

ANTIBACTERIAL EFFICIENCY OF FIVE PROPOLIS EXTRACTS ON PLANKTONIC AND ADHERENT MICROBIAL STRAINS

ABBAS HAZEM¹, CARMEN VIOLETA POPESCU^{3,4*}, IULIANA CRIȘAN³, MARCELA POPA², MARIANA CARMEN CHIFIRIUC², GRAȚIELA GRADIȘTEANU PIRCALABIORU², DUMITRU LUPULIASA¹

¹“Carol Davila” University of Medicine and Pharmacy, Faculty of Pharmacy, Bucharest, Romania

²Research Institute of the University of Bucharest, ICUB, Bucharest, Romania

³S.C. HOFIGAL Export Import S.A., Bucharest, Romania

⁴“Vasile Goldiș” Western University, Faculty of Pharmacy, Arad, Romania

*corresponding author: popescu_carmen88@yahoo.com

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Abstract

Due to its complex composition which includes esters, flavonoids, terpenes and coumaric acids, propolis harbours a wide array of biological activities. Among these, the antimicrobial activity is well established. We used reference strains represented by *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922 and we determined the MIC (minimal inhibitory concentration) and MBEC (minimum biofilm eradication concentration) values for different propolis extracts (30% aqueous propolis solution, 50% aqueous propolis solution and 30% aqueous propolis solution, buffered to pH = 6.8; 30% propolis tincture and 50% propolis tincture). The results of the quantitative tests revealed the antimicrobial and anti-biofilm characteristics of the propolis extracts, highlighting their potential use as antimicrobial agents. Importantly, the more diluted aqueous and alcoholic extracts (30%), were the higher efficiency compared to the concentrated ones (50%), had thus suggesting the need for an aqueous medium to optimally solubilize the antimicrobial compounds.

Rezumat

Datorită compoziției complexe ce include esteri, flavonoide, terpene și acizi cumarici, propolisul are o gamă largă de activități biologice. Dintre acestea, activitatea antimicrobiană este foarte intens studiată. În cadrul acestui studiu, am utilizat tulpini microbiene de referință (*Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922) și am determinat valorile CMI (concentrația minimă inhibitoare) și CMEB (concentrația minimă de eradicare a biofilmului) pentru diferite extracte de propolis (soluție apoasă de propolis 30%, soluție apoasă de propolis 50%, soluție apoasă de propolis 30%, tamponată la pH = 6,8, tinctură de propolis 30% și tinctură de propolis 50%). Rezultatele testelor cantitative au evidențiat caracteristicile microbicide și anti-biofilm ale extractelor de propolis sugerând posibila utilizare a acestora ca agenți antimicrobieni. Extractele diluate apoase și alcoolice (30%) au avut o eficiență mai mare comparativ cu cele concentrate (50%), sugerând astfel necesitatea unui mediu apos pentru solubilizarea optimă a compușilor antimicrobieni.

Keywords: propolis extract, antimicrobial, anti-biofilm

Introduction

In the past decades, products made from medicinal herbs or concentrated propolis extracts, as well as semisynthetic drugs obtained from specific phyto-component raw materials were commonly used in different branches of medicine for both prophylactic and curative purposes [6, 32]. Nowadays, the emergence of antibiotic resistance has led to increased research to find alternative treatments to currently available antibiotics, which have become largely or, in some cases, totally ineffective. Plant extracts and plant derived compounds were shown to have important antimicrobial characteristics, whilst having the advantages of reduced toxicity and low risk of resistance selection. Many studies have shown that

propolis is one of the most important natural products containing bioactive components with a range of biological actions and important therapeutic effects for the maintenance of health including broad spectrum antimicrobial activity which explains its action against the harmful effects of microorganisms on bees.

The antibacterial properties of propolis and its constituents were compared to some known chemotherapeutic agents and showed to be as effective as streptomycin and penicillin [14, 28, 30]. The antimicrobial action of propolis is attributed to flavones, flavanones, phenolic acids, and phenolic acid esters. The qualities of flavonoids are related to their low toxicity and even to the absence of pharmacological resistance of germs for these substances. In addition,

propolis, compared to synthetic antibiotics, has no undesirable side effects. On the other hand, the antimicrobial properties of propolis can be attributed to components such as galangin, pinocembrin, chrysin, tectochryzine, pinobanksin, pinobanksin-3-acetate, p-coumaric acid benzyl ester, and caffeic acid esters. Of all the chemical agents present in propolis, galangin (3,5,7-trihydroxyflavone) was found to be one of the most active antibacterial agents [12, 15, 34-36]. The antibacterial activity of propolis is also attributed to catechinic tannins which are involved in the interaction with proteins having a denaturing action on albumin and serum proteins (characterized by changes in absorbance at 280 nm and release of SH-groups) [23]. The antimicrobial mechanisms of propolis are multiple and complex, being determined by the synergistic effects of phenolic compounds and other biologically active components. The antimicrobial mechanisms include disruption of the cytoplasmic membrane and of the bacterial cell wall, partial bacterial lysis, inhibition of bacterial motility, inhibition of cell division with formation of pseudo-multicellular colonies, and inhibition of protein synthesis [20-22]. Regarding the synergistic activity with various antibiotics, some authors have pointed out that propolis acts on the bacterial ribosome (together with chloramphenicol, tetracycline, neomycin), as well as on the bacterial cell wall by facilitating the penetration of antibiotics (amoxicillin, ampicillin, cefalexin) into the bacterial cell [24, 25]. In this context, the purpose of this study was to investigate the antimicrobial and antibiotic activity of water-soluble and hydro-alcoholic extracts from propolis, in order to obtain possible antimicrobial drug formulations.

Materials and Methods

Propolis samples. The following samples were used for testing: 30% aqueous propolis solution, 50% aqueous propolis solution and 30% aqueous propolis solution, buffered to pH = 6.8; 30% propolis tincture and 50% propolis tincture. The physico-chemical characterization of these extracts was performed [1]. For testing the antimicrobial activity, activity of propolis, we selected several microbial reference strains represented by Gram-positive bacteria (*Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 6538), Gram-negative bacteria (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853). Glycerol stocks were streaked on LB agar in order to obtain 24 h cultures to be used for all further studies. The qualitative screening of the microbial strains susceptibility to propolis extracts was done using a diffusimetric method. The inoculum was represented by a suspension in sterile physiological water harbouring a 0.5 McFarland microbial density. From all the different propolis products, 10 μ L were

spotted on the inoculated microbial plates. In the next step, the plates were allowed to stand at room temperature for adsorption of the solution drop into the medium, after which they were incubated at 37°C for 24 hours. The quantitative testing was performed using the Mueller-Hinton liquid micro-dilution method. Specifically, 96-well plates were used to determine the MIC (Minimal Inhibitory Concentration) which represents the minimum amount of propolis extract capable of inhibiting the microbial growth. Serial binary dilutions of the stock solution of the compound were made in 100 μ L medium. From the first well, 100 μ L was transferred to the second, 100 μ L of the second well was transferred to the third and so on until the last well, from which 100 μ L was discarded. Subsequently, the wells were seeded with 20 μ L of microbial suspension with a MacFarland density of 0.5. Microbial suspensions were made in sterile physiological water from 24 hours cultures. Each test was also run with a microbial culture control (a row of wells containing only culture medium inoculated with a microbial suspension) and a control of medium sterility. After incubating the plates at 37°C for 24 hours, the results obtained were analysed by macroscopic examination. In the growth control well, the medium was cloudy as a result of microbial growth. The mandatory sterility control well showed no bacterial growth, the liquid content remaining clear, transparent. The concentration of the propolis extract at which culture development was no longer observed was the MIC (μ g/mL) value [5].

The development of microbial biofilms on inert substrate. Microbial cells were cultured in 96-well plates with nutrient broth and were incubated at 37°C for 24 hours in the presence of the propolis compound. Plates were emptied and washed twice with sterile physiological water, followed by fixation with 130 μ L of 70% methanol (for 5 minutes) and by staining of the adherent cells with 1% violet crystallized alkaline solution (130 μ L/well) for 15 minutes. After washing twice with tap water, the microbial biofilms formed were re-suspended in 33% acetic acid and the intensity of the coloured suspension was measured by reading the absorbance at 492 nm. MBEC (Minimum Biofilm Eradication Concentration) was calculated as the smallest concentration of compound required to eradicate a microbial biofilm.

Results and Discussion

In the present study, the antimicrobial activity of the tested propolis extracts was performed by a qualitative and quantitative methodological approach, including experimental models for assessing the efficiency against planktonic, but also, adherent, biofilm embedded microbial strains. The results of

the qualitative screening highlighted a clear bactericidal activity, revealed by the occurrence of a clear inhibition zone around the spot deposited on the surface of the culture medium, only for the alcoholic propolis extracts. The results obtained after testing the antimicrobial activity of aqueous and alcoholic propolis extracts are shown in Figures 1 - 5. The quantitative analysis of the antibacterial activity of propolis and its extracts has shown that they are active against a broad spectrum of Gram-positive bacteria but have a lower activity or are inactive against Gram-negative bacteria. Our studies showed that among the aqueous solutions tested, the most effective was the aqueous propolis solution 30% (Figure 1), compared to 50% (Figure 2) and buffered aqueous solution (pH 6.8) (Figure 3). For the aqueous solutions, MIC values ranged from 1.56 to 70 µg/mL, the most sensitive being *S. aureus* and *P. aeruginosa*. *S. aureus* proved to be highly sensitive to the propolis aqueous solution 50%, while *P. aeruginosa* showed the best MIC for the propolis aqueous solution 30% and the buffered solution. Importantly, both of these microbial strains are known for their opportunistic and nosocomial potential.

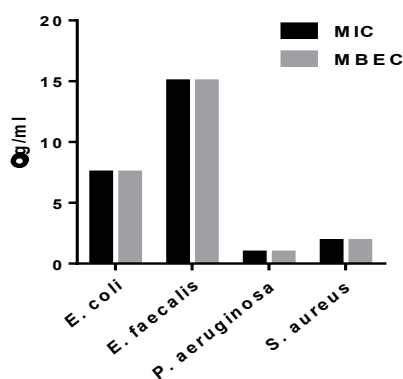


Figure 1.

The MIC and MBEC values for the propolis aqueous solution 30%

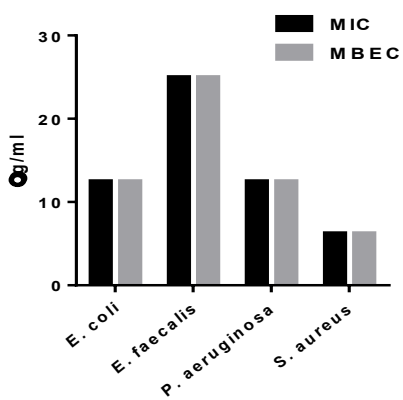


Figure 2.

The MIC and MBEC values for the propolis aqueous solution 50%

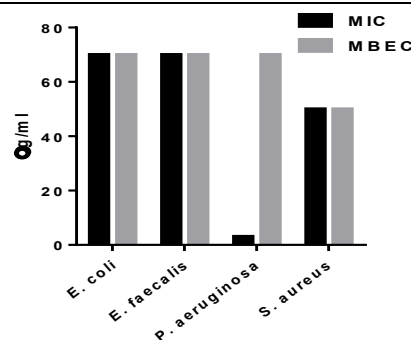


Figure 3.

The MIC and MBEC values for the buffered aqueous propolis 50%

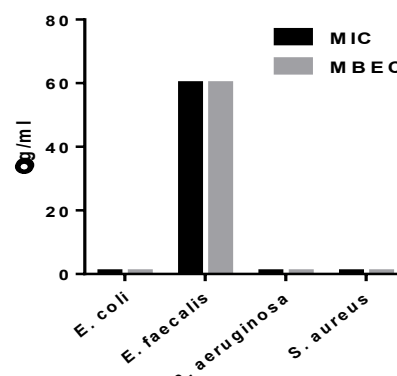


Figure 4.

The MIC and MBEC values for the propolis tincture 30%

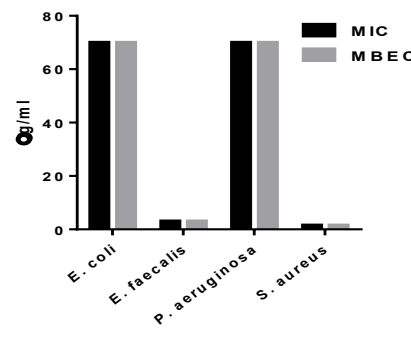


Figure 5.

The MIC and MBEC values for the propolis tincture 50%

Except for the propolis buffered solution, where the MIC value was lower than the MBEC for the *P. aeruginosa* strain, the MIC and MBEC values were identical in all other cases, for all the bacterial strains tested. These results demonstrate the potential of the aqueous solutions to be used in the adjuvant treatment of infection-associated biofilms. As for the analysed alcoholic extracts, the 30% propolis tincture (Figure 4) was more effective than the 50% tincture (Figure 5), and the effectiveness on planktonic and adherent microorganisms was similar. The most sensitive microorganisms were in this case the Gram-

positive *Enterococcus faecalis* (for the propolis tincture 50%) and *Staphylococcus aureus* (for the propolis tincture 30%) strains. Our results are also supported by other researchers who showed the bactericidal effect of ethanolic propolis extract on Gram-positive pathogens: *Staphylococcus aureus*, *Bacillus cereus*, *Enterococcus sp.*, *Streptococcus sp.* and increased resistance of Gram-negative bacteria, requiring higher concentrations of ethanolic extract to be inhibited [11, 13, 27, 31].

For example, the inhibition of *Escherichia coli* and *Pseudomonas aeruginosa* strains with high propolis concentrations (250 - 500 mg/mL) could be obtained. Grange and Davey [11] showed that *Pseudomonas aeruginosa* and *Escherichia coli* were partially inhibited, while the growth of the *Klebsiella pneumoniae* pathogen was not influenced by such an extract. Orsi *et al.* showed that limited propolis activity on Gram-negative bacteria may be due to the structural differences and chemical composition of the Gram-negative and Gram-positive bacterial cell wall [24, 25]. In this direction, some researchers have formulated two hypotheses related to the resistance of Gram-negative bacteria to propolis action: (i) the blocked penetration of propolis constituents into the bacterial cell due to the efflux pumps present in the plasma membrane of the Gram-negative bacteria; (ii) in the propolis composition there are resinous plant-derived substances with a role in protecting them against Gram-positive pathogenic bacteria.

The higher activity of the diluted aqueous and alcoholic solutions observed in our studies can be explained by the fact that the aqueous medium favours the dissolution of compounds with antimicrobial activity as well as the contact with the bacterial cells of the active compounds but also the volatility of the active compounds from propolis. Since the ethanol propolis extract was shown to have an anti-pathogenic effect as it inhibits *S. aureus* virulence by inhibiting coagulase and lipase [32], it is believed that the reduction of these microbial virulence factors is an important target of Gram-positive infection therapy. Our studies have also demonstrated the anti-pathogenic activity of propolis, highlighted by the inhibition of biofilm production, which has a paramount role in microbial pathogenesis. The formation of microbial biofilms protects the microorganisms from the host immune response and antimicrobial substances [7], therefore explaining the involvement of biofilms in 80% of total infections [4, 8, 16-18, 26, 33]. Within this context, the propolis's ability to inhibit the formation of microbial biofilms is poorly investigated. Was analysed the chemical composition of propolis, highlighting that it contains compounds that can inhibit N-acyl homoserine lactone mediated signalling in *P. aeruginosa*.

Studies conducted by Duarte *et al.* highlighted that propolis inhibits growth of microorganisms present in the oral cavity as well as streptococcal glucose-transferase (GTF) activity responsible for glycan synthesis that promotes bacterial adherence and dental plaque formation [10]. The ability of different propolis derivatives (flavonoids, cinnamic acid and terpenoids) to inhibit GTF enzymes from *Streptococcus mutans* and *Streptococcus sanguis* was also investigated. It was also suggested that apigenin (4,5,7-hydroxy-flavone) would be the most effective inhibitor of GTF in both the solution and the surface of the hydroxyapatite beads covered by the salivary film. Stan *et al.* showed that the ethanolic propolis extract from Romania exerted an inhibitory effect on the adhesion of the Gram-positive *S. aureus* and observed a positive correlation between the propolis tincture concentration and the decrease in the bacterial cell adhesion. In addition, the use of propolis in combination with some antibiotics had a synergistic effect against antibiotic resistant strains, a synergism which caused lower MIC values for several bacterial strains [24, 29].

Therefore, antibacterial drugs that have become ineffective as a result of antibiotic resistance could be used simultaneously with propolis at the onset of infections [29].

By use of some antibiotics, the efficacy and duration of action of propolis extract is much more pronounced and thus microorganisms do not develop antibiotic resistance. It was also observed that different propolis samples with a different chemical composition exhibit identical antimicrobial activity, suggesting that propolis has the same biological activity determined by different combinations of bioactive components independently of the geographical area and chemical composition [13, 37].

This hypothesis was confirmed by studies on propolis samples from Europe and Brazil, where antimicrobial activities were similar even though there were differences in terms of chemical composition.

Conclusions

Taken together, the results of the quantitative tests have highlighted the antimicrobial and anti-biofilm characteristics of the propolis extracts, demonstrating their potential for use as antimicrobial agents, as adjuvants for current antibiotics or as preservatives in the pharmaceutical, cosmetic and food industry. Generally, more dilute aqueous and alcoholic extracts (30%) were more efficient than the concentrated ones (50%), hence demonstrating the need for an aqueous medium to solubilize the antimicrobial compounds. The MIC values obtained had a better effect on the Gram-positive bacteria compared to the Gram-negative strain, which confirms other

studies from the literature. In most cases, aqueous extracts and alcoholic propolis have shown similar inhibitory action against microbial cells in the planktonic and adherent growth stage, demonstrating their anti-biofilm effect and potential use in the management of chronic infections associated with biofilm formation.

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