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The Study of the Antimicrobial Activity of Transylvanian (Romanian) Propolis

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Abstract

The aim of the present study was to investigate the antibacterial activity of propolis samples collected from various regions in Transylvania (Romania) against six bacterial strains: three Gram-positive bacteria (*Staphylococcus aureus* ATCC 6538P, *Bacillus cereus* ATCC 14579, *Listeria monocytogenes* ATCC 7644), two Gram-negative bacteria (*Escherichia coli* ATCC 10536, *Pseudomonas aeruginosa* ATCC 27853) and one yeast strain (*Candida albicans* ATCC 90028). Ethanolic extracts of the studied samples were prepared for the evaluation of antimicrobial activity, which was appreciated by measuring the inhibition zones diameters. The methodology used was agar diffusion technique inside Petri dishes with nine wells. Enroxil was used as control. A better sensibility of the Gram-positive bacteria (*Staphylococcus aureus*, *Bacillus cereus*, *Listeria monocytogenes*) was noticed; the diameter of the inhibition zones ranged between 8 mm and 17 mm. As for Gram-negative bacteria, results were different for every tested bacterial strain. Inhibition zones diameter for *E. coli* ranged between 7 mm and 12 mm, while for *P. aeruginosa* a total resistance was noticed. Inhibition zones diameters for *Candida albicans* showed a variation which ranged between 0 mm and 10 mm. Minimum inhibitory concentration (MIC) was evaluated for sample PS 20 due to the positive results showed against *Staphylococcus aureus*. According to the results obtained, it might be concluded that the tested propolis samples showed good results against Gram-positive bacteria.

Keywords: antimicrobial activity, flavonoids, minimum inhibitory concentration, polyphenols, propolis

Introduction

Propolis or bee glue is a resinous hive product collected by honeybees from plant exudates and contains more than 160 constituents (Mohammadzadeh *et al.*, 2007). It has been used as natural remedies in traditional medicine from ancient times in many countries and has been extensively studied in Eastern European countries (Ahn *et al.*, 2007; Bankova *et al.*, 2000). Propolis is also used extensively in food and beverages to improve health and prevent different diseases such as inflammations, diabetes (Burdock, 1998).

Propolis is considered responsible for the low incidence of bacteria and moulds within the hive (Marghitas, 2005). The action against microorganisms is an essential characteristic of propolis, and people have used it for centuries for its pharmaceutical properties. Besides its antibacterial, antifungal and antiviral properties, propolis presents many other beneficial biological activities such as antioxidant, anti-inflammatory, antitumor, hepatoprotective, local anesthetic, immunostimulatory, antimutagenic (Bankova *et al.*, 2000; Ghisalberti, 1978; Marghitas, 2005).

Novel treatments for infectious diseases are urgently needed especially with the increase in the emergence of pathogens, which have developed resistance to current antibiotics (Seidel *et al.*, 2008).

Various constituents of propolis, such as flavonoids, display antimicrobial activity, but the precise mode of the antimicrobial action of propolis is still unclear (Seidel *et al.*, 2008).

The antimicrobial activity of propolis has been widely reported. Several studies showed that ethanolic extracts of propolis inhibited the growth of *Staphylococcus aureus* (Gonsales *et al.*, 2006; Lu *et al.*, 2005; Muli and Maingi, 2007).

The purpose of this work was to investigate antimicrobial activity of a selection of propolis samples collected in different areas form Transylvania. Six bacterial strains were used, such as the following: three Gram-positive bacteria (*Staphylococcus aureus* ATCC 6538P, *Bacillus cereus* ATCC 14579, *Listeria monocytogenes* ATCC 7644), two Gram-negative bacteria (*Escherichia coli* ATCC 10536, *Pseudomonas aeruginosa* ATCC 27853) and one yeast strain (*Candida albicans* ATCC 90028). An antibiotic, Enroxil (5 μg enrofloxacin/disc) (KRKA, Slovenia, lot no. 231579) was also tested and its antimicrobial activity was compared with the one of the studied propolis samples.

Materials and methods

Propolis samples

Propolis samples used in this study were obtained directly from beekeepers from Transylvania. All samples were from stationary apiaries and were harvested by scraping the propolis from the frames. After harvesting, the samples were kept in the freezer (-20°C) until further analysis. Tab. 1 shows the origin of twenty propolis samples used for the present study.

Tab. 1. Origin of the Romanian propolis samples

Sample	Collection site	Latitude	Longitude
PS 1	Bucea, Cluj county	46°57' N	22°41.4' E
PS 2	Cuiesd, Bihor county	47°6.6' N	22°17.4' E
PS 3	Bocsita, Salaj county	47°18.4' N	23°2.9' E
PS 4	Zimbor, Salaj county	47°0.1' N	23°15.5' E
PS 5	Poenita, Sibiu county	45°47.2' N	24°8.6' E
PS 6	Ocnisoara, Alba county	46°15.1' N	23°52.4' E
PS 7	Ciucea, Cluj county	46°57.2' N	22°48.5' E
PS 8	Padureni, Sibiu county	45°55.7' N	24°13.1' E
PS 9	Halmeu, Satu-Mare county	47°58.8' N	23°0.8' E
PS 10	Tiream, Satu-Mare county	47°37' N	22°28' E
PS 11	Bobalna, Hunedoara county	45°52.4' N	23°7.5' E
PS 12	Cergau Mare, Alba county	46°5.8' N	23°55.3' E
PS 13	Arinis, Maramures county	47°30.1' N	23°13.4' E
PS 14	Arad, Arad county	46°10' N	21°19' E
PS 15	Ineu, Arad county	46°25.5' N	21°50.2' E
PS 16	Ludus, Mures county	46°28.7' N	24°5.8' E
PS 17	Targu Mures, Mures county	46°32.7' N	24°33.8' E
PS 18	Fersig, Maramures county	47°32.4' N	23°22.9' E
PS 19	Targu Lapus, Maramures county	47°27.2' N	23°51.7' E
PS 20	Biia, Alba county	46°14' N	24°0.3' E

Extraction of active principles from propolis

Propolis samples used for this study were frozen at -20°C, and grounded into a fine powder. The ground propolis was then extracted with ethanol 70% (5 g of propolis with 30 ml ethanol) and left over night at room temperature and continuous stirring. The suspension obtained was then filtered on qualitative filter paper (ø 90 mm, ROTH) and the extraction was repeated two times. Extracts were combined and adjusted to 100 ml with ethanol 70% in a volumetric flask. The final concentration of propolis extracts used in this study was 5%. All extracts were kept in the dark, at room temperature prior to antibacterial testing.

Flavonoid and polyphenols content determinations

Propolis ethanolic extracts used for the quantification of flavonoids and polyphenols were diluted with ethanol 70% to a final concentration of 1% (test solution).

Flavonoid content was determined by quantification of flavones/flavonols and flavanone/dihydroflavonols.

From the test solution 1 ml and 0.5 ml of 5% aluminium chloride in methanol were mixed in a flask containing 10 ml methanol. The volume was adjusted to 25 ml with methanol and after 30 minutes the absorbance was measured at 425 nm against the blank in order to quantify flavones/flavonols (Bonvehi *et al.*, 1994; Popova *et al.*, 2004).

An aliquot of the test solution (1 ml) and 2 ml dinitrophenilhydrazine (DNP) solution (1 g of DNP was mixed with 2 ml of 96% sulphuric acid and diluted to 100 ml with methanol) were heated at 50°C for 50 minutes. After cooling at room temperature, the solution was diluted to 10 ml with 10% potassium hydroxide methanolic solution. 0.5 ml of the solution was transferred into a volumetric flask and the volume was adjusted to 25 ml with methanol. The absorbance was measured at 486 nm against the blank in order to quantify flavanones/dihydroflavonols (Nagy and Grancai, 1996; Popova *et al.*, 2004).

Stock standard solutions of galangin (0.04 mg/ml) for flavones/flavonols and pinocembrin (1 mg/ml) for flavanone/dihydroflavonols were prepared in order to construct the calibration curves. Series of dilutions within the range 0.005-0.04 mg/ml galangin and 0.13-1 mg/ml pinocembrin were prepared and the protocols described above were followed in order to construct the calibration curves. The following equations and correlation coefficients were obtained: $Y = 1.13397 * X - 0.00443$; $r^2=0.99916$ (flavones/flavonols) and $Y = 0.24135 * X + 0.00089$; $r^2=0.99954$ (flavanone/dihydroflavonols).

Blank solutions were prepared by replacing the sample with an equivalent aliquot of methanol which was carried out through all the steps of the procedure.

Total polyphenolic content was determined by the Folin-Ciocalteu colorimetric method. A reference mixture was prepared of pinocembrin and galangin (2:1, w/w), which was further diluted into series of appropriate concentrations that were used for the calibration curve. Standard curve equation was: $Y=0.54005*X-0.00512$; $r^2=0.99900$. 1 ml of the test solution was added into a volumetric flask containing 15 ml of distillate water. 4 ml of Folin-Ciocalteu reagent and 6 ml of 20 % sodium carbonate were also added in the flask. The volume was adjusted to 50 ml with distillate water. All solutions were kept in the dark for 2 h and the absorbance was measured at 760 nm (Popova *et al.*, 2004).

Microorganism

All bacteria were provided from the collection of Faculty of Veterinary Medicine Cluj-Napoca (Romania), Laboratory of Microbiology. Six bacterial strains were used: three Gram-positive bacteria (*Staphylococcus aureus* ATCC 6538P, *Bacillus cereus* ATCC 14579, *Listeria monocytogenes* ATCC 7644), two Gram-negative bacteria (*Escherichia coli* ATCC 10536, *Pseudomonas aeruginosa* ATCC 27853) and one yeast strain (*Candida albicans* ATCC 90028). Each reference strain, except for *C. albi-*

cans and *L. monocytogenes* were replicated in 5ml nutrient broth (Biolab, Hungary). The nutrient broth used for *C. albicans* and *L. monocytogenes* was enriched with glucose solution 10% (Hemopharm Beogradski, Vršac, Serbia and Montenegro), so that the final concentration was 1‰. All bacterial strains were sowed in test tubes and incubated at 37°C for 24 hours.

In vitro study of the antimicrobial activity of propolis extracts

Petri dishes (90 mm diameter) were filled with 15 ml nutrient agar. *C. albicans* and *L. monocytogenes* required an additional glucose solution 10% to the nutrient agar, having the final concentration of 1‰. After the solidification of the nutrient broth, in each Petri dish were realized 8 peripherical wells and one central with a vertical perforator (5 mm diameter). Each Petri dish was sowed by inundation with 1.5 ml bacterial strain solution (10 µl culture solution was dissolved in 2 ml physiological serum). All Petri dishes were than left for 10-15 minutes in order to dry the surface of the broth. In each of the well 20 µl of propolis extracts 5% were added. After the addition of propolis extracts, all Petri dishes were incubated in vertical position at 37°C for 24 hours. Ethanol 70% was tested for antimicrobial activity as control.

Antimicrobial activity of one antibiotic, Enroxil (5 µg enrofloxacin/disc) (KRKA, Slovenia, lot no. 231579) was also tested according to the protocol described above.

Antimicrobial activity of tested propolis ethanolic extracts antibiotic was appreciated by measuring the inhibition zone diameters, in mm.

Minimum inhibitory concentration (MIC) determination

The sample having the best *in vitro* antibacterial activity against *S. aureus* was selected for MIC (minimum inhibitory concentration) determination. Micro dilutions of the ethanolic propolis extract were performed in round-bottomed 96-well plates containing 100 µl nutrient broth. Propolis ethanolic extracts dilutions were in the range of 2.5% ÷ 0.0012%. After the addition of propolis extract, each of the wells was sowed with 10 µl of the tested bacterial strain. All plates were covered and incubated at 37°C for 24 hours. MIC was defined as the lowest concentration of the sample that caused a clear (1-3 mm) zone of inhibition. In order to avoid any reading mistakes bacterial growths were confirmed using Petri dishes divided into 12 sectors. Petri dishes were filled with glucose agar 1‰. Each of the sectors was sowed by knurling from each well containing the dilutions mentioned above. Petri dishes were incubated at 37°C for 24 hours.

All tests were carried out in triplicate and the results were averaged.

Statistical analysis

The mean values and standard deviation were calculated from the data obtained in triplicate using Excel program. Also results were analyzed using Analysis of variance (ANOVA). The probability of 0.05 was considered as significant level.

Results and discussion

Most propolis samples were dark brown, with amber-reddish pieces and intense aromatic resin flavour. It is generally accepted that propolis from temperate zones, like Europe, originate mainly from the bud exudates of *Populus* species, which are rich in flavonoids, phenolic acids and theirs esters (Bankova *et al.*, 2002; Bankova *et al.*, 2000).

Total polyphenols content in our propolis samples varied within the range 23.25-63.23% with an average of 41.03±11.23% (Tab. 2). The sample PS 20 having the highest content in total polyphenols resulted also with the best antimicrobial activity against *Staphylococcus aureus*. Our samples shoed also various amounts of flavones/flavonols and flavanones/dihydroflavonols. These constituents in propolis are generally regarded as responsible for the antimicrobial activity of propolis (Lu *et al.*, 2005; Sforcin *et al.*, 2000).

Tab. 2. Flavonoid and polyphenols content (in %) of studied propolis samples

Sample	Flavonoids content*		Total polyphenols content*	% flavonoids from total polyphenols
	Flavones/ Flavonols content	Flavanone/ Dihydroflavonols content		
PS 1	1,38±0,04	3,48±0,13	26,07±1,33	18,64
PS 2	1,17±0,11	4,89±0,09	29,53±1,13	20,52
PS 3	2,44±0,14	4,17±0,44	38,86±0,67	16,99
PS 4	8,91±0,31	7,48±0,36	48,67±2,01	33,66
PS 5	0,64±0,07	5,01±0,19	41,61±0,84	13,57
PS 6	1,67±0,33	3,88±0,02	36,32±1,98	15,28
PS 7	1,09±0,18	3,59±0,12	32,58±0,13	14,38
PS 8	0,71±0,00	4,43±0,13	43,09±0,42	11,92
PS 9	3,92±0,22	3,48±0,12	30,57±1,25	24,19
PS 10	7,46±0,07	5,50±0,04	36,95±0,55	35,05
PS 11	4,13±0,11	4,33±0,17	41,69±0,21	20,30
PS 12	2,99±0,17	3,11±0,06	23,25±3,20	26,24
PS 13	4,16±0,00	4,37±0,02	32,94±1,18	25,89
PS 14	7,44±0,10	4,68±0,06	58,39±2,50	20,76
PS 15	6,15±0,17	4,50±0,17	31,73±1,09	33,56
PS 16	4,43±0,10	4,04±0,09	49,56±2,23	17,09
PS 17	7,88±0,19	5,15±0,09	56,38±0,75	23,12
PS 18	1,16±0,00	3,52±0,24	45,20±1,32	10,35
PS 19	7,10±0,25	4,07±0,15	54,01±3,23	20,69
PS 20	11,45±0,33	5,40±0,14	63,23±2,44	26,65

*results are expressed as mean of three determinations ± standard deviation

Antibacterial activity was evaluated as inhibition zone diameters of all 20 propolis samples collected in Transylvania (Romania) against five bacterial strains and one yeast strain. Tab. 3 shows the values that were obtained.

According to data presented in Tab. 1, it can be noticed a better sensibility of the Gram-positive bacteria (*Staphy-*

lococcus aureus, *Bacillus cereus*, *Listeria monocytogenes*); the diameter of the inhibition zones ranged between 8 mm and 17 mm (Fig. 1). Extracts considered most active to this test had an inhibition zone diameter within 13 and 17 mm. Data reported in literature (Gonsales *et al.*, 2006) show that propolis samples from Brazil had inhibition zones against *S. aureus* within the range of 8-13 mm.

As for Gram-negative bacteria, results were different

Tab. 3. Results of the antimicrobial activity (in mm) investigation

Sample code	Diameter of the inhibition zones					
	Gram-positive bacteria			Gram-negative bacteria		Yeast strain
	Staphylococcus aureus ATCC 6538P	*Bacillus cereus* ATCC 14579	*Listeria monocytogenes* ATCC 7644	*Escherichia coli* ATCC 10536	*Pseudomonas aeruginosa* ATCC 27853	*Candida albicans* ATCC 90028
PS 1	11	10	9	8	0	0
PS 2	12	10	8	8	0	0
PS 3	13	12	9	9	0	0
PS 4	15	15	9	7	0	8
PS 5	12	10	9	7	0	0
PS 6	12	12	10	8	0	6
PS 7	10	10	9	8	0	0
PS 8	10	10	9	8	0	0
PS 9	13	14	10	9	0	10
PS 10	12	13	9	10	0	6
PS 11	11	13	9	9	0	6
PS 12	12	11	8	9	0	6
PS 13	11	11	7	9	0	6
PS 14	12	15	13	8	0	6
PS 15	11	13	12	12	0	8
PS 16	11	11	12	10	0	6
PS 17	12	13	11	9	0	7
PS 18	10	12	11	7	0	6
PS 19	12	12	15	8	0	6
PS 20	17	14	17	8	0	8

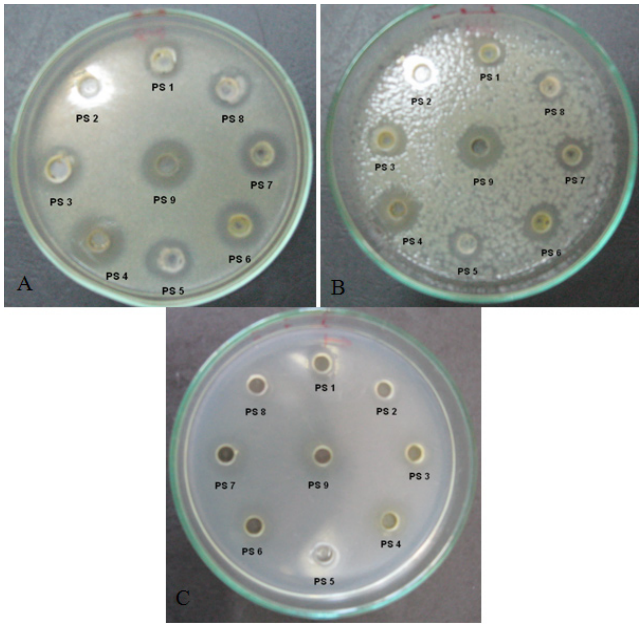

Fig. 1. The inhibition zones for propolis samples tested on Gram-positive bacteria (*Staphylococcus aureus* (A), *Bacillus cereus* (B), *Listeria monocytogenes* (C))

for every tested bacterial strain. Inhibition zones diameter for *E. coli* ranged between 7 mm and 12 mm, while for *P. aeruginosa* a total resistance was noticed. *P. aeruginosa* is a bacteria known for its resistance to antibiotics. Inhibition zones diameters for *Candida albicans* showed a variation which ranged between 0 mm and 10 mm.

Inhibition zones diameter obtained for Enroxil were as follows: 27 mm for *S. aureus*, 24 mm for *B. cereus*, 25 mm for *L. monocytogenes*, 32 mm for *E. coli* and 15 mm for *P. aeruginosa*. *Candida albicans* showed resistance to the tested antibiotic.

The investigation of antimicrobial activity of propolis and also of the tested antibiotic shows interesting results. Some propolis sample show antimicrobial activity against *C. albicans*, while the antibiotic, Enroxil proved to be inefficient. As for the other bacterial stains the antibiotic seems to have a higher antimicrobial capacity than tested propolis samples. Control (ethanol 70%) had no inhibitory effect on the studied bacterial strains.

The differences between the inhibition zone diameters were not statistically significant ($p > 0.05$).

Minimum inhibitory concentration was evaluated for all six tested bacterial strains. Sample PS 20 was chosen for MIC evaluation due to its positive results showed *in vitro* against *Staphylococcus aureus* (Tab. 4).

Numbers from 1 to 12 indicated in the Tab. 4 represents the range of dilutions that were used in order to investigate MIC. These concentrations are as follows: 2.5%; 1.25%; 0.625%; 0.3125%; 0.156%; 0.078%; 0.039%; 0.019%; 0.009%; 0.004%; 0.0024% and 0.0012%.

Tab. 4. Minimum inhibitory concentration (MIC) of sample PS 20

Microorganism	1	2	3	4	5	6	7	8	9	10	11	12
S. aureus	-	-	-	-	-	-	-	-	+	+	+	+
B. cereus	-	-	-	-	-	-	-	-	-	+	+	+
L. monocytogenes	-	-	-	-	-	-	-	-	-	+	+	+
E. coli	-	-	-	+	+	+	+	+	+	+	+	+
P. aeruginosa	-	-	-	+	+	+	+	+	+	+	+	+
C. albicans	-	-	-	-	-	+	+	+	+	+	+	+

Legend: "-" – well in which the growth of the microorganism was absent;
"+" – well in which the growth of the microorganism was present

Several studies regarding antimicrobial activity of propolis ethanolic extracts showed a positive correlation between flavonoids content and antibacterial properties of propolis (Gonsales *et al.*, 2006). The composition of raw propolis depends upon the plan source, bud exudates of different trees, generally *Populus* in the temperate zone (Gregoris and Stevanato, 2010). Propolis contains a wide variability of active principles (flavonoids and phenolic acids). Variations in the flavonoid content of propolis are mainly attributable to the difference in the preferred regional plants visited by honeybees (Ahn *et al.*, 2007).

Conclusions

It may be concluded from this work that tested ethanolic propolis extracts show a good antibacterial activity against Gram-positive bacteria (*Staphylococcus aureus*, *Bacillus cereus*, *Listeria monocytogenes*). As for the Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*) the results obtained are very different. Propolis samples taken into study showed no antibacterial activity on *Pseudomonas aeruginosa*, bacteria also known for its resistance to antibiotics.

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