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ASSESSMENT OF THE ANTIOXIDANT AND ANTIBACTERIAL POTENTIAL OF SOME PROPOLIS-BASED NATURAL PRODUCTS

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Abstract

Propolis has been used for centuries as a medical remedy in both humans and animals. Propolis can be found as a single basic product or as an additional compound in standardized drug formulations. The present research aimed to assess the antioxidant and antibacterial activity of four propolis-based natural products. The investigations were carried out on the following products: a propolis tincture; an aqueous propolis extract; an aqueous propolis extract with colloidal silver; and an ointment consisting of propolis, olive oil, and propolis wax. According to the obtained results, of the four products, the ointment showed the highest antioxidant activity. A higher antibacterial potential has also been demonstrated by the ointment compared to the other investigated formulas.

Key words*: antibacterial, antioxidant, propolis-based products.*

INTRODUCTION

Propolis is commonly referred to as "bee glue", a colloquial term for the resinous material that bees gather from various plant kinds. The Greek word "propolis" has a particular sense, "pro" meaning "defense" and "polis" signifying "community" (Castaldo & Capasso, 2002).
The bioactive components of propolis provide both antioxidant and immune-protective qualities. Propolis is made up of several different compounds, which vary depending on the geographical area. Therefore, propolis contains a variety of chemicals, such as phenolic acids, alcohols, aromatic aldehydes, lignans, flavonoids, esters, diterpenes, sesquiterpenes, amino and fatty acids, as well as minerals and vitamins (Batista et al., 2012). The main flavonoid components of propolis, galangin, pinocembrin, and pinostrobin, are linked to its antibacterial action. They interfere with the DNA genetic encoding of microorganisms and enhance the membrane's permeability of bacteria (Cornara et al., 2017). Moreover, flavonoids can function as powerful anti-bacterial compounds by blocking the secretion of nucleic acids, the adhesion and production of biofilms, and the pathogens' metabolism (Freires et al., 2016).
There are two aspects to take into account regarding the propolis's antibacterial activity. Firstly, it is associated with the immediate impact on the microbe itself, and secondly, with the immune system boost that triggers the organism's inherent defense mechanisms (Sforcin & Bankova, 2011).
The present paperwork aimed to study four natural Romanian propolis-based products, by assessing their antioxidant and antibacterial potential.

MATERIALS AND METHODS

The biological material used in this study was represented by four Romanian propolis-based products and their form of presentation and composition are shown in Table 1. To prevent any alteration or change of the product's composition, the samples were stored in their original, hermetically sealed glass containers for tinctures and aqueous extracts, and plastic containers for ointments, respectively. Additionally, all products were kept out of direct sunlight in dry space until further analyses were carried out.

Table 1. The investigated apitherapeutic products

No. crt.	Product	Form of presentation	Composition
P1	Aqueous propolis extract with colloidal silver	Aqueous extract	Aqueous propolis extract, colloidal silver 70 ppm
P2	Propolis tincture	Tincture	Ethyl alcohol, propolis
P3	Aqueous propolis extract	Aqueous extract	Aqueous propolis extract
P4	Propolis and olive oil-based ointment	Ointment	Propolis, olive oil, propolis wax

The DPPH (2, 2-Diphenyl-1-picrylhydrazyl) radical scavenging activity of the products was assessed spectrophotometrically according to Bankova et al. (2016), with certain adjustments made within the Laboratory for Quality Control of Bee Products and Diagnosis in Bee Diseases (APHIS-DIA) USAMV Cluj-Napoca because of the specifics of the studied products. This test is a widely used technique for evaluating the antioxidant capacity of different products. To conduct this analysis, the samples were prepared as follows: for the liquid products, 1/10 dilutions were made, using water as a solvent for P1 and P3, and 80% ethanol for P2, respectively. In regards to the ointment (P4) containing propolis, olive oil, and propolis wax, solubilization was required first using the following solvents: pure hexane; a mixture of hexane and methanol 70% 1:1; a mixture of hexane and ethanol 1:1; and dimethylsulfoxide (DMSO). The purpose of using several substances was to compare the degree of solubilization of the product, considering its oily-viscous consistency. Based on the results, the mixture containing methanol and hexane has been used in the study's subsequent stages. The DPPH solution was prepared extemporaneously; thus, 1 mg of solid DPPH were dissolved in 50 ml of pure methanol, obtaining a solution with a concentration of 0.02 mg/ml; then, 0.5 ml of each diluted sample and 2.5 ml of DPPH solution were added. The blank sample consisted of 0.5 ml of methanol and 2.5 ml of DPPH solution. The samples' absorbance was read at the wavelength of λ = 517 nm. The antioxidant activity of the samples was expressed as a percentage of inhibition (% inhibition) and IC50 (the sample concentration required to inhibit 50% of free DPPH radicals).

The obtained data regarding both % inhibition and IC50 for all the investigated products (P1, P2, P3, and P4) was tested for normal distribution using the Shapiro-Wilk test, followed by ANOVA one-way and Tukey's multiple comparisons test (alpha was set 0.05).

The broth microdilution method, which has been standardized by the Clinical and Laboratory Standards Institute (2012) and well documented in the literature (Patton et al., 2006; Drago et al., 2007; Cremers et al., 2020), was utilized to evaluate the antibacterial activity. The lowest dose of an antimicrobial drug at which no microbe growth was detected in agar or broth dilution techniques is known as the minimum inhibitory concentration, or MIC (Clinical and Laboratory Standards Institute, 2012). However, the specificity of the products under study required certain modifications.

The medium used to cultivate the bacterial isolates and assess their susceptibility to the investigated products was Mueller Hinton broth (MHB).

The tested bacterial strains were clinically isolated from various skin wounds of canine, feline, and equine patients within the Microbiology Department, Faculty of Veterinary Medicine, USAMV Cluj-Napoca. The bacterial strains along with their source of origin are listed in Table 2.

Table 2. The provenance of the isolated *Staphylococcus* spp. strains

Bacterial strain	Species	Wound type
307	Canine	Avulsion of the left forelimb
199A	Canine	Skin wound of the left flank region
403C	Feline	Skin wound around the tail base
375	Equine	Postoperative abdominal skin wound (umbilical hernia)
272	Equine	Skin wound on the left stifle
273	Equine	Skin wound of the left fetlock

The bacterial suspensions of the isolated strains were realized in MHB in a 0.5 McFarland turbidity.
Four 96-well plates were inoculated as shown in Figure 1 for the antibacterial activity testing.

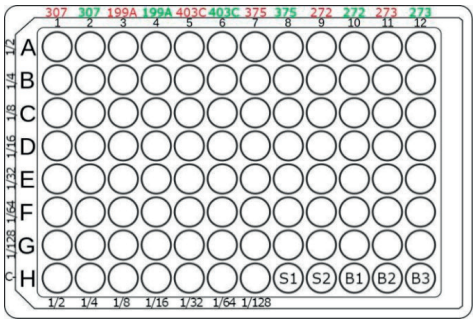

Figure 1. Plate inoculation model for determining the minimum inhibitory concentration; the red numbers represent the test columns and the green numbers the positive control columns; H row 1-7 (C-) negative control consisting in dilution of the product with sterile broth instead of bacterial suspension row; S1, S2 – saline sterility control; B1, B2, B3 – broth sterility control

The two-fold serial dilutions method was used to dilute the products (vertically from A to G); for liquid products (P1, P2, P3), the solvent was sterile saline. In the case of the ointment (P4), the product was first dissolved in 96° alcohol (1/2), after which the two-fold serial dilutions were done in sterile saline. Regarding the H row, wells 1 to 7 were named negative control wells (C-) in which 100 µL of two-fold serial dilution of the product and 100 µL of sterile broth were added. Wells H 8-12 served as sterility controls, wells S1 and S2 for the saline used for products' dilutions, and wells B1, B2, and B3 for the broth. In the odd columns (1, 3, 5, 7, 9, 11, rows A to G) representing the test columns, over 100 µL of the serial dilution of the product, 100 µL of bacterial suspension was added. In the even columns (2, 4, 6, 8, 10, 12, rows A to G), referred to as positive control columns, over the 100 µL of sterile saline used instead of diluted product, 100 µL of bacterial suspension was added. In the case of P4, two-fold serial dilution of the 96° alcohol in saline was used instead of saline in the positive control columns. After adding the bacterial suspensions, the plates were incubated at 37°C for 24 hours.

RESULTS AND DISCUSSIONS

The DPPH method was used to assess the antioxidant capacity of the investigated products. When DPPH radical reacts with various hydrogen donors, like antioxidants, it decolorizes from its dark purple hue to pale yellow (Baliyan et al., 2022). The color change was monitored using UV spectrophotometry. Determinations were made according to the following formulas:

$$\% \text{ Inhibition} = [(\text{Absorbance blank} - \text{Absorbance sample})/\text{Absorbance blank}] \times 100;$$

$$\text{IC50 sample} = [(50 \times \text{sample concentration } (\%)]/ \% \text{ Inhibition}$$

The final value for each propolis-based product is the mean of three independent determinations ± Standard deviation, as seen in Figure 2 and Figure 3.
The ointment (P4) consisting of propolis, olive oil, and propolis wax, exhibited the highest radical scavenging, with a mean % inhibition value of 94.15% ± 0.16; however, the propolis tincture's (P2) mean value of % inhibition was quite similar to P4, namely 89.24 % ± 0.29. The lowest antioxidant activity was observed among the aqueous extracts, as follows: the aqueous propolis extract (P3) recorded a mean % inhibition value of 51.27±0.84%, whereas the aqueous propolis extract with colloidal silver (P1), registered a mean value of 38.87±5.16%.

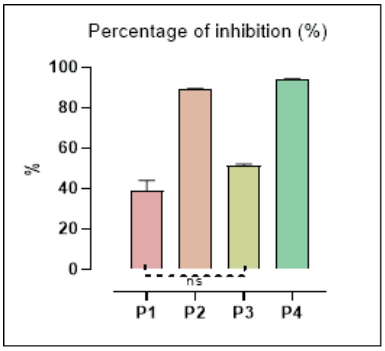

Figure 2. Graphical representation of the % inhibition of the samples

The IC50 value expresses the sample concentration required to inhibit 50% of the free DPPH radicals present in the solution. The lower this value, the higher the antioxidant

capacity of the sample. As expected, the lowest mean value of IC50 was found in P4 (0.53 ± 0.0009), followed by P2 (0.56 ± 0.0019), P3 (0.98 ± 0.0158), and P1 (1.30 ± 0.1612), respectively. Overall, statistically significant differences were observed when comparing the antioxidant activity (% inhibition and IC50) of the tested samples. The results of the ANOVA one-way test indicated that there is a statistically significant difference between the compared values (Percentage of Inhibition: $F = 60.18$; $p = 0.0157$ and IC50: $F = 325.7$; $p = 0.0028$). Further analysis using Tukey's multiple comparison test revealed that there were no significant differences between the antioxidant potential of products P1 and P3 regarding both the % Inhibition ($p = 0.1477$) and the IC50 values ($p = 0.1895$). This suggests that, according to Tukey's test, the antioxidant effects of products P1 and P3 were similar and not statistically different from each other.

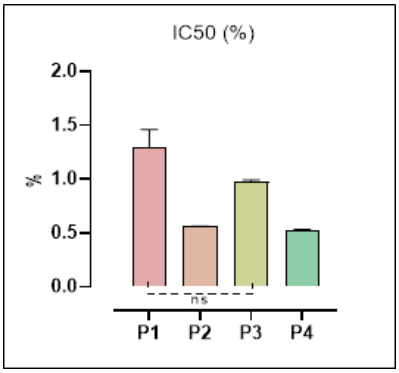

Figure 3. Graphical representation of the IC50 of the samples

The antibacterial activity of the investigated products was initially evaluated by optical density (OD) read using a multichannel spectrophotometer.

In the case of P1 and P4, the minimum inhibitory concentrations were obtained for each bacterial strain, by calculating the difference between the OD of the serially diluted product inoculated with the bacterial strain and the absorbance of the negative control (template) of the same concentration. The obtained result was compared with the corresponding positive control. The last dilution where the difference value was less than half of the positive control value was considered to be the minimum inhibitory concentration of these products.

Regarding P2 and P4, the MIC could not be determined using the calculation method described above. Both these products have a more intense color than the aqueous extracts, and if bacterial growth occurs in the wells, due to the higher turbidity, the sample will lose its initial absorbance.

Due to the difficulties in the interpretation of the obtained OD, the antibacterial activity of the investigated products was evaluated by a naked-eye assessment of the medium's turbidity. The results for P1 and P3 were comparable using both assessments. The minimum inhibitory concentrations (% of propolis-based products in saline) are presented in Table 3.

Table 3. The minimum inhibitory concentrations (%)

Product	*Staphylococcus spp.* bacterial strains (MIC; %)					
	307	199A	403C	375	272	273
P1 (Aqueous propolis extract with colloidal silver)	6.25	3.12	3.12	6.25	12.5	6.25
P2 (Propolis tincture)	3.12	12.5	3.12	6.25	3.12	6.25
P3 (Aqueous propolis extract)	6.25	25	25	25	25	12.5
P4 (Propolis and olive oil-based ointment)	1.56	6.25	6.25	3.12	1.56	1.56

Product P4 emphasized the best inhibitory activity against the tested bacterial strains, the 1.56% concentration having an inhibitory effect on the growth of 3 bacterial strains (307, 272, 273), and that of 3.12% on a single strain (375); the 199A and 403C strains proved to be the most resistant, but even so, they were inhibited by a concentration of 6.25% of the product; additionally, 5 bacterial strains were inhibited at concentrations in the range of 3.12-6.25% in the case of P1 (aqueous extract with silver), while P3 (aqueous propolis extract) inhibited one bacterial strain (307) at the concentration of 6.25% (307) and another strain (273) at the concentration of 12.5%.

As a study limitation, in the plate no. 4 designed for P4, the solvent used to dilute the product might have interfered with the bacterial growth. However, considering the increased antioxidant activity of the ointment, it is unlikely that the obtained results regarding the antibacterial potential are due to the presence of

the solvent and not to the bioactive compounds of the product.

All investigated formulas contained propolis, and the latter is described in the literature as a therapeutic agent due to its curative properties. Available data indicate that propolis exhibits a wide range of significant biological actions as a result of the presence of biologically active components, including antibacterial, antifungal, anti-inflammatory, antiviral, antitumor, hepatoprotective, cardioprotective, and immunomodulatory properties (Farooqui and Farooqui, 2012; Jansen-Alves et al., 2019; Rivero-Cruz et al., 2020; Yuan et al., 2019; Ozdal et al., 2019; Asem et al., 2020; Bhadauria et al., 2010). In addition, flavonoids and phenolic acids, recognized as protective agents against reactive oxygen species are responsible for propolis's antioxidant action. However, the average amounts of polyphenolic components in Romanian propolis extracts have been found to present considerable variation depending on the geographical area (Gatea et al., 2015; Mărghitaş et al., 2014; Dezmirean et al., 2017), the procedures used in beekeeping (Stan et al., 2011), and harvesting period (Mărghitaş et al., 2013). The extraction technique also influences the amount of polyphenols found in propolis (Oroian et al., 2020a; Oroian et al., 2020b).

The studied products displayed notable radical scavenging activity, with P4 providing the best antioxidant potential. This aspect may be due to its unique composition; olive oil is known for its phenolic content, which exerts strong antioxidant effects (Tuck & Hayball, 2002). In addition, most bioactive chemicals are poorly soluble in water, and phenolic substances are ten times less abundant in aqueous propolis extracts than they are in ethanolic propolis extracts (Mello et al., 2010; Moura et al., 2009). These findings were in accordance with the results of our study; the aqueous propolis extracts showed lower antioxidant activity than the propolis tincture (Figure 2; Figure 3).

The ability of propolis to prevent microbial growth, such as yeasts, molds, and both Gram-positive and Gram-negative bacteria, is well acknowledged (Bankova et al., 2014; Benhanifia et al., 2014; Nedji & Loucif-Ayad, 2014; Özcan et al., 2004; Anjum et al., 2019). In general, propolis has greater effects against Gram-positive than Gram-negative bacteria. The secretion of enzymes that degrade the constituents of propolis, as well as the particular structure of the outermost membrane of Gram-negative bacteria, contribute to this aspect (Sforcin, 2016; Kędzi & Hołderna-Kędzia, 2013).

The testing of the products' antibacterial activity highlighted again the strongest potential of the P4 product, namely the ointment based on propolis, olive oil, and propolis wax. This characteristic is also associated with the presence of polyphenols in olive oil, which possess antibacterial effects as well (Capasso et al., 1995). Regarding the antibacterial activity of the aqueous propolis extracts, P1, which has colloidal silver in addition to P3, recorded better values. The antibacterial activity of colloidal silver against both Gram-positive and Gram-negative bacteria was postulated by Vila-Domínguez et al. (2020). According to Barras et al. (2018), this may be explained by the damaging effect of silver on microorganisms by adhering to the chemical structures found on their surface.

CONCLUSIONS

Based on the results obtained when assessing the antioxidant and antibacterial potential of the investigated products, we consider that the use of apitherapeutic formulas in medical practice may offer a viable substitute for diminishing the incidence of antibiotic resistance. We also appreciate that the form of presentation of propolis-based products has a direct influence on the biologically active compounds' content and implicitly on the bioactive properties of the products. In addition, during the study we encountered difficulties regarding the dilution of propolis-based products with a viscous-oily consistency; therefore, conducting more research on finding the most suitable solvents for the solubilization of this type of products is crucial for obtaining conclusive results.

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