aromatic acids and their esters, aliphatic acids and their esters, terpenoids, bioactive compounds responsible for the biological effects of propolis (Kujumgiev et al. [7]). Since ancient time propolis has been used in folk medicine, being one of the most effective natural remedies. Pharmacologically active compounds of propolis are present in varying amounts depending on the propolis sample, the mode of dosage and the nature of extraction method (Ugur şi Arslan [8]). The ratio of organic compounds in propolis is very important for the biological effects (Fokt et al. [2]). The flavonoid compounds are considered to be responsible for its main therapeutic actions. The researches on the antibacterial activity of propolis and its extracts have revealed that they were active against a broad spectrum of Gram-positive bacteria, but exhibited a lower activity or were inactive against Gram-negative bacteria. One of the main opportunistic and nosocomial pathogens is *Staphylococcus aureus,* causing skin, gastrointestinal tract, genital tract, urinary tract infections, hard to treat due to its increasing resistance to antimicrobials and specific and nonspecific host defense mechanisms (Lee et al. [9]).. Moreover, *S.aureus* could develop biofilms both on living tissue and the inert surfaces of medical devices and implants. The pathogenesis of *S. aureus* is facilitated by multiple virulence factors which are implicated in cellular adherence and in invasion and dissemination of infections. The cell surface factors, termed the microbial surface component recognizing adhesive matrix molecules (MSCRAMMs) are involved in the initiation and colonization process (Foster and Hook [10], Speziale et al. [11]). Also, the virulence factors secreted during different stage of infections play an important role in the virulence of *S. aureus*. Enzymatic factors such as lipases, nucleases, proteases, exotoxins may help *S. aureus* to avoid the immune system, to destroy the host tissue and thus facilitate the bacterial spreading (Lowy [12], Dinges et al. [13], Foster [14], Gordon and Lowy [15]). Coagulase-negative staphylococci (CoNS) are opportunistic pathogens that colonize the skin and mucosa normally and can cause serious infections in immunocompromised hosts. *S. haemolyticus, S. sciuri* and *S. warneri* are CoNS species could produce clinically significant infections difficult to treat because of antibiotic resistance and their ability to form biofilms associated with the insertion of medical devices (de Allori et al. [16]). Skin lesions favor the development of acute or chronic skin infections. The process of wound healing is more difficult due to the presence of chronic wounds associated biofilms. The increased incidence of infections caused by bacteria of the genus *Staphylococcus* and their resistance to conventional antibiotic treatment require finding new effective solutions to prevent and to stop the progress of these infections. In recent years there have been extensive studies on the chemical composition and biological effects of natural product, including propolis in order to introduce them in the treatment of various diseases. The purpose of this work was to evaluate the anti-pathogenic effect of eight ethanolic propolis extracts (EEP) from different Romanian regions on 11 *S. aureus* clinical strains.

2. Materials and Methods

Preparation of ethanolic extracts of propolis

We used in our study raw propolis collected from eight Romanian regions. Propolis samples were initially kept in a freezer at -18 ° C and subsequently grounded to obtain a fine powder. In order to obtain the ethanol extract of propolis, there were added 70 ml of 96% ethanol over 30 g of propolis powder. The obtained mixture was kept for 7 days at room temperature while stirring occasionally. The obtained solution was then filtered through filter paper with a pore size of 0.5 mm. (Tagliacollo and Orsi [17]). The extraction was repeated twice, washed with 96% ethanol; the obtained filtrates were combined and the total volume was adjusted to 100 ml with the same extraction solvent. The concentration of the ethanolic

extract obtained was 30%. Ethanolic extracts of propolis were stored in brown tightly closed bottles at room temperature.

Bacterial strains

For testing antipathogenic activity of propolis eight *Staphylococcus aureus* strains, one *Staphylococcus haemolyticus* strain, one *Staphylococcus sciuri* strain and one *Staphylococcus warneri* strain were used. These bacterial strains were isolated from wound secretions found in skin infections.

Assessment of flavanols and flavonols in propolis by the spectrophotometric method

To quantify flavonoids there were used ethanolic extracts of propolis (concentration 30%) obtained from propolis samples of the 8 Romanian counties (Dâmbovița, Calarasi, Dolj Mehedinti, Mures, Arad, Bihor and Cluj). Propolis ethanolic extracts were diluted with ethanol 96% v / v to obtain solutions with a final concentration of 1%. These solutions were then used to determine the content of flavones and flavonols from propolis. The content of flavones and flavonols were determined by the method of aluminum chloride which consist in the development of a color reaction due to the formation of a complex between the aluminum ion Al (III) and the carbonyl and hydroxyl groups of flavonoids (Gomez-Caravaca et al. [18]). In a 25 ml flask were mixed 1 ml of ethanolic extract of propolis concentration 1%), 1 ml of 5% $AlCl_3$ and 10 ml of methanol. The volume was then adjusted to 25 ml with methanol. The resulting solution was kept for 30 minutes at room temperature and the absorbance at 425 nm was measured using a UV-VIS spectrophotometer Shimadzu. For preparing the blank solution the sample was replaced with the same volume of methanol (Popova et al. [19]). The quercetin (3,3-, 4-, 5,7-pentahydroxyflavondihydrate) (Merck) was used as a reference standard for calibration. Calibration was achieved by a series of dilutions from the stock standard solution of quercetin in methanol (0.8 mg / ml), obtaining five standard solutions with concentrations ranging from 0.1- 0.8 mg / ml.

Determination of total polyphenols from propolis by Folin-Ciocalteu method

The method used to determine the total polyphenol content of propolis of the eight ethanolic extracts diluted with 96% ethanol for obtaining working solutions with a concentration of 1% was Folin-Ciocalteu colorimetric method. In a 50 ml flask containing 15 ml of distilled water was added 1 ml of working solution (concentration 1%), 4 ml of Folin-Ciocalteu reagent and 6 ml of 20% sodium carbonate (w/v). The final volume was adjusted to 50 ml with distilled water. The solution was kept for 2 hours in the dark. After 2 hours the absorbance was measured at 760 nm. Blank solution was prepared by replacing the sample with an equal volume of distilled water. (Popova et al. [19]). Gallic acid was used as the standard reference for the calibration curve. There were prepared five standard solutions by dilutions of the gallic acid stock standard solution (1.2mg / ml). The concentrations of these standard solutions were ranged from 0.3 to 1.2 mg / ml.

Antibiofilm assay

In 96-well plates with nutrient broth (TSB Tryptic Soy Broth) there were cultivated bacterial suspensions of 0.5 McFarland (20 µl) density obtained from fresh bacterial cultures. Also, different concentration of EEP (0.058 - 30 mg/ml) were added to the inoculated plates and incubated at 37°C for 24 hours. After incubation, the plates were gently washed twice and the adhered cells were fixed with cold methanol 80% (100µl) for 5 minutes. After this period

Evaluation of the expression of virulence factors of microbial strains tested in the presence of ethanolic extracts of propolis

An important target of infections therapy is to reduce microbial virulence factors. It has been shown that EEP could inhibit the expression of coagulase and lipase in S*taphylococcus sp*. (Scazzocchio et al. [34]). In the present study, all tested EEP had various inhibitory effects on the soluble virulence factors production, depending on the tested bacterial strain. Eight soluble enzymatic virulence factors were tested: hemolysins, lipase, lecithinase, amylase, gelatinase, caseinase, DN-ase and esculinase. None of tested *Staphylococcus* strains expressed gelatinase and amylase. DN-ase was produced by only one bacterial strain (*Staphylococcus haemolyticus* P1).

Table 2. Effect of Romanian EEP on the soluble virulence factors expression of bacterial strains: *S.aureus* 3956 (1), *S.aureus* 4085 (2), *S.aureus* 4115 (3), *S.aureus* 4192 (4), *S.aureus* 4258 (5), *S. haemolyticus* P1(6), *S.aureus* P2 (7), *S.sciuri* P4 (8), *S.warneri* P5 (9).

Bacterial strains	EEP Arad	EEP Bihor	EEP Cluj	EEP Mures	EEP Calarasi	EEP Dambovita	EEP Dolj	EEP Mehedinti
	Lecithinase							
1	-	-	-	-	-	-	-	+
2	+	-	+	-	-	-	-	-
3	-	-	+	-	-	-	-	-
4	-	-	+	-	-	-	-	-
5	-	-	+	-	-	-	-	-
8	+	+	-	-	-	-	-	-
	Lipase							
1	-	-	-	-	-	-	-	+
2	+	-	+	-	-	-	-	-
3	-	-	+	-	-	-	-	-
6	++	++	++	++	++	-	-	-
7	-	-	+	-	+	+	-	+
8	+	-	-	-	-	-	+	-
9	-	-	-	+	-	-	-	-
	Caseinase							
1	-	-	-	-	-	-	-	+
3	-	-	+	-	-	-	-	-
7	-	-	-	-	++	-	-	-
8	++	++	-	-	+	-	+	-
9	-	-	-	+	-	-	-	-
	DN-ase							
6	-	++	-	++	-	-	+	++
	Haemolysins							
1	-	-	-	-	-	-	-	+
2	-	-	+	+	-	-	-	-
3	-	-	+	-	-	-	-	-
4	-	-	+	-	-	-	-	-
5	-	-	+	-	-	-	-	-
9	-	+	-	-	-	-	-	-
	Esculinase							
9	-	+	-	-	-	+	-	-

Legend: „+ " partial inhibition, „++" complete inhibition

In table 2 there is presented the influence of tested EEP on the expression of soluble virulence factors. We observed that EEP Bihor inhibited partially or totally the expression of all soluble virulence factors. Also, EEP Mehedinti and Cluj were efficient, decreasing the virulence factors production. In the case of *S. aureus* P3 strain production of four virulence factors (lecithinase, lipase, caseinase and hemolysin) was not inhibited by any of the eight tested propolis extracts. Our results indicated that some of EEP repressed the expression of soluble virulence factors implicated in bacterial dissemination from invasive infections: lipase determines pore formation in host membrane cell, caseinase contributes to cell damage and invasion and DN-ase is responsible for lesions in host cell and bacteria spreading.

Conclusions

Our results showed that all studied propolis ethanolic extracts affected the ability of various microbial strains to form biofilms on inert substrates, with MBEC values ranging from 0.2343 mg / ml to 15 mg / ml. The antimicrobial activity of the tested propolis extracts varied depending on the geographical area and the chemical composition of propolis. The propolis ethanolic extracts with an increased polyphenols content increased (EEP Arad - 17.62% and EEP Cluj - 21.54%) showed an enhanced inhibitory activity on the microbial biofilms development with the lowest MBEC values (0.2343 mg / ml). Ethanolic extracts of propolis inhibited completly or partially the secretion of soluble enzymatic factors involved in the virulence of the tested *Staphylococcus* strains, except for one *S. aureus* strain. The most frequent inhibited soluble virulence factors were: lipases, caseinases and DN-ases. Also, there was a change in the adherence to the cellular substratum index and pattern of tested bacterial strains in the presence of EEP. In this respect it was found that three of the eight propolis extracts (EEP Arad, EEP Bihor and EEP Dolj) were the most effective with a strong inhibitory effect on *Staphylococcus* sp. strains adherence to the cellular substrate. The other extracts of propolis have induced a decreased adherence index. The Romanian propolis extracts, by inhibiting the analyzed microbial strains adherence capacity to eukaryotic cells and decreasing the production of soluble virulence factors, may be used in the development of effective strategies to prevent colonization and infectious process initiation and/or progress. The propolis extracts can be particularly used in the treatment of opportunistic infections caused by antibioresistant bacterial strains with the ability to develop microbial biofilms.

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