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ANTIMICROBIAL ACTIVITY, CYTOTOXICITY AND TOTAL PHENOLIC CONTENT OF DIFFERENT EXTRACTS OF PROPOLIS FROM THE WEST POMERANIAN REGION IN POLAND

ANNA WIECZYŃSKA[1]*, JOANNA WEŻGOWIEC[2], WŁODZIMIERZ WIĘCKIEWICZ[3], ANNA CZARNY[4], JULITA KULBACKA[5], DANUTA NOWAKOWSKA[2], ROMAN GANCARZ[1] and KAZIMIERA A. WILK[1]

[1]Department of Organic and Pharmaceutical Technology, Faculty of Chemistry, Wrocław University of Science and Technology, Wybrzeże Wyspiańskiego 27, 50-370 Wrocław, Poland
[2]Department of Dental Materials, [3]Department of Prosthodontics, Wroclaw Medical University, Krakowska 26, 50-425 Wrocław, Poland
[4] Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Rudolfa Weigla 12, 53-114 Wrocław, Poland
[5]Department of Medical Biochemistry, Wroclaw Medical University, Chałubinskiego 10, 50-368 Wrocław, Poland

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Propolis, or "bee glue", is a complex material of plant origin, collected and processed by bees to seal hives and to protect them against pathogenic microorganisms. Due to a broad spectrum of biological properties it could be applied in different disciplines of medicine and dentistry, as an antibacterial, antifungial, antioxidant, anti-inflammatory, antiproliferative and immunomodulatory agent (1). More than 300 chemical compounds have been identified in propolis. They include resins, waxes, volatile oils, aromatic acids, vitamins, proteins, amino acids and sugars. The major group of biologically active compounds are polyphenols, including phenolic acids and flavonoids (2, 3). The most characteristic chemical compounds present in propolis originating from the region of temperate climate are flavonoids such as chrysin, galangin, pinocembrin, pinobanksin (4). Numerous studies discovered that chemical composition and biological activity of different propolis samples may differ significantly. This diversity is a result of the plant origin of propolis and it strongly depends on the geographic region and climatic conditions of the site of propolis collection, the botanical origin and the bee species. In Europe, propolis originates mainly from the resinous exudates of the buds of poplar trees. Propolis from tropical regions, such as Brazil or Cuba, has different chemical composition and attracts attention of many research groups. In spite of the considerable diversity of chemical composition, the biological activity of propolis of different origin remains similar (5). Currently, bee glue is used in cosmetic industry as an ingredient of anti-acne creams, body lotions and preparations for oral hygiene (1). The wide area of potential application of propolis covers a treatment of various diseases, such as colds, wounds, acne, rheumatism, heart diseases, diabetes, dental caries and even cancer (4). For this purposes, different formulations (capsules, ointment, creams, pastes, rinses, powder) have been proposed for various applications (2). The final components are determined by the method of extraction, the time of extraction and the type of solvent used. The most popular form is extraction with 70% ethanol to obtain ethanolic extract of propolis (EEP). In 2013, all known commercially available propolis-based products were prepared with EEP (6). However, the possibility of the application of other solvents have been also investigated (7). In spite of the numerous studies concerning properties of propolis, many questions

* Corresponding author: e-mail: anna.wieczynska@pwr.edu.pl

remain still open. The aim of the presented study was to achieve samples of Polish propolis by means of different extraction methodologies. The products obtained through the ethanolic, hexane and water extraction were evaluated in terms of their chemical components and therapeutic efficacy. The comparative analysis included investigation of the phenolic content of different extracts of Polish propolis, their antimicrobial properties and safety to normal cells (human gingival fibroblasts, HGFs).

EXPERIMENTAL

Propolis origin

Raw propolis was collected manually from beehive located in the north west region of Poland (West Pomeranian). Before processing it was stored at a room temperature in a dark conditions.

Preparation of propolis extracts

Ethanolic and hexane extracts of Polish propolis (EEPP and HEPP) were prepared: 5 g of propolis sample was chopped into small pieces, dissolved in 50 mL of 96% ethanol (POCH, Poland) and stirred at the room temperature in a dark conditions for 24 h, using a Big-squid magnetic stirrer (IKA, Germany). Then, the sample was centrifuged at 10500 rpm for 15 min at room temperature using a 5804 centrifuge (Eppendorf, Germany), obtained supernatant was the ethanolic extract of propolis no. 1 (EEPP 1). The extraction procedure was repeated three more times to obtain EEPP 2 – EEPP 4. Afterwards, the residue was extracted twice with hexane (POCH, Poland), to obtain hexane extracts (HEPP 1 and HEPP 2) and then twice with distilled water to obtain water extracts (WEPP 1 and WEPP2). The final, post-extraction residue (material not dissolved and not marked as WEPP 2) was left for the further analysis as RPP (residue of Polish propolis). Due to the precipitation observed in some of the supernatants, all the samples were centrifuged again and the obtained residues were left for further analysis (EEPP-R 1-3, HEPP-R 1, WEPP-R 2). All the obtained supernatants were analyzed *via* TLC method. Similar fractions were combined, in order to obtain EEPP 1&2 (a combination of EEPP 1 and EEPP 2), EEPP 3&4 (a combination of EEPP 3 and EEPP 4), HEPP 1&2 (a combination of HEPP 1 and HEPP 2) and WEPP 1&2 (a combination of WEPP 1 and WEPP 2). The EEPP 1 and 2, EEPP 3 and 4 and HEPP 1 and 2 were evaporated to dryness at 40^{o}C using a RV 10 rotary vacuum evaporator (IKA, Germany) and kept at 4^{o}C in the dark until further use. WEPP 1&2 was not used in the further studies due to ineffective evaporation, (resulting from the low temperature 40^{o}C) applied to avoid thermal degradation of the sample. For further analysis the samples were dissolved in DMSO (POCH, Poland) at a concentration of 10 mg/mL (for TPC assay) or 100 mg/mL (for biological studies). For biological studies they were subsequently diluted with the appropriate culture medium.

Determination of total phenolic content (TPC)

The content of phenolics was analyzed *via* Folin-Ciocalteu assay (8) and expressed as gallic acid equivalent. The reaction mixture was prepared by mixing 20 μL of DMSO solution of the propolis extract (the concentration of 1 mg/mL) and 100 μL of the Folin-Ciocalteu's reagent dissolved in 1.58 mL of distilled water. Each solution was mixed for 5 min. Then, 300 μL of sodium carbonate (Na_2CO_3) was added to the reaction mixture. Thereafter, the samples were incubated in a thermostat at 40^{o}C for 30 min. The absorbance of the samples at 765 nm was measured with a UV/Vis SP 8001 spectrophotometer (Metertech Inc., Taiwan). The samples were prepared in triplicate for each analysis, mean value of absorbance was calculated. TPC was determined on the basis of the calibration curve of gallic acid ($y = 0.0001X$; $R^2 = 0.99$) and expressed as a gallic acid equivalent (GAE) in 1 g of the propolis sample.

Determination of antimicrobial activity

Antimicrobial activity of the extracts was evaluated *via* the bacterial growth inhibition assay. The experiments were conducted on strains from the Polish Collection of Microorganisms (PCM) – IITD, PAN, Wrocław (*Escherichia coli* PCM 2057, *Staphylococcus aureus* PCM 2054), from American Type Culture Collection (*Streptococcus mutans* ATCC 25175, *Candida albicans* ATCC 90028) and clinical strains (*Proteus mirabilis*, *Citrobacter freundii*) derived from patients of clinical hospital in Wrocław. The cells were grown in culture media: nutrient broth, nutrient agar, brain heart broth, blood agar, Sabouraud broth, Sabouraud agar and 0.9% NaCl. The media were purchased from BTL Ltd., Łódź, Dept. of Enzymes and Peptones (Poland). The preliminary studies were aimed at the selection of most active propolis extracts, based on the method proposed by Czarny et al (9). The precultures of *E. coli, Proteus mirabilis, Citrobacter freundii* and *S. aureus* in nutrient broth, and the precultures of *Streptococcus mutans* in brain hearth broth were incubated at 37^{o}C. The precultures of *C. albicans* in Sabouraud's broth were incubated at 28^{o}C. After an overnight incubation the cultures were 10× diluted

and then the colony-forming units of bacteria and fungal cells in sample were determined (number of CFU for: *E. coli* 9.8 × 10^7/mL *Proteus mirabilis* 1.7 × 10^7/mL, *Citrobacter freundii* 2.8 × 10^7/mL, *S. aureus* 2.5 × 10^7/mL, *Streptococcus mutans* 3.1 × 10^7/ml, *C. albicans* 7.1 × 10^7/mL). The suspensions of the microorganisms (100 µL) were seeded into plates containing nutrient agar, blood agar or Sabouraud's agar. After drying of plates, 20 µL of the studied propolis solutions at the concentrations of 1000 µg/mL, 200 µg/mL and 100 µg/mL were added. The samples of propolis were initially dissolved in DMSO at 100 mg/mL concentration and then subsequent dilutions were prepared in 0.9% NaCl. Microorganisms on control plates were treated with appropriate dilutions of DMSO (concentrations did not exceed 5% v/v). After 24 h incubation with propolis, the growth of bacterial or *Candida* colonies was evaluated qualitatively. Antimicrobial activity was demonstrated as a clear zone of growth inhibition observed on the agar.

Cell culture of human gingival fibroblasts

HGF's (human gingival fibroblasts) were derived from patients with healthy periodontium undergoing tooth extraction in the Department of Dental Surgery of Wroclaw Medical University. The cells were isolated according to the procedure described and patented by Saczko et al. (Patent no. P 3812045). HGFs were grown in DMEM (Lonza, BioWhittaker, Switzerland) supplemented with 10% fetal bovine serum (Lonza, BioWhittaker, Switzer-

Table 1. The antimicrobial effects of selected propolis extracts.

Extracts of propolis		Strains of microorganisms					
Abbrev.	Concentration [µg/mL]	*Escherichia coli* PCM 2057	*Proteus mirabilis*	*Citrobacter feundi*	*Staphylococcus aureus* PCM 2054	*Streptococcus mutans* ATCC 25175	*Candida albicans* ATCC 90028
Control (5% DMSO)		-	-	-	-	-	-
EEPP 1&2	1000	++	++	++	++	++	+
	200	++	++	++	++	++	-
	100	++	++	++	+	++	-
EEPP 3&4	1000	++	++	++	++	++	+
	200	++	++	++	+	++	-
	100	+	++	+	+	+	-
HEPP 1&2	1000	+	++	++	+	+	++
	200	-	+	-	-	-	+
	100	-	-	-	-	-	+
EEPP -R1	1000	++	++	++	+	++	-
	200	+	++	+	-	+	-
	100	+	+	+	-	+	-
EEPP -R2	1000	+	++	++	-	+	-
	200	-	+	+	-	+	-
	100	-	+	-	-	-	-
EEPP -R3	1000	++	++	+	-	+	-
	200	+	+	-	-	-	-
	100	-	-	-	-	-	-
HEPP -R1	1000	+	-	-	-	-	+
	200	-	-	-	-	-	-
	100	-	-	-	-	-	-
RPP	1000	+	+	+	-	-	-
	200	+	+	-	-	-	-
	100	-	-	-	-	-	-

Degree of the antimicrobial effectiveness: - = inactive, + = weak active (opaque zone) ++ = active (clear zone of growth inhibition).

land) and penicillin/streptomycin (Lonza, BioWhittaker, Switzerland) as a monolayer plated on a plastic flask 25 cm^2 (Nunc, Denmark). They were maintained in a humidified atmosphere at 37°C and 5% CO_2. For experimental purposes, the cells were detached by trypsinization (trypsin 0.025% EDTA 0.02% solution, Lonza).

Determination of cytotoxicity of propolis extracts (MTT assay)

The cytotoxic effect of the analyzed systems on human gingival fibroblasts was assessed *via* MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The cells were seeded into 96-well microculture plates (200 µL of the cell suspension in the culture medium per well) (Nunc, Denmark). After 24 h, a fresh culture medium with the proper concentration of propolis extract was added to each well. From one part of samples the medium with propolis was removed after 3 min and the cells were incubated 24 h with a culture medium without propolis. The second part of samples was incubated with propolis for 24 h. Then, MTT assay was performed to evaluate cells mitochondrial func-

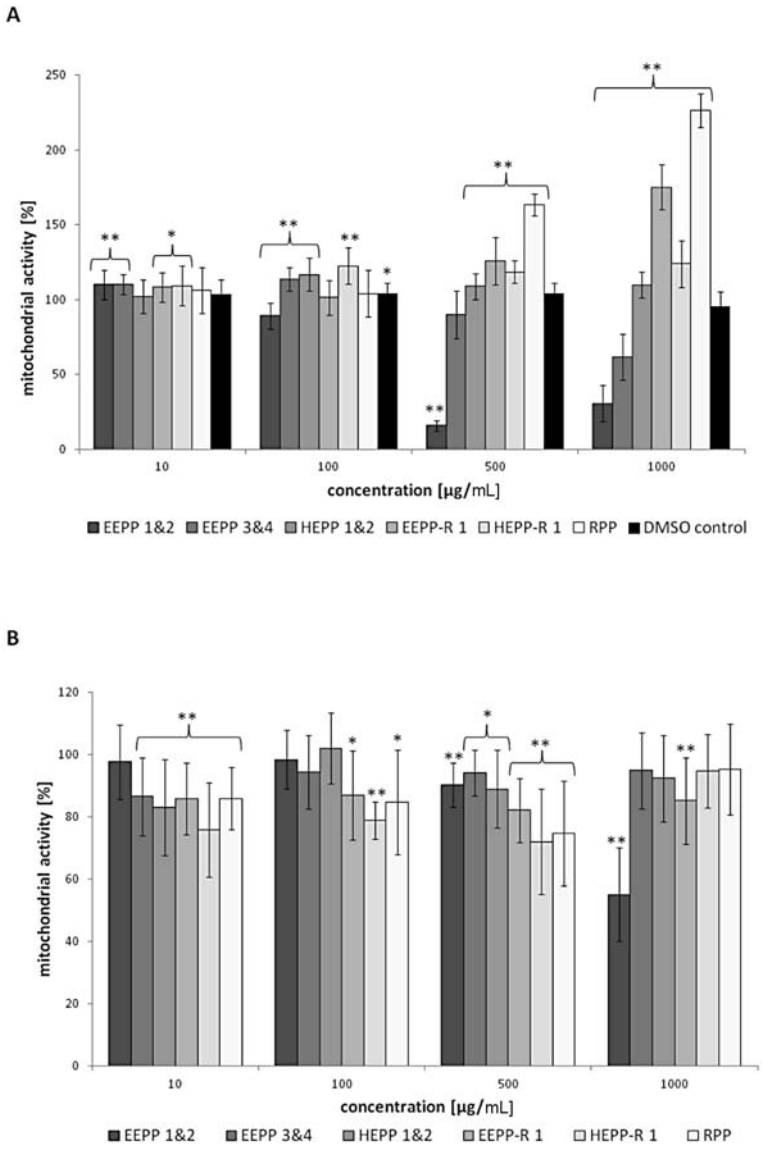

Figure 1. Cytotoxicity of propolis extracts on human gingival fibroblasts (HGFs); (A) 24 h after a 3 min of incubation, (B) after 24 h of incubation; *$p < 0.05$, **$p < 0.005$

tion. Cells were incubated with 100 µL of MTT reagent (Sigma, Poland) at 37°C for 90 min. Then, formazan crystals were dissolved with addition of 100 µL of acidic isopropanol (1 : 250 of HCl in isopropanol, Stanlab, Poland) and mixed. The absorbance was measured at 570 nm using multiwell plate reader (EnSpire Multimode Reader; Perkin Elmer, USA). The results were expressed as the percentage of mitochondrial function relative to untreated control cells.

Statistical analysis

The results of the quantitative analysis were expressed as the mean ± standard deviation. Three independent experiments per each sample were prepared with at least three replications ($n \geq 9$). For MTT assay the significance of the difference between mean values of different groups and untreated control group was assessed by Student's *t*-test with p-value of $p \leq 0.05$ or $p \leq 0.005$, to show the statistical significance.

RESULTS

Total phenolic content (TPC)

The results of the Folin-Ciocalteu assay used for determination of the total phenolic content expressed as a gallic acid equivalent (GAE) in 1 g of the propolis sample are following in descending order: EEPP 1&2 (150.80 ± 9.56), HEPP 1 and 2 (86.72 ± 13.28), EEPP 3 and 4 (74.09 ± 9.06), HEPP-R 1 (23.52 ± 11.33) and RPP (14.59 ± 10.18).

Antimicrobial activity

The antibacterial activity of propolis products against Gram-positive (*Staphylococcus aureus, Streptococcus mutans*) and Gram-negative (*E. coli, Proteus mirabilis, Citrobacter freundii*) strains, as well as the antifungal activity against *C. albicans* are presented in Table 1. EEPP 1 and 2 and EEPP 3 and 4 fractions exhibited a wide spectrum of antimicrobial activity even at the low concentrations (100 and 200 µg/mL). Hexane fraction (HEPP 1 and 2) was less active against Gram-positive and Gram-negative bacteria. On the other hand, it inhibited the growth of *Candida albicans* even more effectively than the ethanolic fractions. None or weak antimicrobial activity of HEPP-R 1 and RPP was observed.

Cytotoxicity

The cytotoxic influence of the obtained propolis extracts on the human gingival fibroblasts was assessed *via* MTT assay. Figure 1 presents mitochondrial activity of the cells measured 24 h after adding the selected concentrations of propolis products for 3 min (Fig. 1a) or 24 h (Fig. 1b). This study confirmed safety of most of the samples when HGFs were incubated with them for 3 min (Fig. 1a). Only EEPP 1 and 2 at a concentration of 1000 µg/mL induced a cytotoxic effect resulting in a decrease of mitochondrial activity to ca. 55%. After the incubation of the cells with HEPP-R 1 or RPP the mitochondrial activity also decreased below 80%, but it was still above 70%. A prolonged incubation of cells with the studied extracts (24 h) resulted in a stronger cytotoxic effect of the EEPP (Fig. 1b). However, for most of the samples, the mitochondrial activity remained above 80% for all selected concentrations of the propolis products. Only EEPP 1 and 2 and EEPP 3 and 4 at a concentration of 500 µg/mL and 1000 µg/mL caused a significant decrease of the mitochondrial activity, which could be related to the decrease of cellular viability. The incubation of cells with RPP at a concentration of 500 µg/mL and 1000 µg/mL significantly enhanced the mitochondrial activity above 160% of the untreated control.

DISCUSSION AND CONCLUSION

So far, the extraction with ethanol is the most common method of the extraction of propolis. Ethanol extract of propolis (EEP) revealed antibacterial (10), antifungal (11), antioxidant (12), and antiproliferative (13) properties.

Antimicrobial activity

The presented study aimed at the investigation of antimicrobial properties of Polish propolis. The influence of DMSO itself in different concentrations (1, 5, 7.5, 10, 15 and 20%) on the growth of bacteria and fungi was also determined. Incubation of samples with higher concentrations of DMSO (15, 20%) resulted in visible inhibition of growth zones. Lower concentrations of DMSO (10% and below) had no effect on the tested microorganism. According to these results, 5% concentration of DMSO, non-toxic to bacteria and fungi, was selected for further research. Effective inhibition of the growth of the selected Gram negative and Gram positive strains was obtained, particularly after the incubation with ethanolic fractions of propolis. The hexane fraction at the highest concentration (1000 µg/mL) demonstrated also a promising antimicrobial activity, expanding the current knowledge on the properties of Polish propolis. So far, bee glue of this origin has been evaluated by a few research

groups but all of them used ethanol for the extraction. Dziedzic et al. demonstrated the antibacterial activity of EEPP in the inhibition of the growth of *Mutans Streptococci* group bacteria and *Lactobacilli* saliva residents (14). The antibacterial activity of EEPP against 12 *S. aureus* strains was also assessed by Wojtyczka et al. (15). Szliszka et al. identified 37 phenolic ingredients in the EEPP, revealing pinobanksin, chrysin and methoxyflavanone as the major flavonoids. Their biological studies showed that EEPP induced apoptosis of LNCaP prostate cancer cells (16). Another study focused on the anticancer properties of Polish propolis was performed by Kubina et al. (17). Socha et al. studied a composition and antioxidant activity of Polish propolis. They achieved a strong diversity of chemical composition and antiradical activity of samples from different regions of Poland (18).

Cytotoxicity

Apart from the investigation of the antimicrobial activity of the samples of Polish propolis, the safety of this product was simultaneously assessed, since some researchers describe a toxic influence of propolis to normal cells. The presented study indicated that 10 μg/mL and 100 μg/mL concentrations of all the studied propolis extracts were non-toxic, but two ethanolic ones (EEPP 1 and 2 and EEPP 3 and 4) in a concentration of 500 μg/mL or 1000 μg/mL had a cytotoxic influence on the HGFs *in vitro*. On the other hand, the prolonged incubation of the cells with RPP even stimulated HGFs proliferation. A toxicity of propolis to normal cells has been demonstrated also by the other authors. EEP of Portuguese propolis revealed a high cytotoxicity against five tumor cell lines but also against non-tumor cells (13). Similar effect was observed by Lopez et al. in the study focused on the analysis of Brazilian and Cuban red propolis (19). Since propolis is a complex material, a methodology applied for the preparation of the product significantly influence its biological properties. Some researchers tried to use other solvents instead of ethanol in order to obtain more effective product. Maciejewicz isolated 10 flavonoids from Polish propolis, preparing EEP and shaking it with hexane to remove the part of the weak polar compounds (20). The hexane fraction of EEP was also prepared by Castro et al. who isolated prenylated benzophenone, hyperibone A and proved its antiproliferative and antimicrobial activity (21). Rassu et al. extracted raw propolis with n-hexane to assess the wax content and with ethanol to get the flavonoids content (22). In another study, Guo et al. demonstrated a strong antioxidant activity of WEP (23).

Total phenols

Undoubtedly, the most interesting studies combine different solvents or extractive procedures and compare their effectiveness. In this study, the chemical composition, the antimicrobial activity, as well as the cytotoxicity of ethanolic and hexane products and residue after the water extraction of Polish propolis were compared. Total phenolic contents in the examined samples ranged from 14.59 to 150.80 mg GAE/g; the highest concentration of phenols has been found in ethanolic ones. The HEPP-R 1, and RPP contains considerably lower concentrations of phenols. Different solvents used for the extraction of French propolis was compared by Boisard et al. (24). They used six systems: (1) boiling water, (2) 95% EtOH, (3) 70% EtOH, (4) MeOH, (5) DCM proceeded by a cyclohexane wax elimination and (6) a mixture of DCM, MeOH and water proceeded by cyclohexane wax elimination. Total polyphenol content ranged between 238.6 mg GAE/g for MeOH and 292.1 mg GAE/g, for water extraction. On the other hand, total flavone/flavonol content, total flavanone/dihydroflavonol content and the extraction yield were the lowest for WEP samples. Quantitative analysis of 12 major compounds revealed the highest amount of these components in DCM samples and the lowest in water and MeOH ones. Moreover, WEP exhibited good antioxidant activity but weak antiglycation potential. The extracts obtained for mixed solvents and for ethanol had both a good antioxidant and antiglycation properties (24). Teerasripreecha et al. prepared methanol product of Thai propolis. Then, the residual propolis was extracted with dichloromethane followed by hexane. The three samples varied in appearance and antiproliferative activity. Hexane and dichloromethane extracts revealed a strong cytotoxicity in five cancer cell lines, but the antiproliferative effect of methanol product was much weaker. The authors isolated two phenolic lipids with the strongest anticancer activity (cardanol and cardol) from the HEP (25). In another study, conducted by Banskota et al., Brazilian propolis was extracted with distilled water and then the insoluble portion was extracted with methanol and $CHCl_3$. Methanol sample shown the highest cytotoxicity against human fibrosarcoma and murine colon carcinoma cells. It was fractionated into EtOAc soluble and insoluble fractions and the EtOAc soluble fraction was the most effective in the inhibition of the growth of cancer cells (26). Neves et al. evaluated the antimicrobial activity of Brazilian red propolis. The powdered sample and the EEP was effective against all tested Gram-positive and Gram-negative bacteria and yeast strains.

The acetate fraction exhibited a weaker antibacterial activity, but stronger antifungal activity. The hexane fraction possessed a similar or better antibacterial activity and similar antifungal activity in comparison with the acetate fraction. The methanolic fraction demonstrated similar antifungal activity and weaker antibacterial activity than the hexane and acetate fractions. The authors found that formononetin and pinocembrin were the major compounds identified in the samples (27). A comparison of the WEP with the EEP was performed by Rocha et al. However, WEP was obtained through the resolubilization of the hydroalcoholic extract with water. Both studied products had similar chemical composition, but WEP had a better anti-oxidant and antibacterial activity (28). Kubiliene et al. compared chemical composition and biological activity of propolis extracts prepared in different conditions. Ethanolic sample has the highest total content of phenolic compounds, but the results obtained for the samples prepared at 70^{o}C with PEG and water or with PEG, water and olive oil were not much lower. Results of the analysis of the antioxidant and antimicrobial activity confirmed the effectiveness of these methods of the extraction of propolis and indicated the low effectiveness of the process with water or olive oil at room temperature (29). The procedure of propolis extraction may differ not only with a solvent but also with technical details. De Lima et al. compared three methods: maceration, Soxhlet and ultrasound-assisted extraction to obtain phenolic components from Brazilian propolis. They revealed that hydrogels loaded with propolis obtained *via* ultrasound-assisted extraction gave the most effective inhibition of bacteria growth (30). Machado et al. compared properties of propolis products obtained by supercritical extraction with CO_2 and ethanolic extraction. Extraction with supercritical CO_2 was more effective in order to obtain the products with a higher content of p-coumaric acid and artepillin C (31).

The studies discussed above and the presented research allow to conclude that it is not possible to clearly indicate one best procedure of the extraction of biologically active compound from propolis. This may partially result from the differences of chemical composition between propolis originated from different regions and from a diversity of biological functions of different chemical constituents. Our results indicate the significant influence of the applied procedure of extraction on the properties of the obtained product. Ethanolic fractions of propolis had the highest total phenolic content and demonstrated better antimicrobial activity. However, at the higher concentrations, they could also induce a cytotoxic effect in HGFs. The obtained results encourage further studies focused on the comparison of the propolis samples of different origin being analyzed in terms of its chemical composition and biological activity. Additional studies with a larger group of bacterial strains and human cells are needed. A combination of propolis extracts with standard antibiotics in order to increase their bactericidal effect is also an interesting direction for the future research.

Moreover, the issue of propolis safety should be also considered in more detail. In the presented study, the concentrations of propolis products which were effective against the selected microorganisms were simultaneously safe to the normal cells. Nevertheless, it is obvious that potential toxicity of this material should be taken into consideration. It would be valuable to propose some solutions for the improvement of sample preparation in order to achieve better selectivity of propolis. Such approach could enhance a therapeutic potential of this natural product.

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