Propolis Extracts Enhance the Antimicrobial Activity of Slovenian: Chestnut, Forest and Flower Honeys

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Abstract: Because of the overuse and misuse of antibiotic, an increase in antibiotic resistance of pathogenic bacteria is evolving. Attention should be focused on natural alternatives to antibiotics, like propolis and honey. They all have strong antibacterial properties because of the active substances they contain. In the present study, the influence of P1 (water-soluble propolis (WSP) Greit 120) or P2 (phosphate buffer saline (PBS) propolis extract Lj. Center pH = 8.0) on antimicrobial activity of different Slovenian: chestnut honey (CH), forest honeys (FoH), and flower honey (FIH) against various Gram-positive or Gram-negative bacteria and yeast *Candida albicans* are shown. Gram-positive bacteria used in the experiments were: methicillin-resistant *Staphylococcus aureus*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus agalactiae* and *Streptococcus mutans*. The yeast was *Candida albicans*. Gram-negative bacteria used were: *Escherichia coli*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. P1, P2, FoH, CH, FIH and their combinations were prepared in saline as 10% solutions. The composition of both propolis extracts and the three kinds of honey was determined by reverse-phase high-performance liquid chromatography (RP-HPLC). It expressed the antimicrobial activity as a minimal inhibitory concentration (MIC) in mg/mL. Each of the two propolis extracts showed antimicrobial activity when applied alone. Also, the application of each of honey yielded an antimicrobial activity in decreasing order: CH, FoH and FIH honey. The combination of any of the propolis extract with any of the honey showed the additive antimicrobial effect on either Gram-positive or Gram-negative bacteria and on yeast *Candida albicans*. The effectiveness of P1 or P2 depends on their nature and from the sort of honey and is the highest in the case of CH followed by FoH and FIH.

Key words: Honey, propolis, flavonoids, antimicrobial activity, enhancement.

1. Introduction

Propolis (sometimes also referred to as bee glue) is a resinous natural product, produced by bees (*Apis mellifera*), from vegetable parts and secretions. Bees use it mainly as a sealer to cover the hive interior and to repair fissures as well as to protect against pathogen

microorganisms. Propolis is normally composed of 45% resins, 30% waxes and fatty acids, 10% essential oils, 5% pollen, and 10% organic compounds and minerals [1, 2]. More than 300 compounds, among them flavonoids, glycans, phenolic acids and their esters, phenolic aldehydes, alcohols, ketones, terpenes, steroids, sugars, and amino acids have been detected in raw propolis, but the proportion of each compound differs greatly with botanical and geographical factors, as well as the collection season [3-5]. The compounds

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present in propolis are the explanation for some of its biological activities as antimicrobial and antioxidant one. They are responsible for the great interest by the pharmaceutical industry and health-food stores, being used in foods, beverages, cosmetics and medicine to improve health and prevent diseases [6, 7]. As propolis is a hard resinous substance, it cannot be consumed in its natural form. Its usual way of preparation is by powdering the resinous, followed by extraction in an alcoholic or aqueous medium.

Flavonoids are one of the most important constituents and can represent around 50% of the propolis contents, depending on the region where it is collected, since its characteristics are influenced by botanical, geographical factors and weather. The antioxidant activity of propolis from various geographic origins was studied and different activities were found for each region. Its properties were investigated confirming its antibacterial, antiviral, antifungal and anti protozoal activities among others. Due to its antimicrobial activity, ethanol propolis extracts inhibit the growth of *Streptococcus* sp. [8] and are more active against Gram-positive bacteria than against Gram-negative ones [9].

The effect of the pH variation during the extraction of flavonoids and polyphenols has been studied working with different vegetal species. The pH variation could have a positive or a negative effect on extraction, depending on the interaction of the polyphenols with other constituents of each plant.

The most common propolis in Brazil is usually collected in the southeast region, characterized as group 12 (Brazil has 12 different groups of propolis, with distinct characteristics), and presents a great number of soluble substances with antimicrobial activity against *Staphylococcus aureus* and *Str. mutans*. Activity of propolis originating from various geographic origins was studied and different activities were found specific for each region. Its properties were investigated confirming its antibacterial, antiviral, antifungal and anti protozoal activities.

Considering the widespread use of propolis, the objective was to evaluate the effect of pH variation on propolis extraction, prepared with water as a solvent.

Different pH values were tested to water as a solvent, and were compared with samples without pH variation. Final water extracts of propolis were quantified regarding flavonoids and phenol contents to verify the relation between the pH and water in the extraction efficiency. Analyses of antimicrobial and antioxidant activities show the strongest antimicrobial activity of propolis extract at pH = 8.0 [10].

Honey is a complementary remedy for the treatment of infected wounds particularly where conventional modern therapeutics fails. Among honey types, certainly, the most effective Manuka honey was reported to exhibit antimicrobial activity against pathogenic bacteria like: *S. aureus* and *Helicobacter pylori* making it promising agent for the treatment of wounds or even stomach ulcer [11]. The beneficial role of honey is attributed to the beneficial property that is comprised of high osmolarity, acidity (low pH) and content of hydrogen peroxide (H_2O_2) as well as non-peroxide components, i.e., the presence of phytochemical components, like methylglyoxal (MGO). The H_2O_2 is the most important antimicrobial agent in the honey. Its concentration is determined by relative levels of glucose oxidase, synthesized by the bee and catalase originating from flower pollen [12, 13]. Most types of honey generate H_2O_2 when diluted, because of the activation of the enzyme glucose oxidase that oxidizes glucose to gluconic acid and H_2O_2 , which thus attributes the overall antimicrobial activity [14].

The healing properties of honey can be ascribed to the fact that it offers the antibacterial activity, maintains a moist wound environment to promote healing and has a high viscosity that helps to form a protective barrier to prevent infection [15, 16]. On the other hand, proteins with glycosidase activity can induce tumor necrosis factor-alpha (TNF- α) secretion

by macrophages. This cytokine is known to induce the mechanism of wound healing.

In the present study, the influences of P1 (water-soluble propolis (WSP) Greit 120) or P2 (phosphate buffer saline (PBS) propolis extract Lj. Center pH = 8.0) on the antimicrobial activity of different Slovenian honeys: chestnut honey (CH), forest honey (FoH) and flower honey (FlH) against various Gram-positive or Gram-negative bacteria and yeast *Candida albicans* are shown.

2. Materials and Methods

2.1 Samples

2.1.1 Species of Microorganisms Used in the Experiments

Different clinical isolates of Gram-positive bacteria: methicillin-resistant *S. aureus* (MRSA), *S. aureus*, *Str. pyogenes*, *Str. agalactiae*, *Str. mutans* and yeast *C. albicans* were used. The Gram-negative clinical isolates were: *Escherichia coli*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. All clinical isolates used in the experiments, were obtained from microbe collection of the Institute for Microbiology and Immunology, Medical Faculty in Ljubljana, Slovenia. All microbes were first cultivated on Mueller-Hinton agar at 37 °C for 48 h and afterward, were transferred to Mueller-Hinton broth, until the concentration of 0.5 McFarland was obtained.

2.1.2 Propolis

Two types of propolis extracts were used in this study. P1 by BNatural, Corbetta, Italy was used in dilution 1:10 w/v in saline. The P2 was prepared in the laboratory and also used in dilution 1:10 w/v in saline.

2.2 Methods

2.2.1. P1

By the new technology developed by Fachini and Volpi [17, 18] the P1 was extracted. This new multi-step extraction technology is called dynamic multi extraction (M.E.D.). It allows modifying some

parameters such as solvent degree, temperature, pH, etc., during the extraction phase thanks to a series of modular equipment and according to the raw material to be worked out. The results are then standardized extracts richer in integral polyphenols (phenolic acids, bioflavonoid aglycons and glucosides). The propolis samples (P1) were processed as follows: (1) aqueous extraction, to remove waxes and impurities from raw materials, using a 1:1 solvent/propolis ratio, at 80 °C for 10 h and with 100 watt ultrasounds. After cooling at 8 °C, the solution was filtered with a 30 µm filter; (2) three hydro-alcoholic extractions, one for each insoluble residue of the preceding extraction step, carried out using different alcoholic degrees and temperatures, from 4 h to 36 h, with a fixed 1:1 solvent/propolis residue ratio. Each extraction step was followed by a sample cooling step at circa 15 °C, a filtration step with 30-50 µm filters and a concentration step using a rotating evaporator, to obtain a soft mixture; (3) concentration: the combined extracts were mixed and concentrated to a residual humidity value ranging from 15% to 20% by weight; (4) dissolution in glycerin: the concentrated extracts were evaporated to remove ethanol. The dough was mixed with hot glycerin and water for 2 h within a mixer and then cooled at 10 °C to give a non-alcoholic liquid. After precipitation the solution was filtered twice, using a 30 µm and a 10 µm filter.

2.2.2 P2

The P2 was prepared as follows: (1) propolis sample "Lj. center" (10 g) was frozen at -40 °C for 3 d; (2) partially refrozen sample was grounded in mortar and resuspended in 100 mL PBS with pH 8.0; (3) the suspension was shaken at 37 °C for 5 d and it was grounded in mortar and shaken at 37 °C for additional 3 d; (4) the suspension was put into 50 mL tubes and then put into microwave oven at 300 MW for three times × 2 min with 10 min break; (5) the P2 suspension was centrifuged at 5,000 rpm for 20 min, +4 °C; (6) supernatant was sterilized by filtration

through 0.2 μm syringe filters and stored at $-20\text{ }^{\circ}\text{C}$ till use.

2.2.3 Quantity of Caffeic Acid, Chrysin, Pinocembrin and Galangin in P1 and P2

The amounts of the caffeic acid, chrysin, pinocembrin and galangin in P1 and P2 were determined by the method of Urushisaki *et al.* [19] as follows: 1.0 mg of caffeic acid, chrysin, pinocembrin and galangin were put and diluted to 10.0 mL with methanol. From this solution, 150 μL of the sample was transferred into a vial and loaded with 1.350 μL of methanol. Samples filtered through a 0.45 μm filter were injected by 20 μL into the reverse-phase high-performance liquid chromatography (RP-HPLC) column Purospher® Star RP-18 end-capped (5 μm). Their separation was achieved with acetonitrile gradient in RP-HPLC column.

The P2 was analyzed on the Purospher® STAR RP-18 end-capped (5 μm) RP-HPLC column. Its

separation measured at 290 nm, with acetonitrile gradient is presented in Fig. 1. The quantity of total flavonoids, caffeic acid, chrysin, pinocembrin and galangin in the experimental sample of P2 was calculated in comparison to standards.

2.2.4 Estimation of Total Flavonoid Content by Folin-Ciocalteu (FC) Colorimetric Method

The FC was performed by the method of Blainski *et al.* [20] and Filipič *et al.* [21] as follows: phenol compounds react with an FC reagent in a basic medium and colored product is formed. The absorbance is measured at 750 nm. Results are expressed as g of caffeic acid per 100 g of the sample (g/100 g). Procedure goes like: pipette 200 μL of sample or standard and adds 1.0 mL of FC reagent and 1.0 mL of 10% Na_2CO_3 . Mix well and after 1 h the absorbance at 750 nm is measured against water. Each sample and standard is analyzed in duplicate. The results are expressed as caffeic acid in mg/mL.

Fig. 1 HPLC-UV-ESI-MS504971 of flavonoid's profile of an average sample of P1.

1 = phenolic acids (caffeic, coumaric, ferulic, isoferulic); 2 = quercetin; 3 = pinobanksin 5-methyl ester; 4 = quercetin 3-methyl ester; 5 = pinobanksin; 6 = apigenin; 7 = kaempferol; 8 = isorhamnetin; 9 = luteolin 5-methyl ester; 10 = quercetin 5-7-dimethyl ester; 11 = galangin 5-methyl ester; 12 = quercetin 7-methyl ester; 13 = chrysin; 14 = pinocembrin; 15 = galangin; 16 = pinobanksin-3-*O*-acetate; 17 = caffeic acid phenethyl ester (CAPE); 18 = metoxychrysin; 19 = pinobanksin-3-*O*-propionate; 20 = caffeic acid cinnamyl ester; 21 = pinobanksin-3-*O*-butyrate; 22 = pinobanksin-3-*O*-pentenoate; 23 = other pinobanksin derivative; 24 = pinobanksin-3-*O*-hexanoate; 25 = other pinobanksin derivative.

2.2.5 Determination of the Minimal Inhibitory Concentration (MIC)

The MIC values determinations were performed according to Resazurin method [22, 23] as follows. Here, 96 well “U” profile microtitre plates (8 × 12 wells) were used. Into the 2nd column (eight wells) until the 12th column, the 50 µL of saline was put. Into the 1st column (eight wells), 100 µL of samples was put as follows: in the 1st plate samples, in wells 1-4 and into wells 5-8 in the 1st column; in the 2nd plate samples, in wells 9-11 + untreated control in the 1st column; into the 3rd plate, penicillin, streptomycin, gentamycin and nystatin were put into wells in duplicate in the 1st column. Then, 50 µL was transferred from the 1st column into the 2nd, 3rd and until 11th, where 50 µL was discharged. Into each plate on each well, 50 µL of test microorganisms in the concentration of 0.5 McFarland was added, and the plate was wrapped into aluminum foil and incubated for 48 h at 37 °C. After incubation is finished, 20 µL of Resazurin (Sigma-Aldrich) (0.0028 g of Resazurin dissolved in 10 mL of distilled water, to which 90 mL of eagle’s minimal essential medium (EMEM) was added) and plates were wrapped into aluminums foil and incubated at room temperature for 4 h. The following is the growth inhibition of bacteria after the staining with Resazurin. The Resazurin has a blue color. The absence of microbe’s growth inhibition results in the change of the color of Resazurin from blue to pink. MIC was determined as the highest dilution, resulting in no or the minimal change in color. For example, in the 7th well, there is a change from pink to blue. So, color change in the 7th well with

dilution 1:64, MIC = $1 \times 10/64 = 0.156$ mg/mL.

2.2.6 Percent of Microbial Growth Inhibition

The percent of microbial growth inhibition was measured by the two-fold dilution of the substance, from the 1st to 11th well in the row, and then different microbes were added (0.5 McFarland). After 24 h of incubation at 37 °C, 20 µL of Resazurin was added. The plates were incubated and wrapped in aluminums foil for 4 h at room temperature. After the incubation, it was detected, in which well the growth inhibition occurred. The per cent of microbial growth inhibition was calculated as follows: if the growth inhibition was found in the 4th well, the percent of bacterial/fungal growth inhibition = $4/12 \times 100 = 33.33\%$.

2.2.7 Scheme of the Experiments

The following combinations between P1, P2 and Slovenian CH, FoH and FIH were used in the experiments (Table 1).

2.3 Statistical Analysis

The statistical significance was calculated by the two-tailed Student’s *t*-test. In order to compare the difference in susceptibility between honey alone and honey with the addition of P1 or P2, the MICs for different honey samples were pooled.

3. Results

3.1 Molecular Composition of P1 Determined by HPLC-UV-ESI-MS 504971

Fig. 1 shows the HPLC-UV-ESI-MS504971 profile and in Table 2, the molecular composition of P1 in a very precise way.

Table 1 Scheme of the experiments.

		P1 ^a	P2 ^b
		10% solution in saline	10% solution in saline
CH ^c	10% solution in saline	90% of CH + 10% of P1	90% of CH + 10% of P2
FoH ^d	10% solution in saline	90% of FoH + 10% of P1	90% of FoH + 10% of P2
FIH ^e	10% solution in saline	90% of FIH + 10% of P1	90% of FIH + 10% of P2

^a WSP Greit 120; ^b PBS extract of propolis Ljubljana Center pH 8.0; ^c chestnut honey; ^d forest honey; ^e flower honey.

Table 2 The molecular composition of P1 determined by HPLC-UV-ESI-MS504971.

Number on chromatogram	Polyphenols species	Greit 120 (% w/v)
1	Phenolic acids (caffeic, coumaric, ferulic, isoferulic)	0.5
2	Quercetin	1.5
3	Pinobanksin 5-methyl ester	1.4
4	Quercetin 3-methyl ester	5.0
5	Pinobanksin	4.0
6	Apigenin	1.1
7	Kaempferol	3.8
8	Isorhamnetin	3.3
9	Luteolin 5-methyl ester	2.1
10	Quercetin 5-7-dimethyl ester	1.7
11	Galangin 5-methyl ester	1.1
12	Quercetin 7-methyl ester	3.2
13	Chrysin	5.0
14	Pinocembrin	2.9
15	Galangin	7.5
16	Pinobanksin-3- <i>O</i> -acetate	9.5
17	CAPE	0.3
18	Metoxychrysin	1.1
19	Pinobanksin-3- <i>O</i> -propionate	1.2
20	Caffeic acid cinnamyl ester	0.2
21	Pinobanksin-3- <i>O</i> -butyrate	5.7
22	Pinobanksin-3- <i>O</i> -pentenoate	2.6
23	Other pinobanksin derivative	0.7
24	Pinobanksin-3- <i>O</i> -hexanoate	0.3
25	Other pinobanksin derivative	0.4
26	Other pinobanksin derivative	3.3
27	Other pinobanksin derivative	0.5
28	Other pinobanksin derivative	0.2
29	Other pinobanksin derivative	0.7
Total identified polyphenols		70.8
Phenolic acids and derivatives		1.0
Flavones and flavonols		36.4
Flavanones and dihydroflavonoles		33.4

Source: data were kindly provided by Prof. Dr. Nicola Volpi (Department of Life Sciences, University of Modena & Reggio Emilia, Modena 41125, Italy).

3.2 Quantity of Caffeic Acid, Chrysin, Pinocembrin and Galangin in P2

The P2 was analyzed on the Purospher® STAR RP-18 end-capped (5 µm) RP-HPLC column.

The quantity of total flavonoids, caffeic acid, chrysin, pinocembrin and galangin in mg/mL is shown in Fig. 2 and are as follows: total flavonoids 10.42 ± 1.23 mg/mL, caffeic acid 2.44 ± 0.18 mg/mL, caffeic

acid 1, 1.43 ± 0.11 mg/mL, chrysin 0.15 ± 0.08 mg/mL, pinocembrin 0.46 ± 0.04 mg/mL and galangin 0.62 ± 0.05 mg/mL.

3.3 Physico-Chemical Properties of Slovenian Honeys: FoH, CH and FIH

The physico-chemical properties of Slovenian CH, FIH and FoH were obtained from doctoral dissertations of Bertoncelj [24].

Fig. 2 The presence of different flavonoids in the P2.

1 = caffeic acid; 2 = caffeic acid 1; 3 = cresin; 4 = pinocembrin; 5 = galangin.

3.4 Antimicrobial Activity of P1, P2, FoH, CH and FIH

3.4.1 Antimicrobial Activity of P1

Recently, P1 became important because of being a buffer for problems with respiratory health. In the treatment of upper respiratory tract the hydroglyceric extract of P1 is used. Besides, P1 shows also quite strong antimicrobial activity, which is stronger against Gram-positive bacteria than against Gram-negative bacteria. The antimicrobial activity of P1 is present in Table 3 and Fig. 3. For the Gram-positive bacteria, the range of MIC is between 0.007 ± 0.001 mg/mL and 0.015 ± 0.007 mg/mL. Against Gram-negative bacteria, MIC of P1 lies between 0.015 ± 0.007 mg/mL and 0.031 ± 0.015 mg/mL. For *C. albicans*, the MIC is 0.015 ± 0.007 mg/mL. The antibacterial activity of propolis is usually strong against Gram-positive bacteria and *C. albicans*.

3.4.2 Antimicrobial Activity of P2

Propolis extract P2 presented in Table 3 and Fig. 3 shows the antimicrobial activity for the Gram-positive bacteria where the range of MIC (mg/mL) lays between 0.007 ± 0.0035 and 0.031 ± 0.015 . For the Gram-negative bacteria, the MIC (mg/mL) is between 0.031 ± 0.015 and 0.062 ± 0.015 . MIC (mg/mL) for *C. albicans* is 0.031 ± 0.015 .

3.5 Antimicrobial Activity of Slovenian Kinds of Honey

In Table 3 the antimicrobial activities of different Slovenian kinds of honey are shown.

3.5.1 CH

In concentration of 10% in saline, the antibacterial activity of Slovenian CH was stronger (Fig. 4) for Gram-positive bacteria with MIC (mg/mL) of 0.015 ± 0.007 and 0.062 ± 0.015 , than for Gram-negative bacteria, where the value of MIC (mg/mL) lies between 0.015 ± 0.007 and 0.031 ± 0.015 . The MIC (mg/mL) for *C. albicans* is 0.015 ± 0.007 .

3.5.2 FoH

In concentration of 10% in saline, the antibacterial activity is surprisingly the same for Gram-positive and Gram-negative bacteria (Fig. 4). MIC (mg/mL) values are between 0.015 ± 0.007 and 0.062 ± 0.015 . Quite strong the FoH is against *C. albicans* with MIC (mg/mL) 0.007 ± 0.0035 .

3.5.3 FIH

The weakest among Slovenian honeys is the FIH (Fig. 4). The MIC (mg/mL) values for Gram-positive bacteria lays between 0.007 ± 0.0035 and 0.250 ± 0.030 and for Gram-negative bacteria between 0.031 ± 0.015 and 0.125 ± 0.015 . The MIC (mg/mL) value for *C. albicans* is also weak 0.062 ± 0.015 .

Table 3 MIC (mg/mL) and growth index (GI)-Inhibition % for different microbes depending on P1, P2, CH, FoH, FIH and combinations of CH + P1, FoH + P1, FIH + P1, CH + P2, FoH + P2, FIH + P2, penicillin, streptomycin, gentamycin and nystatin.

10% sample of:	Gram-positive bacteria										Gram-negative bacteria						Yeast	
	MRSA ^a		<i>Staphylococcus aureus</i>		<i>Streptococcus pyogenes</i>		<i>Str. agalactiae</i>		<i>Str. mutans</i>		<i>Escherichia coli</i>		<i>Pseudomonas aeruginosa</i>		<i>Acinetobacter baumannii</i>		<i>Candida albicans</i>	
	MIC (mg/mL)	GI-Inh. (%)	MIC (mg/mL)	GI-Inh. (%)	MIC (mg/mL)	GI-Inh. (%)	MIC (mg/mL)	GI-Inh. (%)	MIC (mg/mL)	GI-Inh. (%)	MIC (mg/mL)	GI-Inh. (%)	MIC (mg/mL)	GI-Inh. (%)	MIC (mg/mL)	GI-Inh. (%)	MIC (mg/mL)	GI-Inh. (%)
P1 ^b	0.007 ± 0.001	66.66	0.015 ± 0.007	58.0	0.015 ± 0.007	58.0	0.015 ± 0.007	58.0	0.007 ± 0.001	66.66	0.031 ± 0.015	50.0	0.015 ± 0.007	58.3	0.031 ± 0.015	50.0	0.031 ± 0.015	41.6
P2 ^c	0.015 ± 0.007	58.3	0.031 ± 0.015	50.0	0.031 ± 0.007	50.0	0.007 ± 0.0035	66.66	0.015 ± 0.007	58.3	0.015 ± 0.007	58.3	0.015 ± 0.007	58.3	0.015 ± 0.007	58.3	0.031 ± 0.015	50.0
CH ^d	0.031 ± 0.007	50.0	0.015 ± 0.007	58.0	0.015 ± 0.007	58.0	0.031 ± 0.015	50.0	0.062 ± 0.015	41.6	0.031 ± 0.007	50.0	0.031 ± 0.015	50.0	0.015 ± 0.007	58.3	0.015 ± 0.007	58.3
FoH ^e	0.062 ± 0.015	41.6	0.015 ± 0.007	58.0	0.031 ± 0.007	50.0	0.015 ± 0.007	58.3	0.031 ± 0.015	50.0	0.031 ± 0.007	50.0	0.062 ± 0.015	41.6	0.015 ± 0.007	58.3	0.007 ± 0.0035	66.66
FIH ^f	0.250 ± 0.030	25.0	0.031 ± 0.007	50.0	0.031 ± 0.007	50.0	0.007 ± 0.0035	66.6	0.125 ± 0.015	33.3	0.062 ± 0.0015	41.6	0.125 ± 0.015	33.3	0.031 ± 0.031	41.6	0.062 ± 0.015	41.6
CH + P1	0.003 ± 0.0015	75.0	0.007 ± 0.0035	66.6	0.003 ± 0.0015	75.0	0.007 ± 0.0035	66.6	0.031 ± 0.007	50.0	0.007 ± 0.0035	66.6	0.003 ± 0.0015	75.0	0.003 ± 0.0015	75.0	0.003 ± 0.0035	66.6
FoH + P1	0.003 ± 0.0015	75.0	0.007 ± 0.0035	66.6	0.007 ± 0.0035	66.6	0.007 ± 0.0035	66.6	0.015 ± 0.007	58.0	0.003 ± 0.0015	75.0	0.007 ± 0.0035	66.6	0.007 ± 0.0035	66.6	0.003 ± 0.0015	75.0
FIH + P1	0.015 ± 0.007	58.3	0.015 ± 0.007	58.3	0.031 ± 0.015	50.0	0.031 ± 0.015	50.0	0.031 ± 0.015	50.0	0.062 ± 0.015	41.6	0.015 ± 0.007	58.3	0.062 ± 0.015	41.6	0.031 ± 0.015	41.6
CH + P2	0.015 ± 0.007	58.3	0.015 ± 0.007	58.3	0.007 ± 0.0036	66.6	0.003 ± 0.0015	75.0	0.015 ± 0.007	58.3	0.031 ± 0.015	50.0	0.007 ± 0.0035	66.6	0.003 ± 0.0015	75.0	0.015 ± 0.007	58.3
FoH + P2	0.007 ± 0.0035	66.6	0.003 ± 0.0015	75.0	0.007 ± 0.0036	66.8	0.007 ± 0.0035	66.6	0.003 ± 0.0015	75.0	0.007 ± 0.0036	66.6	0.003 ± 0.0015	75.0	0.007 ± 0.0035	66.6	0.003 ± 0.0015	75.0
FIH + P2	0.031 ± 0.007	50.0	0.015 ± 0.007	58.3	0.015 ± 0.007	58.3	0.031 ± 0.007	50.0	0.031 ± 0.007	50.0	0.015 ± 0.007	58.3	0.015 ± 0.007	58.3	0.062 ± 0.015	41.6	0.015 ± 0.007	58.3
Penicillin (1:10)	5.000 ± 0.016	16.66	2.500 ± 0.260	25.00	0.312 ± 0.040	50.00	2.500 ± 0.260	25.00	1.897 ± 0.174	28.00	0.156 ± 0.040	58.33	0.625 ± 0.016	41.66	0.312 ± 0.040	50.00		
Streptomycin (1:10)	0.019 ± 0.004	83.33	0.009 ± 0.004	91.66	0.019 ± 0.004	83.33	0.009 ± 0.004	91.66	0.009 ± 0.004	91.66	0.039 ± 0.008	75.00	0.009 ± 0.004	91.66	0.009 ± 0.004	91.66		
Gentamycin (1:10)	0.019 ± 0.004	83.33	0.009 ± 0.004	91.66	0.039 ± 0.008	75.00	0.009 ± 0.004	91.66	0.009 ± 0.004	91.66	0.078 ± 0.008	66.66	0.009 ± 0.004	91.66	0.009 ± 0.004	83.33		
Nystatin (1:10)																	0.019 ± 0.004	83.33

a = methicillin-resistant *Staphylococcus aureus*; b = WSP Greit 120; c = PBS extract Lj. Center pH = 8.0; d = chestnut honey; e = forest honey; f = flower honey.

Fig. 3 The antimicrobial activity (MIC mg/mL) of P1 versus P2 against Gram-positive, Gram-negative bacteria and yeast *C. albicans*.

Fig. 4 The antimicrobial activity (MIC in mg/mL) of Slovenian CH, FoH and FIH against Gram-positive, Gram-negative bacteria and yeast *C. albicans*.

3.6 Enhancing Effect of P1 on Antimicrobial Activity of Slovenian Honeys

3.6.1 Enhancing Effect of P1 on Antimicrobial Activity of CH

The combination of the P1 with Slovenian CH shows the following data (Fig. 5) about MIC (mg/mL) values: for Gram-positive bacteria MIC (mg/mL) was between 0.003 ± 0.0015 and 0.031 ± 0.0035 . In the case of Gram-negative bacteria the MIC (mg/mL) lays between 0.003 ± 0.0015 . The value of MIC (mg/mL) for *C. albicans* is 0.007 ± 0.0035 .

3.6.2 Enhancing Effect of P1 on Antimicrobial Activity of FoH

Combination of the P1 and Slovenian FoH shows (Fig. 6) that for Gram-positive bacteria MIC (mg/mL) was between 0.003 ± 0.0015 and 0.015 ± 0.007 . For Gram-negative bacteria the MIC (mg/mL) lays between 0.003 ± 0.0015 and 0.007 ± 0.0035 . MIC (mg/mL) for *C. albicans* is 0.003 ± 0.0015 .

3.6.3 Enhancing Effect of P1 on Antimicrobial Activity of FIH

Combination of the P1 and Slovenian FIH shows (Fig. 7) that for Gram-positive bacteria MIC (mg/mL) was between 0.015 ± 0.0070 and 0.031 ± 0.0035 . For Gram-negative bacteria the MIC lays between 0.015 ± 0.0070 and 0.062 ± 0.015 . MIC (mg/mL) for *C. albicans* is 0.031 ± 0.015 .

3.7 Enhancing Effect of P2 on Antimicrobial Activity of Slovenian Honeys

3.7.1 Enhancing Effect of P2 on Antimicrobial Activity of CH

Combination of PBS extract P2 and CH gives the following MIC (mg/mL) values: for Gram-positive bacteria 0.003 ± 0.0015 and 0.015 ± 0.007 ; for Gram-negative bacteria the MIC (mg/mL) range (Fig. 5) is between 0.003 ± 0.0015 and 0.031 ± 0.0035 . The MIC (mg/mL) for *C. albicans* is 0.015 ± 0.007 .

Fig. 5 Enhancing influence of P1 or P2 on the antimicrobial activity (MIC in mg/mL) of CH against Gram-positive, Gram-negative bacteria and yeast *C. albicans*.

Fig. 6 Enhancing influence of P1 or P2 on the antimicrobial activity (MIC in mg/mL) of FoH against Gram-positive, Gram-negative bacteria and yeast *C. albicans*.

Fig. 7 Enhancing influence of P1 or P2 on the antimicrobial activity (MIC in mg/mL) of FIH against Gram-positive, Gram-negative bacteria and yeast *C. albicans*.

3.7.2 Enhancing Effect of P2 on Antimicrobial Activity of FoH

Combinations of PBS extract P2 and FoH gives the following MIC (mg/mL) values (Fig. 6): for Gram-positive bacteria 0.003 ± 0.0015 and 0.007 ± 0.0035 . The MIC (mg/mL) values for Gram-negative bacteria are between 0.003 ± 0.0015 and 0.007 ± 0.0035 , and are surprisingly the same as for Gram-positive bacteria. The MIC (mg/mL) for yeast *C. albicans* is 0.003 ± 0.0015 .

3.7.3 Enhancing Effect of P2 on Antimicrobial Activity of FIH

The combination of P2 and FIH gives the range of MIC (mg/mL) values (Fig. 7) for Gram-positive bacteria between 0.015 ± 0.007 and 0.031 ± 0.0035 . In the case of Gram-negative bacteria MIC (mg/mL) values are between 0.015 ± 0.007 and 0.062 ± 0.015 . The MIC (mg/mL) for *C. albicans* is 0.015 ± 0.007 .

3.8 Effectiveness of P1 versus P2 Added to CH

The effectiveness of P1 versus CH can be seen in Table 4. The P1 added to CH is better than P2 added to CH as follows (Fig. 8): for Gram-positive bacteria, $9.00\times$ (MRSA), $2.143\times$ (*S. aureus*), $2.334\times$ (*Str. pyogenes*), $0.429\times$ (*Str. agalactiae*), $0.484\times$ (*Str. mutans*); for Gram-negative bacteria, $4.429\times$ (*E. coli*), $2.334\times$ (*Ps. aeruginosa*), $1.000\times$ (*Acin. baumannii*) and for *C. albicans* $2.143\times$.

3.9 Effectiveness of P1 versus P2 Added to FoH

P1 added to FoH is better than P2 added to FoH as follows (Fig. 8): for Gram-positive bacteria, $2.334\times$ (MRSA), $0.492\times$ (*S. aureus*), $1.000\times$ (*Str. pyogenes*), $1.000\times$ (*Str. agalactiae*) and $0.200\times$ (*Str. mutans*); for Gram-negative bacteria, $2.334\times$ (*E. coli*), $0.429\times$ (*Ps. aeruginosa*), $1.000\times$ (*Acin. baumannii*) and for *C. albicans* $1.000\times$.

3.10 Effectiveness of P1 versus P2 Added to FIH

The P1 added to FIH is better than P2 added to FIH as follows (Fig. 8): for Gram-positive bacteria, $2.067\times$

(MRSA), $1.000\times$ (*S. aureus*), $0.484\times$ (*Str. pyogenes*), $1.000\times$ (*Str. agalactiae*) and $1.000\times$ (*Str. mutans*); for Gram-negative bacteria, $0.242\times$ (*E. coli*), $1.000\times$ (*Ps. aeruginosa*), $1.000\times$ (*Acin. baumannii*) and for *C. albicans* $0.484\times$.

4. Discussion

The propolis is classified into two groups according to the differences of their components: one is a Brazilian type rich in *p*-cumaric acid derivatives, the other is a European type rich in flavonoids [25]. This present study reveals that propolis samples taken from different areas in Turkey possess similar characteristics from both groups. The total phenolic content was determined in comparison with standard gallic acid and the results expressed in terms of mg GAE/g propolis. Total phenolic content of propolis samples was found to be 115-210 mg gallic acid/g of propolis extract by using FC method. All the propolis samples, except the Artvin sample (P10), possessed a high level of phenolic compound. Analyzed propolis samples contain an extensive number of phenolic compounds, and each sample has a distinctive profile. The amount and type of phenolic agents depend on the floral origin of propolis and they exhibit a wide range of biological effects and act as natural antioxidants and antimicrobials. The propolis samples contain a great percentage of benzoic acid (7.37-13.94 mg/mL), ferulic acid (22.3-574.11 mg/mL), abscisic acid (31.48-585.57 mg/mL), caffeic acids (125.94-465.81 mg/mL) and *p*-coumaric acid (0.48-1.44 mg/mL). In small amounts, gallic acid (7.01-4.85 mg/mL), vanillic acid (3.16-33.38 mg/mL) *proto*-catechuic acid (4.01-27.08 mg/mL), chlorogenic acid (6.32-53.46 mg/mL) and *p*-hydroxybenzoic acid (41.32-387.13 mg/mL). In minor amounts, syringic acid (0.25-0.48 mg/mL) and *o*-coumaric acid (0.48-1.45 mg/mL) were found. It seems that the ferulic acid exhibits the potent antioxidant capacity *in vitro* and *in vivo*. The analyses of the total phenolic content of Slovenian propolis extract P2 show 102.42 ± 1.23 mg/mL. Analysis of

Table 4 Effectiveness of CH + P2 versus CH + P1, FoH + P2 versus FoH + P1 and FIH + P2 versus FIH + P1.

10% sample of:	Gram-positive bacteria										Gram-negative bacteria						Yeast	
	MRSA ^a		<i>S. aureus</i>		<i>Str. pyogenes</i>		<i>Str. agalactiae</i>		<i>Str. mutans</i>		<i>E. coli</i>		<i>Ps. aeruginosa</i>		<i>Acin. baumanii</i>		<i>C. albicans</i>	
	MIC (mg/mL)	GI-Inh. (%)	MIC (mg/mL)	GI-Inh. (%)	MIC (mg/mL)	GI-Inh. (%)	MIC (mg/mL)	GI-Inh. (%)	MIC (mg/mL)	GI-Inh. (%)	MIC (mg/mL)	GI-Inh. (%)	MIC (mg/mL)	GI-Inh. (%)	MIC (mg/mL)	GI-Inh. (%)	MIC (mg/mL)	GI-Inh. (%)
CH ^b + P2 ^c	0.015 ± 0.007	58.3	0.015 ± 0.007	58.3	0.007 ± 0.0036	66.6	0.003 ± 0.0015	75.0	0.015 ± 0.007	58.3	0.031 ± 0.015	50.0	0.007 ± 0.0035	66.6	0.003 ± 0.0015	75.0	0.015 ± 0.007	58.3
CH + P1 ^f	0.003 ± 0.0015	75.0	0.007 ± 0.0035	66.6	0.003 ± 0.0015	75.0	0.007 ± 0.0035	66.6	0.031 ± 0.015	50.0	0.007 ± 0.0035	66.6	0.003 ± 0.0015	75.0	0.003 ± 0.0015	75.0	0.007 ± 0.0035	66.6
CH + P2/ CH + P1	9.00		2.143		2.334		0.429		0.484		4.429		2.334		1.000		2.143	
<i>t</i> -test	0.025		0.025		0.025		0.025		0.025		0.025		0.025		0.025		0.025	
CI	1.8413		1.9484		1.8983		1.8983		1.9224		1.8428		1.8983		4.3027		1.9484	
FoH ^c + P2	0.007 ± 0.0035	66.6	0.003 ± 0.0015	75.0	0.007 ± 0.0035	66.8	0.007 ± 0.0035	66.6	0.003 ± 0.0015	75.0	0.007 ± 0.0035	66.6	0.003 ± 0.0015	75.0	0.007 ± 0.0035	66.6	0.003 ± 0.0015	75.0
FoH + P1	0.003 ± 0.0015	75.0	0.007 ± 0.0035	66.6	0.007 ± 0.0035	66.6	0.007 ± 0.0035	66.6	0.015 ± 0.007	58.0	0.003 ± 0.0015	75.0	0.007 ± 0.0035	66.6	0.007 ± 0.0035	66.6	0.003 ± 0.0015	75.0
FoH + P2/ FoH + P1	2.334		0.429		1.000		1.000		0.200		2.334		0.429		1.000		1.000	
<i>t</i> -test	0.025		0.025		0.025		0.025		0.025		0.025		0.025		0.025		0.025	
CI	1.8983		1.8983		4.3027		4.3027		1.8413		1.8983		1.8983		4.3027		4.3027	
FIH ^d + P2	0.031 ± 0.007	50.0	0.015 ± 0.007	58.3	0.015 ± 0.007	58.3	0.031 ± 0.015	50.0	0.031 ± 0.015	58.3	0.015 ± 0.007	58.3	0.015 ± 0.007	58.3	0.062 ± 0.015	41.6	0.015 ± 0.007	58.3
FIH + P1	0.015 ± 0.007	58.3	0.015 ± 0.007	58.3	0.031 ± 0.015	50.0	0.031 ± 0.015	50.0	0.031 ± 0.015	50.0	0.062 ± 0.015	41.6	0.015 ± 0.007	58.3	0.062 ± 0.015	41.6	0.031 ± 0.015	41.6
FIH + P2/ FIH + P1	2.067		1.000		0.484		1.000		1.000		0.242		1.000		1.000		0.484	
<i>t</i> -test	0.025		0.025		0.025		0.025		0.025		0.025		0.025		0.025		0.025	
CI	4.3027		4.3027		1.9224		4.3027		4.3027		1.9224		4.3027		4.3027		4.3027	

a = methicillin-resistant *Staphylococcus aureus*; b = chestnut honey; c = forest honey; d = flower honey; e = PBS propolis extract Lj. Center pH = 8.0; f = WSP Greit 120.

Fig. 8 The effectiveness of P1 versus P2 for the antimicrobial activity (MIC in mg/mL) against Gram-positive, Gram-negative bacteria and yeast *C. albicans* of Slovenian CH, FoH and FIH .

phenolic agents show the presence of: caffeic acid 2.44 ± 0.18 mg/mL, caffeic acid 1 1.43 ± 0.11 mg/mL, chrysin 0.15 ± 0.008 mg/mL, pinocembrin 0.46 ± 0.004 mg/mL and galangin 0.62 ± 0.05 mg/mL. In the experiments performed by Mello *et al.* [26] it was shown that water extracts of propolis prepared in pH = 8.0 show stronger antimicrobial activity than those prepared in pH 6.4 and 7.2. This was the basis for the experiments in which the PBS extract of P2 was prepared. This propolis extract has comparable antimicrobial activities with P1 from BNatural.

The antibacterial effect of honey is associated with its high osmolality, low pH and hydrogen peroxide content which is called inhibin factor. Non-peroxide antibacterial substances such as aromatic acids, phenolics, flavonoids are other groups of compounds of antibacterial effectors of honey. The results obtained showed that the total phenolic content (mg GAE/100 g honey) determined by the modified FC method varied among the different types of honey. The total phenolic content of Turkish honey was between 1.50-108.21 mg

GAE/100 g of honey. The total phenolic substances were highest in CH (46.97 ± 17.52 mg GAE/100 g) followed by FoH (24.2 ± 0.6 mg GAE/100 g). The lowest values were found in FIH with 11.11 ± 0.019 mg GAE/100 g. When the Slovenian kinds of honey were analyzed, the most important factors are their flavonoid content and the acidity of honey, with a pH between 3.2 and 4.5. The pH is an important active factor in honey's antibacterial activity since most bacteria grow in a pH range between 6.5 and 7.5. So for CH the flavonoid content is 191.7 ± 26.8 mg/mL and pH 5.51 ± 0.42 . For FoH the flavonoid content is 210.4 ± 36.4 mg/mL and pH 4.77 ± 0.21 . And for FIH the flavonoid content is 138.0 ± 29.7 mg/mL with pH 4.35 ± 0.24 , as it was found by Bertoneclj *et al.* [27] and Strelec *et al.* [28]. The flavonoid content correlates with antioxidant and antimicrobial activity.

The fact that different sorts of propolis or propolis' extracts affect different kind of honey is not new. So it was found that P1 enhances the antimicrobial activity of FoH from Italy and Spain [29]. In additional

experiments, it was studied at first the antimicrobial activity of Slovenian CH, FoH and FIH. Interestingly, quite strong MIC in mg/mL was found for CH and FoH, but very weak MIC in mg/mL was found for FIH. To measure the enhancement with P1 or P2 it was prepared the mixture of different Slovenian honeys: CH, FoH and FIH with 10% of P1 or P2 and then measured their antimicrobial activity and compared this with antimicrobial activity (MIC) in mg/mL of CH, FoH and FIH. The best results were obtained when the CH was mixed with 10% of P1 or P2. The best was the addition of P1 in a way of enhancement of the antimicrobial activity of CH (Fig. 5) followed by mixtures of FoH and FIH with 10% of P2 (Figs. 6 and 7). Finally, the effectiveness of P2 versus P1 was calculated (Fig. 8). It seems that the enhancement of antimicrobial activity depends on the sort of honey and also on the nature of propolis extracts P1 or P2. It was also found by Sarikaya *et al.* [30] that the content of phenolics caffeic acid in CH (0.6 ± 0.02 mg/mL) and much higher in propolis (2.3 ± 1.12 mg/mL), could be the factors of enhancement of antioxidative activity and consequently antimicrobial activity of mixture of CH and propolis. In comparison, in Slovenian CH there was 0.87 ± 0.04 mg/mL of caffeic acid and much higher in P1 3.11 ± 0.92 mg/mL suggesting about the enhancement of antimicrobial activity.

5. Conclusions

From the performed experimental study, the following conclusions may be drawn:

(1) The P1 added to Slovenian CH is better than P2 added to CH as follows: for Gram-positive bacteria, $9.00 \times$ (MRSA), $2.143 \times$ (*S. aureus*), $2.334 \times$ (*Str. pyogenes*), $0.429 \times$ (*Str. agalactiae*), $0.484 \times$ (*Str. mutans*); for Gram-negative bacteria, $4.429 \times$ (*E. coli*), $2.334 \times$ (*Ps. aeruginosa*), $1.000 \times$ (*Acin. baumanii*) and for *C. albicans* $2.143 \times$.

(2) The P1 added to Slovenian FoH is better than P2 added to FoH as follows: for Gram-positive

bacteria, $2.334 \times$ (MRSA), $0.492 \times$ (*S. aureus*), $1.000 \times$ (*Str. pyogenes*), $1.000 \times$ (*Str. agalactiae*) and $0.200 \times$ (*Str. mutans*); for Gram-negative bacteria, $2.334 \times$ (*E. coli*), $0.429 \times$ (*Ps. aeruginosa*), $1.000 \times$ (*Acin. baumanii*) and for *C. albicans* $1.000 \times$.

(3) The P1 added to FIH is better than P2 added to FIH as follows: for Gram-positive bacteria, $2.067 \times$ (MRSA), $1.000 \times$ (*S. aureus*), $0.484 \times$ (*Str. pyogenes*), $1.000 \times$ (*Str. agalactiae*) and $1.000 \times$ (*Str. mutans*); for Gram-negative bacteria, $0.242 \times$ (*E. coli*), $1.000 \times$ (*Ps. aeruginosa*), $1.000 \times$ (*Acin. baumanii*) and for *C. albicans* $0.484 \times$.

(4) The enhancement of antimicrobial activity depends on sort and phenolic composition of honeys and also on the nature of propolis extracts P1 or P2.

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