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Assessment of the antistaphylococcal activity of ethanolic extract of propolis (EEP)

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The aim of this study was to determine *in vitro* conditions the antimicrobial activity of Ethanolic Extract of Propolis (EEP), originating from Poland, in relation to 23 various *Staphylococcus* spp. strains depending on time of its activity. The conducted research shows that examined EEP demonstrated the highest activity in the twentieth hour of research although, all the analyzed strains displayed MIC value lower than 37.5 mg/ml. Summing up the obtained research results, it may be concluded that preparations including appropriate concentration of EEP may constitute an alternative way of treating infections caused by various *Staphylococcus* spp. strains and may also complement antibiotherapy.

Key words: *Staphylococcus* spp., propolis, antimicrobial activity.

INTRODUCTION

Microorganisms making up the genus *Staphylococcus* are very often responsible for a number of infections (Zhang et al., 2004; Morellion et al., 2005; Akcam et al., 2009). Constituting one of the main elements of human physiological flora, staphylococci colonize the skin of every person (Malikova et al., 2007). One of the main pathogenic microorganisms for humans is *S. aureus*, which is responsible for a wide scope of affections connected with nosocomial infections and those that occurred outside the hospital (Luczak–Kadłubowska et al., 2006; Feng et al., 2007). In ca. 20% of healthy individuals *S. aureus* permanently colonizes pharyngo-nasal cavity, and in 30% it is isolated periodically. However, it may also colonize such places as axillae, groins and gastrointestinal tract. Places colonized by staphylococci constitute a reservoir of these bacteria, which in the cases of decreased immunity of an organism, for example, during surgeries, catheterism, assisted breathing or even shaving, may be the source of further infections (Gordon and Lowy, 2008). High risk occurs due to the presence of the methicillin-resistant *S. aureus* (MRSA), one of the reasons being that all these strains are resistant to all beta-lactam antibiotics (Sareyyüpoğlu et al., 2008) and are often resistant to other antibiotics such as aminoglycoside, tetracycline and quinolone antibiotics (Orsi et al., 2012; Akcam et al., 2009; Unal et al., 1994). The second group is made up by coagulase-negative staphylococci, which may also cause severe infections especially in the cases of surgeries and inserting or implanting foreign bodies such as venous catheters, vascular valves, artificial heart valves, vascular protheses, artificial limbs or drainage tubes. Infections are most often caused by contamination of biomaterial during procedures and treatments or as a result of transient bacteremia occurring after a procedure (Gotz, 2002; Bartoszewicz et al., 2005).

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Due to increasing resistance of staphylococci to currently used chemotherapeutic agents more and more often alternative methods of treatment of these infections or methods of supporting antibiotics treatment are being searched for. Usage of preparations of bee origin seems to be one of these methods. Propolis is a resinous substance collected and party converted by bees which has strong bactericidal and bacteriostatic proprieties. Important pharmacologically active components of propolis include flavonoids, phenols and aromatic compounds (Lu et al., 2005; Uzel et al., 2005). The antibacterial proprieties of propolis are predominantly connected with the presence of flavanone-pinocembrin, flavonol-galangin and caffeic acid phenethyl esters; the machanism of their activity is probably connected with inhibition of bacterial activity of RNA polymerase (Uzel et al., 2005). Apart from antibacterial action propolis, thanks to the fact that it contains more than 300 elements from numerous chemical compound groups, is also characterized by antiviral, antifungal (Orozco et al., 2010; Stepanović et al., 2003; Scazzocchio et al., 2006; Velazquez et al., 2007) anti-inflamatory, antioxidant (Moreira et al., 2008; Shimizu et al., 2004), detoxicant, regenerating, cardioprotective (Tringali, 2001; Cushine et al., 2003; Kabała-Dzik et al., 2003), antihepatotoxic (Bhadauria et al., 2008; Shukla et al., 2004) antitumour and radioprotective actions (Szliszka et al., 2011; Oršolić and Bašić, 2005) *inter alia*.

Susceptibility to the action of propolis is indicated by both Gram-positive and Gram-negative bacteria. Gram-positive bacteria susceptible to the action of propolis include various species from the genus *Staphylococcus*, streptococci such as for example, *S. pneumoniae*, *S. mutans*, *S. pyogenes*, *Enterococcus faecalis*. Other microorganisms demonstrating susceptibility to propolis are yeast-like fungi from the *Candida* spp., bacilli *M. tuberculosis* and Gram-negative bacteria: *E. coli*, *K. pneumoniae* and *P. aeruginosa* (Dziedzic et al., 2013; Stepanović et al., 2003; Krol et al., 1993; Scheller et al., 1999; Rahman et al., 2010). The aim of this study was to determine *in vitro* conditions the antimicrobial activity of ethanolic extract of propolis (EEP), originating from the area of southern Poland, in relation to 23 various *Staphylococcus* spp. strains isolated from hospital environment, depending on time of its activity.

MATERIALS AND METHODS

Propolis

Samples of propolis were obtained from an apiary in Kamianna (southern Poland). Ethanolic extract of propolis was obtained according to methods described by Krol et al. (1993), with custom modification. During the first phase propolis was mechanically minced, and then to flat-bottomed flask 10 g of propolis and 100 g 95% ethanol was added in order to obtain alcoholic extract. The flask was placed on a shaker in a dark, closed container for 14 days in room temperature. After this period of time ethanolic extract of propolis was cooled in 4°C temperature for 24 h, and then it was filtered through filter paper (Whatman number 4) in order to precipitate all insoluble substances. Filtrate obtained was evaporated in rotary evaporator in temperature 40°C. Obtained substance was weighed out and dissolved in 96% ethanol till concentration of 75 mg/ml was obtained.

Microorganisms

Twenty three of the *Staphylococcus* spp. strains was examined. Twenty two hospital environmental strains from the Microorganisms Collection of Department and Institute of Microbiology and Virology, Medical University of Silesia in Katowice and reference strain *S. aureus* ATCC 25923 were used. Among analyzed microorganisms, 16 strains belong to the genus *S. aureus*, 3 strains to *S. hominis*, 2 strains to *S. epidermidis* and 2 to *S. xylosus*. Stored in Viabank system (-80°C), bacterial strains were inoculated onto fluid growth medium (Tripticase Soy Broth) and incubated in 35°C for 16 to 18 h ambient air. Then, bacteria were cultured onto agar growth medium with addition of 5% of sheep's blood. In the next stage suspensions of 23 *Staphyloccocus* spp. strains in a 0.9% NaCl solution was prepared in order to obtain turbidity was equal to a 0.5 McFarland standard scale (DensiLaMeter Pliva Lachem, Brno), which equaled 1.0 x 10^8 CFU/ml. From the initial suspension 66.7 µL was drawn and added to 10 ml 0.9% NaCl solution.

Antibacterial activity

Antimicrobial activity of EEP was examined by means of serial dilutions. In order to do so, to 2 ml of Müller-Hinton liquid growth material (Müller-Hinton Broth) 2 ml of ethanolic solution of propolis were added and a series of 6 dilutions of propolis from 37.5 to 1.17 mg/ml were performed. In the next stage, to each test tube from the series of dilutions 50 µL of bacterial suspension was added. In the 15th min, the whole was incubated 18 to 24 h in 35°C and in the 12th and the 24th h the minimal inhibitory concentration (MIC) growth of microorganisms and the lowest concentration of EEP visually inhibiting the growth of bacteria was determined. Using the same method, a control study with 96% ethanol without addition of propolis was performed.

RESULTS

Obtained results of antimicrobial activity of 23 examined *Staphylococcus* spp. strains in the 15th min as well as the 12th and the 24th h of incubation are presented in Table 1. Due to the fact that strain number 7 did not exhibit any growth in the control research, it was excluded from further analyses. In the 15th min of incubation in the cases of 7 (30.43%), *Staphylococcus* spp. strains examined concentrations from 37.5 to 1.17 mg/ml growth inhibition was not noticed. The minimal concentration of EEP inhibiting the growth of microorganisms for the 15 (65.22%) examined strains was within the scope of 9.38 and 37.5 mg/ml. For 13 (56.52%) of examined strains the MIC was 37.5 mg/ml and 1 (4.35%) in the cases of the MIC value 18.7 and 9.38 mg/ml. In concentration 4.68 mg/ml and lower, in the examined times in all analyzed samples growth of microorganisms was noticed (Figure 1).

In the 12th h of research the minimal concentration inhibiting the growth of examined microorganisms *Staphylococcus* spp. was within the scope from 37.5 to 1.17 mg/ml. In 10 (43.48%) of the examined strains in the

Table 1. Minimal concentration of EEP inhibiting the growth of the *Staphylococcus* spp. strains in the 15th min, 12th and 24th h of incubation.

Number of the strain	Number of the strain in the collection	Identification	Concentration 37.5 mg/ml	Concentration 18.75 mg/ml	Concentration 9.38 mg/ml	Concentration 4.69 mg/ml	Concentration 2.34 mg/ml	Concentration 1.17 mg/ml	Growth control
1	12	*S. aureus*	-/-/-	+/-/-	+/-/-	+/-/-	+/-/-	+/-/-	+/+/+
2	16	*S. epidermidis*	+/-/-	+/-/-	+/+/-	+/+/+	+/+/+	+/+/+	+/+/+
3	37	*S. aureus*	+/-/-	+/-/-	+/-/-	+/-/-	+/-/-	+/-/-	+/+/+
4	38	*S. aureus*	+/-/-	+/-/-	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+
5	40	*S. aureus*	-/-/-	+/+/-	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+
6	41	*S. xylosus*	-/-/-	+/-/-	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+
7	46	*S. aureus*	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/+
8	74	*S. aureus*	+/-/-	+/-/-	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+
9	86	*S. aureus*	-/-/-	+/-/-	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+
10	107	*S. aureus*	+/-/-	+/+/-	+/+/-	+/+/+	+/+/+	+/+/+	+/+/+
11	109	*S. aureus*	-/-/-	+/-/-	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+
12	120	*S. hominis*	-/-/-	-/-/-	-/-/-	+/-/-	+/-/-	+/-/-	+/+/+
13	124	*S. epidermidis*	+/-/-	+/-/-	+/-/-	+/-/-	+/+/-	+/+/+	+/+/+
14	130	*S. hominis*	+/-/-	+/-/-	+/+/-	+/+/+	+/+/+	+/+/+	+/+/+
15	155	*S. aureus*	-/-/-	+/-/-	+/-/-	+/-/-	+/-/-	+/-/-	+/+/+
16	156	*S. aureus*	-/-/-	+/-/-	+/-/-	+/-/-	+/-/-	+/-/-	+/+/+
17	168	*S. aureus*	-/-/-	+/-/-	+/-/-	+/-/-	+/-/-	+/+/+	+/+/+
18	170	*S. aureus*	-/-/-	+/-/-	+/-/-	+/-/-	+/-/-	+/-/-	+/+/+
19	195	*S. xylosus*	-/-/-	+/-/-	+/-/-	+/-/-	+/-/-	+/+/-	+/+/+
20	212	*S. aureus*	-/-/-	+/-/-	+/-/-	+/-/-	+/-/-	+/-/-	+/+/+
21	236	*S. aureus*	-/-/-	+/-/-	+/-/-	+/-/-	+/-/-	+/-/-	+/+/+
22	243	*S. hominis*	-/-/-	-/-/-	+/-/-	+/-/-	+/+/-	+/+/-	+/+/+
23	ATCC 25923	*S. aureus*	-/-/-	**+/-/-**	+/-/-	+/-/-	+/-/-	+/-/-	+/+/+
24	Ethanol Control	*S. aureus*	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+

+, Growth of microorganisms; - , lack of growth of microorganisms.

12th h of incubation the minimal concentration inhibiting the growth of microorganisms was 1.17 mg/ml and 2 (8.7%) for concentrations 2.34 and 4.69 mg/ml, 7 (30.43%) for concentration 18.75 mg/ml and 2 (8.7%) for concentration 37.5 mg/ml (Figure 2).

In the 24th hour of examination the scope of minimal concentration of EEP inhibiting the growth of examined *Staphylococcus* spp. strains was from 18.75 to 1.17 mg/ml. In 23 (52.17%) of the examined strains in the 24th h of incubation already in concentration 1.17 mg/ml no microbial growth was noticed; 1 (4.35%) strain in concentration 2.34 and 4.69 mg/ml, 3 (13.04%) srains in concentration 9.38 mg/ml, and 6 (26.09%) strains in concentration 18.75 mg/ml (Figure 3). In the control of antimicrobial activity of ethanol with the use of the reference culture *S.*

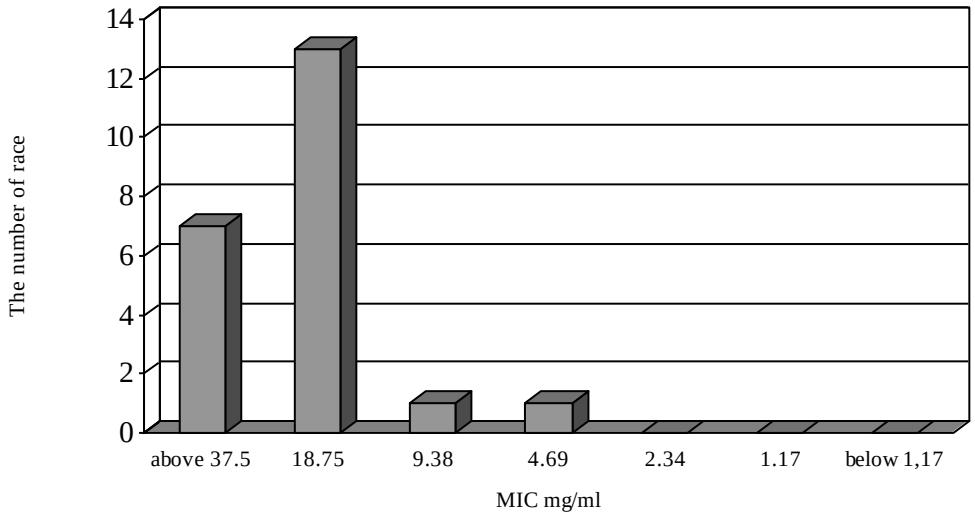

Figure 1. Layout of MIC value of ethanolic extract of propolis with regard to the *Staphylococcus* spp. strains in the 15th minute of incubation.

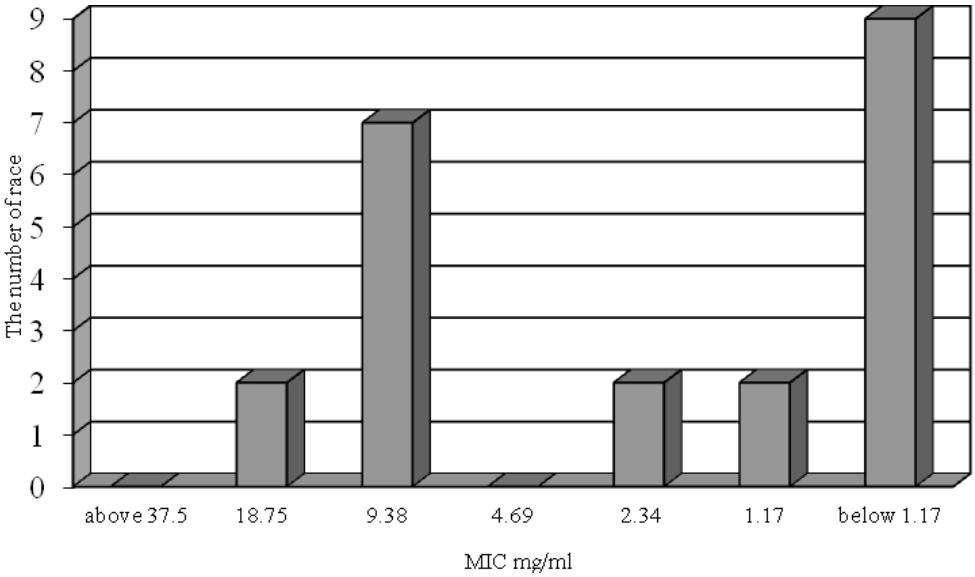

Figure 2. Layout of MIC value of ethanolic extract of propolis with regard to *Staphylococcus* spp. strains in the 12th hour of incubation.

aureus ATCC 25923 the inhibition of growth in none of dilutions in examined times was noticed.

DISCUSSION

At present the problems with antibiotics abuse and difficulties with obtaining new compounds useful in infection treatments lead to the comeback of numerous treatments used in previous years, and a positive effect of propolis action is known since years (Scazzocchio et al., 2006; Miorin et al., 2003). In the research of Hegazi et al. (2000), propolis samples originating from three different regions (Austria, France, Germany) on *S. aureus* and *E. coli* strains were analyzed. It was demonstrated that depending on the place of origin, propolis was showing different activity on examined strains. In the case of *S. aureus* strains the strongest activity was showed by propolis originating from Germany.

In our research, considerable diversity in anti-staphylo-

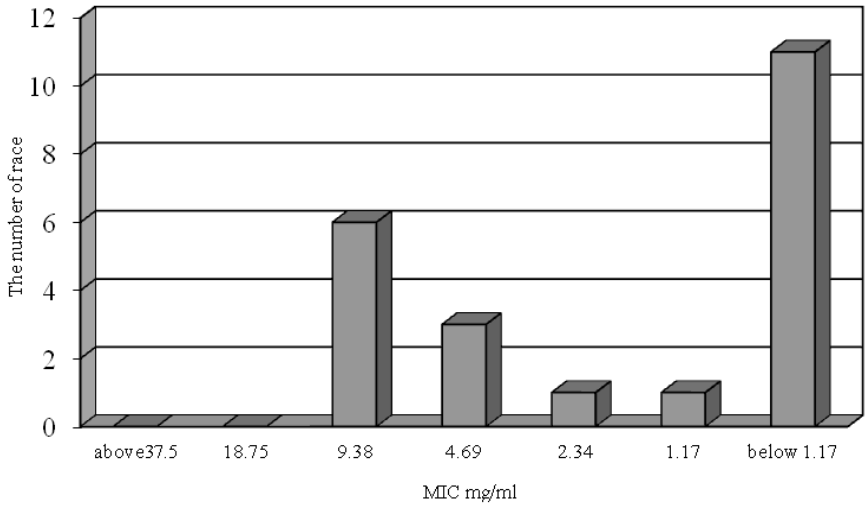

Figure 3. Layout of MIC value of ethanolic extract of propolis with regard to *Staphylococcus* spp. strains in the 24th h of incubation.

coccal activity of ethanolic extract of propolis was noticed. Some of the *Staphylococcus* spp. strains displayed great sensitivity to low concentration of propolis. In the 24th hour of incubation, majority of 23 examined strains demonstrated MIC value lower than 1.17 mg/ml. Similar research results were obtained by Scazzocchio et al. (2006) who described MIC_{50} and MIC_{90} values for 140 clinical strains of *Staphylococcus* spp. In their research they showed that the values of MIC_{50} and MIC_{90} for 35 *S. aureus* strains were for both 1.25 mg/ml and in the cases of 63 other *Staphylococcus* spp. strains respectively 1.25 and 2.5 mg/ml. Klilic et al. (2005) in his research on 3 samples of propolis, originating from Mamak and Kemaliye regions (Turkey), determined the MIC value on *S. aureus* MRSA and *E. faecalis* strains. Klilic et al. (2005) in their research demonstrated that dependence of antibacterial activity effect of EEP is changeable and dependent on propolis fraction and bacteria species. Higher activity of the examined extracts of propolis was gained with regard to S. aureus strains, and depending on the place of origin MIC value were 15.6, 140.4 and 140.6 µg/ml. Obtained results indicate also a considerable diversity in EEP activity with regard to examined Staphylococcus spp. strains. Uzel et al. (2005) in their research drew attention to large differences in propolis activity depending on the genus of bacteria.

Analyzing ethanolic extracts of propolis obtained from 4 samples of propolis on 13 strains of Gram-positive bacteria, Gram-negative bacteria and yeast-like fungi the researchers demonstrated that all of them exhibited antimicrobial activity, and MIC value was within the range from 2 to 256 µg/ml. In the cases of examined *S. aureus* and *S. epidermidis* strains, the MIC value range was from 8 to 3 µg/ml depending on the origin of propolis. In the case of Gram-negative bacteria MIC values were considerably higher. Similar research results were obtained by Stepanovič et al. (2003) examining 13 various ethanolic extracts of propolis from various regions of Serbia the researchers demonstrated that the extracts showed increased activity with regard to Gram-positive bacteria. MIC value of these bacteria was within the range 0.078 and 1.25% EEP. The researchers demonstrated also that despite the considerable diversity of propolis samples, it showed little diversity in its activity.

Heterogenic activity of propolis is connected with the different content of its active substances. The research of Uzel et al. (2005) demonstrated that the highest activity was displayed by samples of propolis with high content of galangin, pinocembrin or caffeic acid derivatives as well as from synergism of these three components. Analyzing the obtained research results a high diversity in MIC values within the analyzed *Staphylococcus* spp. strains may be observed. The conducted research shows that examined EEP demonstrated the highest activity in the 24th h of research although, all the analyzed strains in the 12th h of research displayed MIC value lower than 37.5 mg/ml.

Despite the fact, that, the results presented in this study were promising, the clinical controlled studies are needed to define the validated efficacy. This research would determine the potential medical application of propolis in combination with certain antimicrobial drugs on staphylococci diseases, resistant to standard drugs. Antimicrobial action of the antibiotics can be increased when given along with other highly-reactive agents (for example, organic extracts), which could reduce the early break down of the antibiotic agent by enzyme or synergisti-

cally influence the final results of the pharmacotherapy. The propolis extract may facilitate the antibiotic action by correcting the pharmacokinetic or pharmacodynamic properties and potentiate its biological action.

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