parapsilosis* SZMC 8008, and *C. glabrata* SZMC 1378 (apoptotic cells ≤ 2%). On the other hand, no necrosis was seen in any of the tested strains (necrotic cells ≤ 1%).

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

A significant increase in the viability of CAPE-treated *C. albicans* ATCC 44829, *C. albicans* SZMC 1424, *C. tropicalis* SZMC 1366, and *C. tropicalis* SZMC 1512 that are pre-incubated with pan-caspase inhibitor Z-VAD-FMK was observed; this means that apoptosis in these strains is caspase-dependent. However, the viability of CAPE-treated *C. albicans* SZMC 1423 and *C. parapsilosis* SZMC 8007 was not affected with the pre-incubation with pan-caspase inhibitor Z-VAD-FMK; which means that apoptosis in these strains is caspase-independent.

12. Visualization of apoptotic and necrotic markers

The TEM micrographs of *C. tropicalis*, *C. albicans*, and *C. parapsilosis* mainly revealed typical hallmarks of apoptosis, including nuclear chromatin margination, nuclear blebs, condensation in the nucleus, vacuolization, plasma membrane detachment, huge lysosomes, cytoplasm fragmentation, cell wall distortion, and whole-cell shrinkage. However, very few cells displayed the signs of necrosis, such as membrane disintegration and loss of cytoplasm density. Whereas, the TEM micrographs of *C. glabrata* mainly revealed smaller necrotic signs.

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

The steady-state anisotropy of G-actin was not altered in the presence of 50 μg/mL CAPE, thus the flexibility of the protein matrix did not change which indicates no considerable effect for CAPE on G-actin.

14. Effect of CAPE on actin polymerization

There are no significant differences in the fluorescence signal measured in the absence and presence of different concentrations of CAPE. This means that CAPE has no effect on actin polymerization.

15. Effect of CAPE on the thermal stability of actin

The T_m values of G-actin and F-actin in absence of CAPE were 55.53 °C and 66.09 °C, respectively. While the T_m values of G-actin and F-actin in the presence of CAPE were 56.08 °C and 65.89 °C, respectively. These results indicated that there is no significant effect for CAPE on the thermal stability of both G- and F-actins.

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

The IC_{50} values of the methanolic extract and its subsequent fractions ranged from 25.08 to 337.32 mg/mL. The best free radical scavenging activity (reflected by the smallest IC_{50} value) was exerted by the total methanolic extract followed by the acetone fraction. Whereas the lowest antioxidant activity was observed in the isopropanol fraction. The antioxidant capacity of the different fractions has been found to increase with decreased solvent polarity

V. Conclusions

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

1. PEE has a probiotic product and concentration-dependent effect on the viability and biofilm-forming ability of the probiotics *in vitro*. PEE, in certain cases, can act as a prebiotic at low concentrations, however, at higher concentrations, it may inhibit the planktonic growth and biofilm-forming ability of the probiotics. At low concentrations, PEE can enhance the growth of the biofilms of Normaflore microbes (*B. clausii*), which could be due to the enhanced autoaggregation and the inhibited swarming motility. While, at high concentrations, PEE can eradicate the biofilms of Normaflore microbes (*B. clausii*). Therefore, more attention should be paid for the selection of the appropriate probiotics used for the treatment of dysbiosis, and on the other hand for the simultaneous application of PEE. The present observations showed limitations for the co-application of PEE and probiotics and adumbrated a potential double-face action of PEE on the natural gut microbiota.
2. CAPE has a concentration and strain-dependent antifungal effect on the viability and biofilm-forming ability of the different *Candida* species. Moreover, it has a moderate activity to eradicate the mature biofilms of biofilm-forming strains of *Candida*. CAPE has a species and strain-dependent cell death response in *Candida*. Therefore, CAPE could be considered as a promising drug nominee for antifungal therapy.

3. CAPE has no significant effect on the anisotropy, polymerization, and thermal stability of actin protein.
4. Methanolic extract of *C. arabica* beans and its acetone fraction have relatively high antioxidant properties as compared to the ethyl acetate and isopropanol fractions. Thus, they are considered as interesting sources for natural antioxidant compounds that have many applications in food and pharmaceutical industries.

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- **Publications related to thesis topic**

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

- **Conference proceedings related to thesis topic**

1. **Ibrahim Alfarrayeh**, Csaba Fekete, Zoltán Gazdag, Gábor Papp. Propolis ethanolic extract has double-face in vitro effect on the planktonic growth and biofilm formation of some commercial probiotics. In: Csiszár, B; Hankó, Cs; Kajos, L F; Kovács, O B; Mező, E; Szabó, R; Szabó-Guth, K (eds.). 9th Interdisciplinary Doctoral Conference 2020 Book of Abstracts.

Pécs, Hungary: Doctoral Student Association of the University of Pécs (2020) 384 p. pp. 319-319.,1 p.

2. **Ibrahim Alfarrayeh**, Csaba Fekete, Zoltán Gazdag, Gábor Papp. Antifungal and antibiofilm effects of caffeic acid phenethyl ester on different Candida species. In: Csiszár, Beáta; Hankó, Csilla; Kajos, Luca Fanni; Mező, Emerencia (eds.) Medical Conference for PhD Students and Experts of Clinical Sciences: Book of Abstracts. Pécs, Hungary: Doctoral Student Association of the University of Pécs. (2020). 83 p. pp. 54-54., 1 p.
3. **Ibrahim Alfarrayeh**, Csaba Fekete, Zoltán Gazdag, Gábor Papp. Antimicrobial and antibiofilm effects of propolis ethanolic extract on some commercial probiotics. In: Bódog, Ferenc; Csiszár, Beáta (eds.) VIII. Interdiszciplináris Doktorandusz Konferencia 2019: absztraktkötet=8th Interdisciplinary Doctoral Conference 2019: Book of Abstracts. Pécs, Hungary: Doctoral Student Association of the University of Pécs. (2019). 185 p. pp. 12-12., 1 p.
4. **Ibrahim Alfarrayeh**, Csaba Fekete, Zoltán Gazdag, Gábor Papp. Effect of propolis ethanolic extract on the growth and biofilm formation ability of some commercial probiotics. In: XVI. János Szentágothai Multidisciplinary Conference and Student Competition – Abstracts. Pécs, Hungary: János Szentágothai Scholastic Honorary Society, Faculty of Sciences, University of Pécs (2019) pp. 252-252., 1 p.

- **Publications outside to thesis topic**

  No publications outside the thesis topic

- **Conference proceedings outside thesis topic**

  No conference proceedings outside the thesis topic