Arch. Biol. Sci., Belgrade, 62 (4), 915-934, 2010 DOI:10.2298/ABS1004915I

IN VITRO ANTIBACTERIAL ACTIVITY OF PROPOLIS EXTRACTS ON 12 DIFFERENT BACTERIA IN CONDITIONS OF 3 VARIOUS PH VALUES

S. IVANČAJIĆ , I. MILEUSNIĆ and DESANKA CENIĆ-MILOŠEVIĆ

Faculty of Stomatology, 26000 Pančevo, Serbia

Abstract - This research investigated the effects of propolis extracted by 5 different solvents and aged for 7 days on twelve species of bacteria classified into four groups according to their pathogenicity in slightly acidic (pH=6), neutral (pH=7) and slightly alkaline (pH=8) environments. Propolis extracted by the examined solvents had antibacterial effects. The strongest effects on the growth of all tested microorganisms, except on the bacteria of the *Salmonella* genus, regardless of the pH value of the environment, were exerted by propolis extracted by ether, acetone, toluol and chloroform. In some cases the antibacterial action of propolis was best in a slightly acidic environment (pH=6).

Key words: Bacterial cultures, pH of the environment, propolis, solvents.

UDC 638.135:615.451:615.281

INTRODUCTION

Propolis is a rubbery, sticky, brown, thermoplastic resin collected by honey-bees from tree buds. Honey bees use propolis in their hives as a universal means of repairing crevices, as a surface cover, hardener and preservative. It is probably also used as a repellent since it is applied inside the beehive and around its entrance. The term propolis originates from the Greek words *pro* – meaning before/in front and *polis* – meaning town (Jankovic, 1968) and denotes the fact that bees use propolis to construct the entrance to the beehive – "town". It is also known as "bee glue".

Propolis has bactericidal and fungicidal properties and it is used as an alternative treatment for infections. The wide range of action of propolis on various microorganisms is the result of the combined activities of flavonoids and aromatic compounds.

In principle, active components of a natural preparation can be separated by solvents in the process of extraction. Solvents of different polarity may be used as extractants. For this purpose polar (water, glycerol, methanol), less polar (ethanol, propyl alcohol, acetone, and others) and nonpolar (dichloroethane, chloroform, carbon tetrachloride, diethyl ether, benzol) solvents are used. Today, ethanol is generally the solvent used in the process of extraction of propolis and all published data show the effects of propolis extracts dissolved in ethanol. Since ethanol belongs to the group of less polar solvents, when used as an extractant only less polar active substances are extracted, while flavonoids are extracted in minimal quantities. Using nonpolar solvents, nonpolar flavonoids can better be extracted from the basic substrate, leading to an increase of their concentration in the final preparation. Pharmacologically active constituents of propolis have been discovered in fractions that are soluble in solvents such as ethanol. Several large classes of active substances have been identified in a variety of constituents of propolis. Among these are: flavones, flavonoles and flavanones (known under the collective name of flavonoids), various phenols and aromatics. Flavonoids represent a large group of natural pigments and active compounds of herbal origin. All flavonoids have two benzene rings linked by a triple bond. The most significant phenols are: cinnamyl alcohol, cinnamic acid, vanillin, benzyl alcohol, benzoic acid and caffeic and ferulic acids (which are derivatives of cinnamic acid with an extra hydroxy and/or methyloxy group). Propo-

lis also contains rare compounds such as phenol triglycerides (Popravko et al., 1983), pterostilbene and eugenol and phenethyl ester of caffeic acid. The principal pharmacological constituents of propolis are natural antibiotics – flavonoids which exert most of the healing properties of propolis. The antimicrobial activity of propolis has been well investigated (Ghisalberti, 1979; Grange and Davey, 1990). Flavon pinocembrin affects numerous bacteria, fungi and molds and it is, together with galangin, 3-acetyl pinobanksin and caffeic and ferulic acid, probably responsible for many of the biological activities of propolis (Hegazi and Abd El Hady, 2001; Hegazi and Abd El Hady, 2002a; Hegazi and Abd El Hady, 2002b; Popova et al., 2004).

During the physiological processes in an organism the mechanism of homeostasis maintains the pH value of the internal environment around the neutral level. However, during pathophysiological processes, the chemical reactions that take place decrease the level of pH, making the affected environment slightly acidic.

The principal hypothesis of this study was that the bactericidal and antimicrobial effects of propolis vary depending on: the type of solvent used during the extraction, bacterial species and acidity of the environment.

The aim of this study was to define which preparation of propolis had most antimicrobial activity so that the way propolis is used could be improved. The bactericidal effect of propolis extracted by different solvents (ethanol, ether, acetone, toluol and chloroform) aged for 7 days on cultures of 12 different species of bacteria (*Morganella morgani, Streptococcus faecalis, Achromobacter, Sarcina lutea, Escherichia coli, Aeromonas hydrophila, Salmonella typhimurium, Bacillus subtilis, Salmonella gallinarum, Salmonella cholerae, Staphylococcus aureus, Bacillus cereus*) in varying (pH=6, pH=7 and pH=8) environment conditions, was investigated.

MATERIAL AND METHODS

Standardized pure cultures of bacterial strains procured from the Faculty of Veterinary Medicine, University of Belgrade, were used in this research. The bacterial species were chosen according to the frequency that they were used in various researches and also according to the frequency of infections in humans. Pure cultures of bacterial species belonging to four different groups, according to the classification published in Bergey's Manual of Determinative Bacteriology (Tortora et al., 2004) were used in this research. This classification is one of the best known and most accepted among microbiologists.

I group - bacteria banal: *Proteus morgani* (morganella), *Streptococcus faecalis*, *Achromobacter – Acinetobacter*, *Sarcina lutea* and *Escherichia coli*.

II group - opportunistic pathogenic bacteria: *Aeromonas hydrophila*, *Salmonella typhimurium* and *Bacillus subtilis*.

III group – infectious pathogenic bacteria: *Salmonella gallinarum* and *Salmonella cholerae.*

IV group - exotoxic pathogenic bacteria: *Staphylococcus aureus* and *Bacillus cereus.*

To ensure the homogeneity of the solution, only propolis from one colony and a fixed time period was used in this study.

Five solvents were used to extract the active ingredients in propolis: ethanol (C2H5OH), ether (C2H5-O-C2H5), acetone ((CH3)2CO), chloroform (CHCl3) and toluol (C6H5CH3). The extracted propolis was aged for 7 days and used for experiments that were performed with all test microorganisms. Extraction was performed in the following manner, regardless of the type of solvent. The mix of solvent and water (volume ratio from 60:40 up to 96:4) was poured into a mixer with a double container (used for cooling purposes). Then, when ethanol was used, 500 ml of ethanol was added to 150 g of propolis. The extraction process lasted for 48 h, after which 465 ml of filtrate were obtained with a specific weight of 0.845. The weight of the precipitate was 130 g, the temperature of the water bath was 65°C and the steaming time was 20 min. The weight of the propolis extract obtained was 60 g.

When ether was used for the extraction, 500 ml of ether was added to 150 g of propolis. The extraction process lasted for 48 h, after which 360 ml of filtrate were obtained with a specific weight of 0.782. The weight of the precipitate was 160 g, the temperature of the water bath was 40°C, and the steaming time was 10 min. The weight of the propolis extract was 80 g. When acetone was used, 500 ml of acetone was added to 150 g of propolis. The extraction lasted 48 h yielding 450 ml of filtrate with a specific weight of 0.842. The weight of the precipitate was 130 g, the temperature of the water bath was 55°C, and the steaming time was 30 min. The weight of the propolis extract was 80 g. When extraction was done by chloroform, 500 ml of chloroform was added to 150 g of propolis. The extraction took place for 48 h, yielding 500 ml of filtrate with a specific weight of 1.350. The weight of the precipitate was 120 g, the temperature of the water bath was 55°C and the steaming time was 45 min. The weight of the propolis extract was 150 g. When extraction was done by toluol, 500 ml of toluol was added to 150 g of propolis. The extraction process lasted for 48 h, yielding 486 ml of the filtrate with a specific weight of 0.865. The weight of the precipitate was 160 g, the temperature of the water bath was 65°C, and the steaming time was 15 min. The weight of the propolis extract was 40 g.

Cooling was done at 278 – 288K for 30 min and mixing at the speed of 20 m/sec. The resulting suspension was centrifuged three times in succession. The obtained extract was a clear liquid, dark brown in color.

Test microorganisms were cultivated in a nutritious broth (obtained from Institute of Virology, Vaccines and Sera "Torlak", Belgrade, Serbia).The broth contained peptones, beef extract, sodium chloride and potassium phosphate in a standardized proportion. The dissolved base broth was heated to boiling point until completely dissolved. The dissolved base, having a pH value of 7.3 was poured into test tubes and sterilized in an autoclave for 15 min at 120°C. Broths cultivated by test microorganisms were incubated for 24 h at 30°C. Each test microorganism was re-cultivated in this manner three days in succession before the experiment. In this experiment agar (obtained from Institute of Virology, Vaccines and Sera "Torlak", Belgrade, Serbia) was used as the growth medium. The agar consisted of peptone, beef extract, potassium phosphate and agar in standardized proportions. This medium altogether consisted of peptone, beef extract, potassium phosphate and agar in a standardized proportion. 41.3 g of agar powder was suspended in 1000 ml of cold distilled water and left for 15 min. The medium was then carefully heated to boiling point until completely dissolved and poured into bottles and sterilized in an autoclave for 15 min at 120°C. After sterilization, the pH value of the medium was adjusted to 6, or 7, or 8, by the addition of either 0.1 mol of hydrochloric acid solution or 0.1 mol of sodium hydroxide solution. The melted nutritious base was divided into several parts. After cooling down to 45-50°C each part was cultivated by the culture of one test microorganism.

The cultures of all 12 test microorganisms were diluted ten-fold (down to 0.1 mol) by the addition of sterilized physiological solution and cultivated on 100 ml of nutritious agar medium that had been melted and cooled to 45-50°C. In such a manner nutritious agar having three different pH values could be cultivated with each of the 12 species of test microorganisms. The cultivated agar was poured into plastic, sterile Petri dishes, volume 100 ml, so that the thickness of the base was 2 mm. Three holes, 10 mm in diameter, were drilled in the set agar in each Petri dish.

The antimicrobial activity of the propolis samples was investigated by the method of growth inhibition of the chosen test microorganism in the culture medium. Various propolis extracts were placed in the holes made in the cultivated growth medium from which it could freely diffuse into the environment. The growth of the investigated bacterium was inhibited in the diffusion zone according to its sensitivity to propolis. The width of this inhibition zone showed the degree of sensitivity of the investigated bacterium to propolis, ranging from a narrow or nonexistent inhibition zone, in the case of resistance, to a wider zone, corresponding to certain degrees of sensitivity.

Table 1. Activity of the propolis solution aged for 7 days on *Morganella morgani* in slightly acidic (pH=6), neutral (pH=7) and slightly alkaline (pH=8) cultures.

	Morganella morgani				
	ethanol	ether	acetone	toluol	chloroform
pH=6	7.3	8	7.6	9*	6.3
pH=7	6.6	6.6	7	6.3	5.3
pH=8	6.3	6.3	4.3	5	6.3
SE pH=6	±0.14	±0.42	±0.32	±0.24	±0.15
SE pH=7	±0.35	±0.27	±0.5	±0.25	±0.27
SE pH=8	±0.22	±0.51	±0.27	±0.43	±0.17

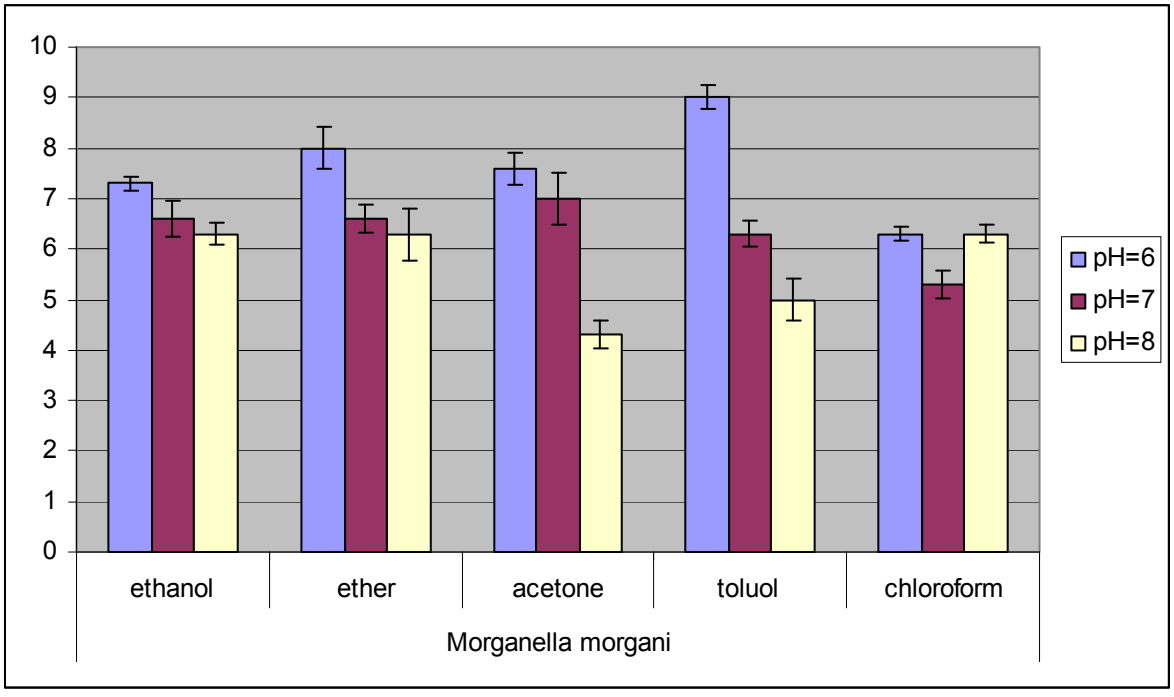

Fig. 1. Activity of the propolis solution aged for 7 days on *Morganella morgani* in slightly acidic (pH=6), neutral (pH=7) and slightly alkaline (pH=8) cultures.

Measuring procedure for antimicrobial activity of propolis

Propolis extracts were heated in a water bath at 50°C until a semi-liquid consistency was reached. Each sample was subsequently poured into holes made in the nutritious agar base. The prepared holes were completely filled by the propolis extracts. The cultures were then incubated for 2 h at 4°C, followed by 18 h at 30°C. The width of the inhibition zone of growth of the tested microorganism was measured from the margin of the hole to its outer border.

The value, expressed in millimeters, was the mean value of measurements around all three holes in one Petri dish.

Since there more than one statistical sample (propolis extracted in 5 types of solvents at 3 pH values), Student T-test according to Bonferroni (Steel RGD et al., 1960) was used to determine the

level of differences between the arithmetical means of the samples' modification.

RESULTS

All results have been statistically processed and shown in tables and figures. Each investigated parameter is represented by the mean value and statistical significance, separately marked.

Results of the inhibitory effect of 5 preparations of propolis in cultures with a pH value of 6, 7 and 8 on bacteria from group I

1. Morganella morgani

Table 1 and Fig. 1 show mean values of the inhibition zones of propolis aged for 7 days on bacterial cultures of *Morganella morgani* in slightly acidic (pH=6), neutral (pH=7) and slightly alkaline (pH=8) cultures.

It was clear that propolis extracted in any solvent had a significant inhibitory effect on the growth of *Morganella morgani*. The propolis extracted in toluol at pH=6 exhibited the most distinct, statistical significant ($p<0.001$) effect, and the propolis extracted by acetone, at pH=8, had the lowest effect on the growth of *Morganella morgani* ($p<0.001$),

The results of the inhibitory effect of propolis extracted by various types of solvents on *Morganella morgani* depending on the pH value of cultures showed that there was a statistically significant ($p<0.05$) stronger effect of propolis extracted by ethanol in cultures with pH=6 than in cultures with a pH value of 8. There was a statistically significant ($p<0.05$) stronger effect of propolis extracted by ether in cultures with pH=6 than in cultures with a pH value of 7 and 8. There was a statistically significant ($p<0.001$) stronger effect of propolis extracted by acetone in cultures with pH=6 than in cultures with a pH value of 8. There was a statistically significant ($p<0.001$) stronger effect of propolis extracted by toluol in cultures with pH=6 than in cultures with a pH value of 7 and 8. There was a statistically significant ($p<0.05$) stronger effect of propolis extracted by chloroform in cultures with pH=6 and pH=8 on bases with pH=6 and 8 than in cultures with a pH value of 7.

2. Streptococcus faecalis

Table 2 and Fig. 2 show mean values of the inhibition zones of propolis aged for 7 days on bacterial cultures of *Streptococcus faecalis* in slightly acidic (pH=6), neutral (pH=7) and slightly alkaline (pH=8) cultures.

The results showed that the effect of propolis extracted by acetone and applied in the growth medium with a pH value of 6 was most distinct and statistically significantly ($p<0.01$) stronger than the inhibitory activity of other types of propolis on the growth of *Streptococcus faecalis*.

The results of the inhibitory effect of propolis extracted by various types of solvents on *Streptococcus faecalis* depending on the pH value of cultures showed that there was a statistically significant ($p<0.001$) stronger effect of propolis extracted by ethanol in cultures with pH=6 than in cultures with a pH value of 8 and a statistically significantly greater effect ($p<0.01$) than in cultures with a pH value of 7. There was a statistically significant ($p<0.001$) stronger effect of propolis extracted by ether in cultures with pH=6 than in cultures with a pH value of 7 and 8. There was a statistically significant ($p<0.001$) stronger effect of propolis extracted by acetone in cultures with pH=6 than in cultures with a pH value of 7 and 8. There was a statistically significant ($p<0.05$) stronger effect of propolis extracted by toluol in cultures with pH=8 than in cultures with a pH value of 6. There was a statistically significant ($p<0.001$) stronger effect of propolis extracted by chloroform in cultures with pH=6 than in cultures with a pH value of 7 and 8.

3. Achromobacter

Table 3 and Fig. 3 show mean values of the inhibition zones of propolis aged for 7 days on bacterial cultures of *Achromobacter* in slightly aci-

Table 2. Activity of the propolis solution aged for 7 days on *Streptococcus faecalis* in slightly acidic (pH=6), neutral (pH=7) and slightly alkaline (pH=8) cultures.

Streptococcus faecalis					
pH=6	pH=6	pH=6	pH=6	pH=6	pH=6
pH=7	pH=7	pH=7	pH=7	pH=7	pH=7
pH=8	pH=8	pH=8	pH=8	pH=8	pH=8
SE pH=6	SE pH=6	SE pH=6	SE pH=6	SE pH=6	SE pH=6
SE pH=7	SE pH=7	SE pH=7	SE pH=7	SE pH=7	SE pH=7
SE pH=8	SE pH=8	SE pH=8	SE pH=8	SE pH=8	SE pH=8

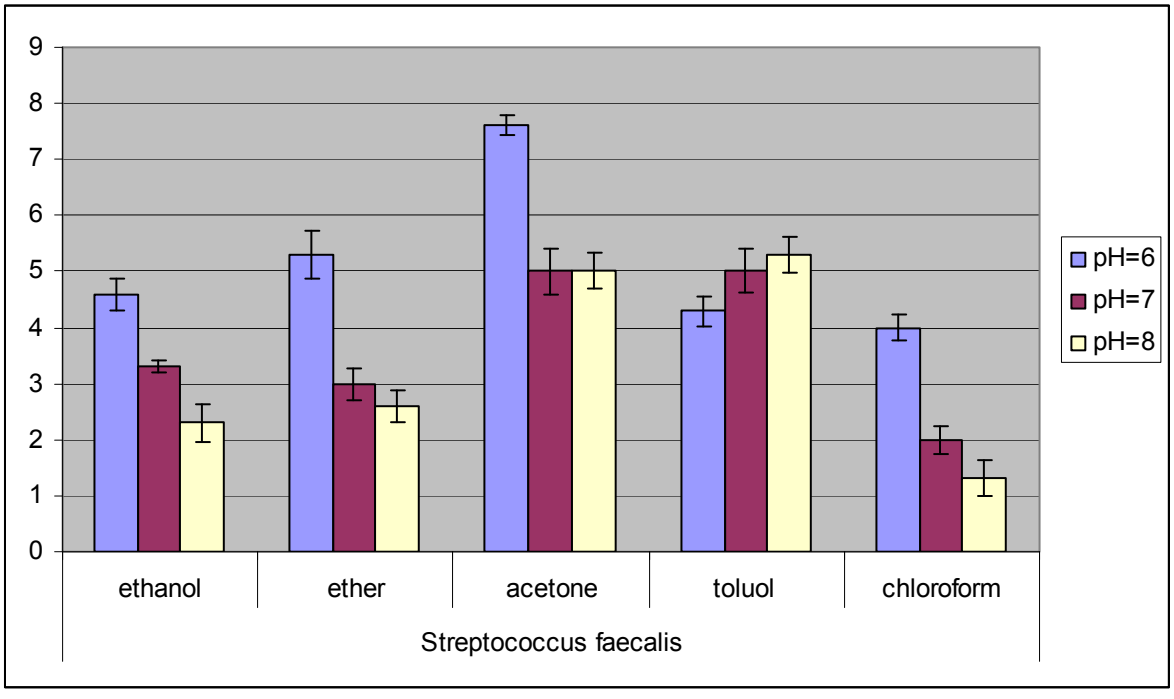

Fig. 2. Activity of the propolis solution aged for 7 days on *Streptococcus faecalis* in slightly acidic (pH=6), neutral (pH=7) and slightly alkaline (pH=8) cultures.

dic (pH=6), neutral (pH=7) and slightly alkaline (pH=8) cultures.

All types of propolis had a distinct inhibitory activity on the growth of *Achromobacter*. However propolis extracted by toluol had a statistically significantly ($p<0.001$) greater inhibitory activity than any other type of propolis, but without statistical significance as regards to the pH of the medium.

4. Sarcina lutea

Table 4 and Fig. 4 show mean values of the inhibition zones of propolis aged for 7 days on bacterial cultures of *Sarcina lutea* in slightly acidic (pH=6), neutral (pH=7) and slightly alkaline (pH=8) cultures.

The results showed that all preparations of propolis had a moderate inhibitory activity on the

Table 3. Activity of the propolis solution aged for 7 days on *Achromobacter* in slightly acidic (pH=6), neutral (pH=7) and slightly alkaline (pH=8) cultures.

Achromobacter					
pH=6	pH=6	pH=6	pH=6	pH=6	pH=6
pH=7	pH=7	pH=7	pH=7	pH=7	pH=7
pH=8	pH=8	pH=8	pH=8	pH=8	pH=8
SE pH=6	SE pH=6	SE pH=6	SE pH=6	SE pH=6	SE pH=6
SE pH=7	SE pH=7	SE pH=7	SE pH=7	SE pH=7	SE pH=7

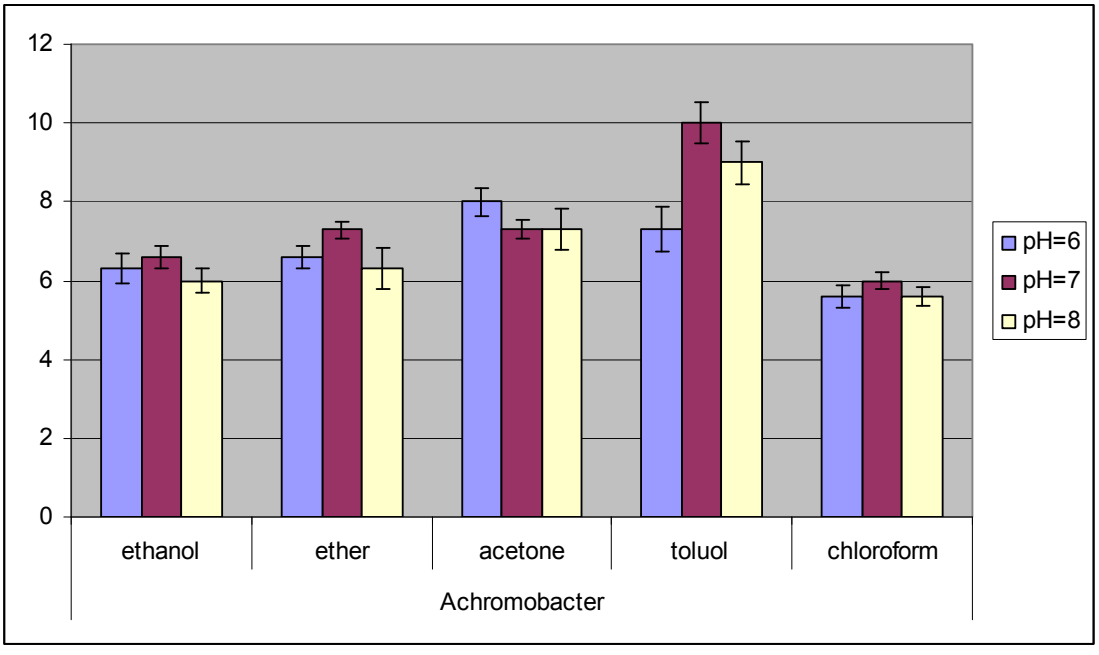

Fig. 3. Activity of the propolis solution aged for 7 days on *Achromobacter* in slightly acidic (pH=6), neutral (pH=7) and slightly alkaline (pH=8) cultures.

growth of *Sarcina lutea*, while there was a statistically significantly ($p<0.01$) stronger inhibitory effect of propolis extracted by acetone and toluol in cultures with a pH value of 6 and 7.

Results of the inhibitory effect of propolis extracted by various types of solvents on *Sarcina lutea* depending on the pH value of cultures showed that there was a statistically significant ($p<0.01$) stronger effect of propolis extracted by ethanol in cultures with pH=6 than in cultures with a pH value of 8. There was a statistically significant ($p<0.05$) stronger effect of propolis extracted by ether in cultures with pH=6 than in cultures with a pH value of 7. There was a statistically significant ($p<0.001$) stronger effect of propolis extracted by toluol in cultures with pH=6 and 7 than in cultures with a pH value of 8. Propolis extracted by acetone or chloroform did not show any significant inhibitory activity on *Sarcina lutea* regardless of the pH value of the medium.

Table 4. Activity of the propolis solution aged for 7 days on *Sarcina lutea* in slightly acidic (pH=6), neutral (pH=7) and slightly alkaline (pH=8) cultures.

Sarcina lutea					
pH=6	pH=6	pH=6	pH=6	pH=6	pH=6
pH=7	pH=7	pH=7	pH=7	pH=7	pH=7
pH=8	pH=8	pH=8	pH=8	pH=8	pH=8
SE pH=6	SE pH=6	SE pH=6	SE pH=6	SE pH=6	SE pH=6
SE pH=7	SE pH=7	SE pH=7	SE pH=7	SE pH=7	SE pH=7

* statistically significant ($p<0.01$)

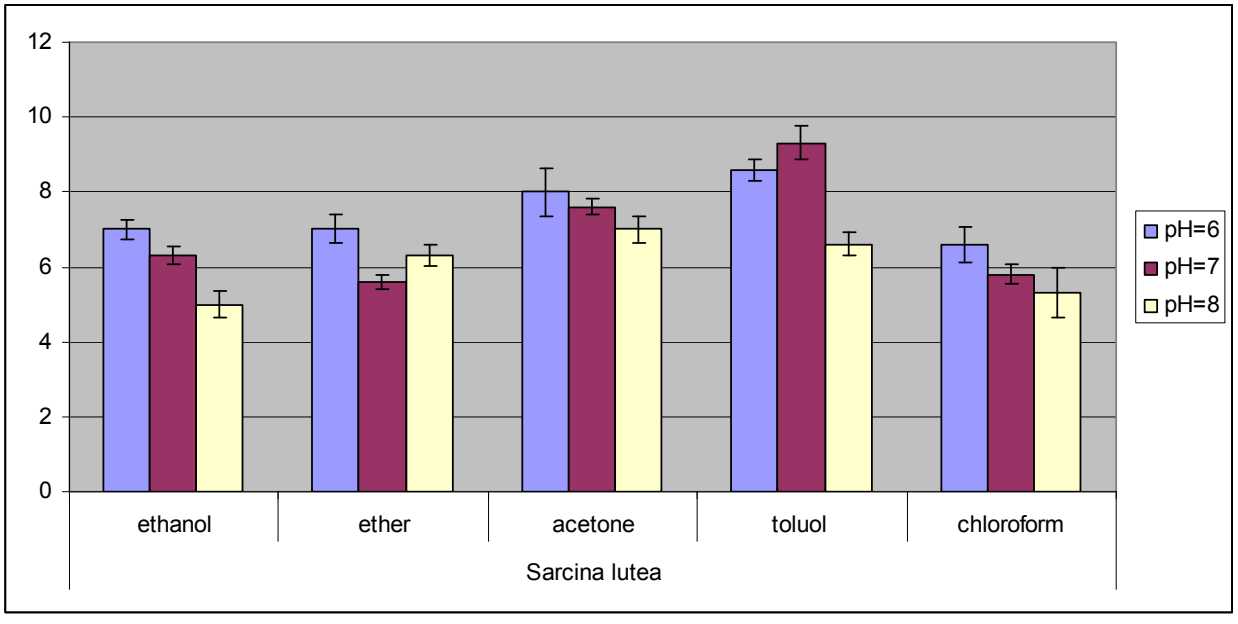

Fig. 4. Activity of the propolis solution aged for 7 days on *Sarcina lutea* in slightly acidic (pH=6), neutral (pH=7) and slightly alkaline (pH=8) cultures.

5. Escherichia coli

Table 5 and Fig. 5 show mean values of the inhibition zones of propolis aged for 7 days on bacterial cultures of *Escherichia coli* in slightly acidic (pH=6), neutral (pH=7) and slightly alkaline (pH=8) cultures.

The results showed that inhibitory activity of all extracts of propolis on the growth of *Escherichia coli* was distinct, and that propolis aged 7 days extracted by toluol and chloroform had a statistically significantly ($p<0.001$) stronger inhibitory effect than other extracts of propolis, in a slightly acidic environment (pH=6).

Results of the inhibitory effect of propolis extracted by various types of solvents on *Escherichia coli* depending on the pH value of cultures showed that there was a statistically significant ($p<0.01$) stronger effect of propolis extracted by ethanol in cultures with pH=6 than in cultures with a pH value of 7 and a statistically significantly ($p<0.001$) greater inhibitory effect than in cultures with a pH value of 8. There was a statistically significant ($p<0.05$) stronger effect of propolis extracted by

Table 5. Activity of the propolis solution aged for 7 days on *Escherichia coli* in slightly acidic (pH=6), neutral (pH=7) and slightly alkaline (pH=8) cultures.

Escherichia coli					
pH=6	pH=6	pH=6	pH=6	pH=6	pH=6
pH=7	pH=7	pH=7	pH=7	pH=7	pH=7
pH=8	pH=8	pH=8	pH=8	pH=8	pH=8
SE pH=6	SE pH=6	SE pH=6	SE pH=6	SE pH=6	SE pH=6
SE pH=7	SE pH=7	SE pH=7	SE pH=7	SE pH=7	SE pH=7

* statistically significant ($p<0.01$)

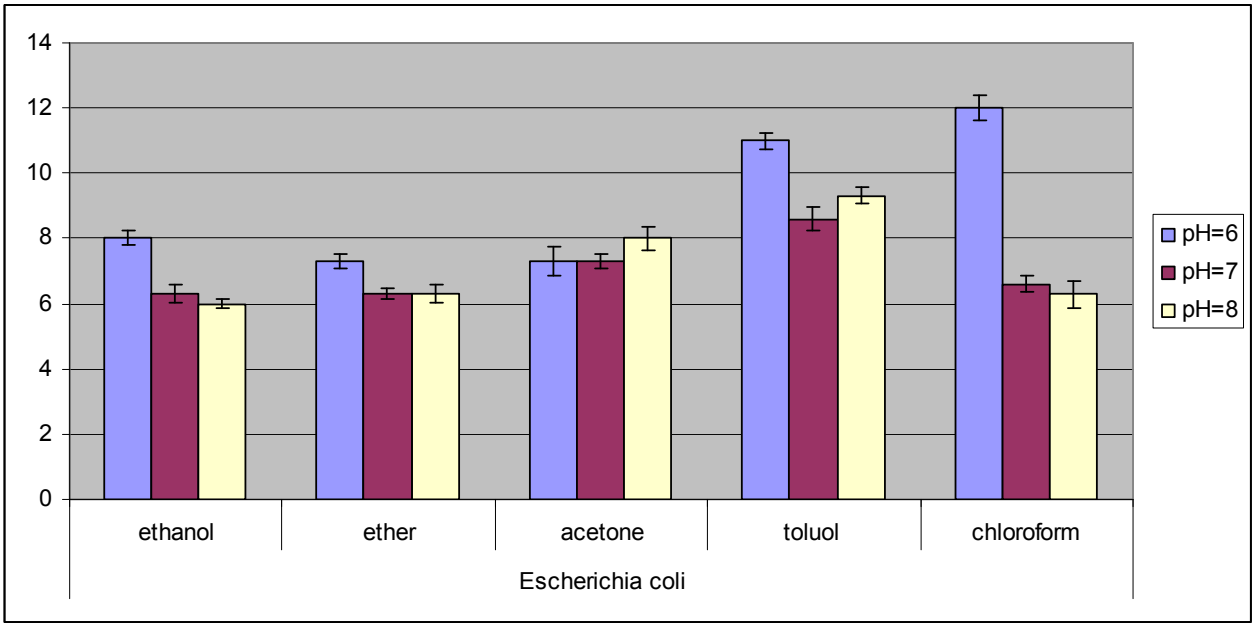

Fig. 5. Activity of the propolis solution aged for 7 days on *Escherichia coli* in slightly acidic (pH=6), neutral (pH=7) and slightly alkaline (pH=8) cultures.

ether in cultures with pH=6 than in cultures with pH values of 7 and 8. Propolis extracted by acetone did not show any significant inhibitory activity on *Escherichia coli* regardless of the pH value of the medium. There was a statistically significant ($p<0.001$) stronger effect of propolis extracted by toluol in cultures with pH=6 than in cultures with pH values of 7 and 8. There was a statistically significant ($p<0.001$) stronger effect of propolis extracted by chloroform in cultures with pH=6 than in cultures with pH values of 7 and 8.

Results of the inhibitory effect of 5 preparations of propolis in cultures with pH values of 6, 7 and 8 on bacteria from group II

1. Aeromonas hydrophila

Table 6 and Fig. 6 show mean values of the inhibition zones of propolis aged for 7 days on bacterial cultures of *Aeromonas hydrophila* in slightly acidic (pH=6), neutral (pH=7) and slightly alkaline (pH=8) cultures.

Table 6. Activity of the propolis solution aged for 7 days on *Aeromonas hydrophila* in slightly acidic (pH=6), neutral (pH=7) and slightly alkaline (pH=8) cultures.

Aeromonas hydrophila					
pH=6	pH=6	pH=6	pH=6	pH=6	pH=6
pH=7	pH=7	pH=7	pH=7	pH=7	pH=7
pH=8	pH=8	pH=8	pH=8	pH=8	pH=8
SE pH=6	SE pH=6	SE pH=6	SE pH=6	SE pH=6	SE pH=6
SE pH=7	SE pH=7	SE pH=7	SE pH=7	SE pH=7	SE pH=7

* statistically significant ($p<0.01$)

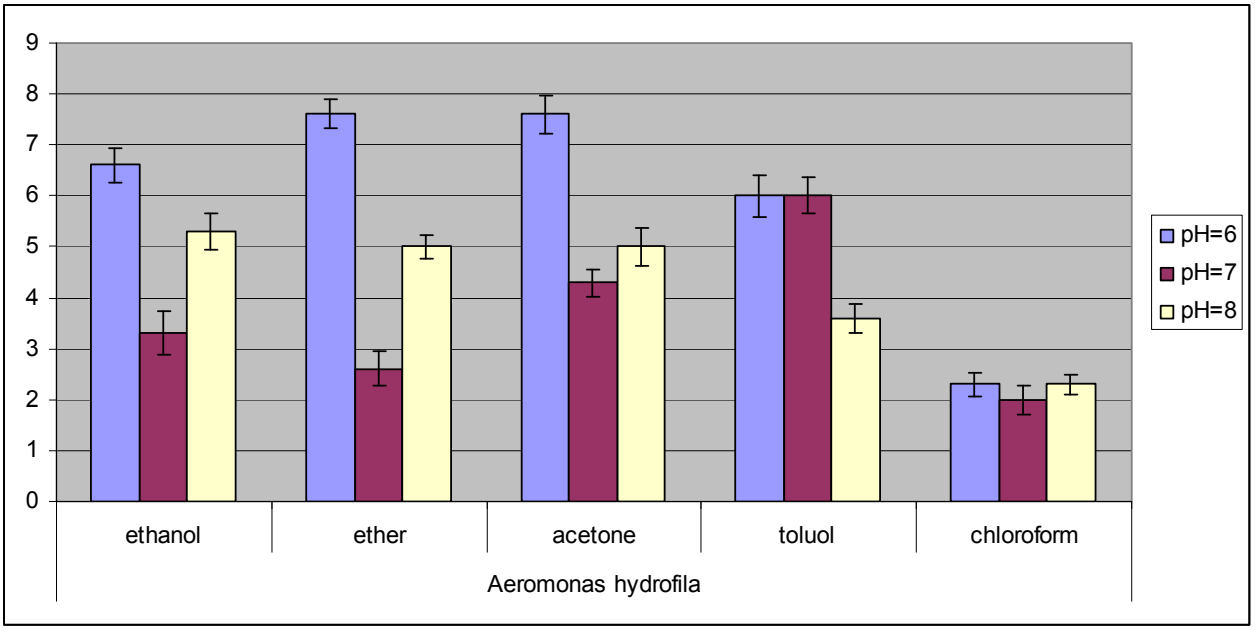

Fig. 6. Activity of the propolis solution aged for 7 days on *Aeromonas hydrophila* in slightly acidic (pH=6), neutral (pH=7) and slightly alkaline (pH=8) cultures.

All propolis preparations had a moderate inhibitory activity on the growth of *Aeromonas hydrophila*. Statistically significantly greater ($p<0.01$) inhibitory activity was exhibited by propolis extracted by ether and by acetone in cultures with a pH value of 6 compared to the other extracts used.

Results of the inhibitory effect of propolis extracted by various types of solvents on *Aeromonas hydrophila* depending on the pH value of cultures showed that there was a statistically significant ($p<0.001$) stronger effect of propolis extracted by ethanol in cultures with pH=6 than in cultures with a pH value of 7. There was a statistically significant ($p<0.001$) stronger effect of propolis extracted by ether in cultures with pH=6 than in cultures with pH values of 7 and 8. There was a statistically significant ($p<0.001$) stronger effect of propolis extracted by acetone in cultures with pH=6 than in cultures with pH values of 7 and 8. There was a statistically significant ($p<0.001$) stronger effect of propolis extracted by toluol in cultures with pH=6 than in cultures with pH values of 7 and 8. Propolis extracted by chloroform did not show any significant inhibitory activity on *Aeromo-*

Table 7. Activity of the propolis solution aged for 7 days on *Salmonella typhimurium* in slightly acidic (pH=6), neutral (pH=7) and slightly alkaline (pH=8) cultures.

Salmonella typhimurium					
pH=6	pH=6	pH=6	pH=6	pH=6	pH=6
pH=7	pH=7	pH=7	pH=7	pH=7	pH=7
pH=8	pH=8	pH=8	pH=8	pH=8	pH=8
SE pH=6	SE pH=6	SE pH=6	SE pH=6	SE pH=6	SE pH=6
SE pH=7	SE pH=7	SE pH=7	SE pH=7	SE pH=7	SE pH=7

* statistically significant ($p<0.01$)

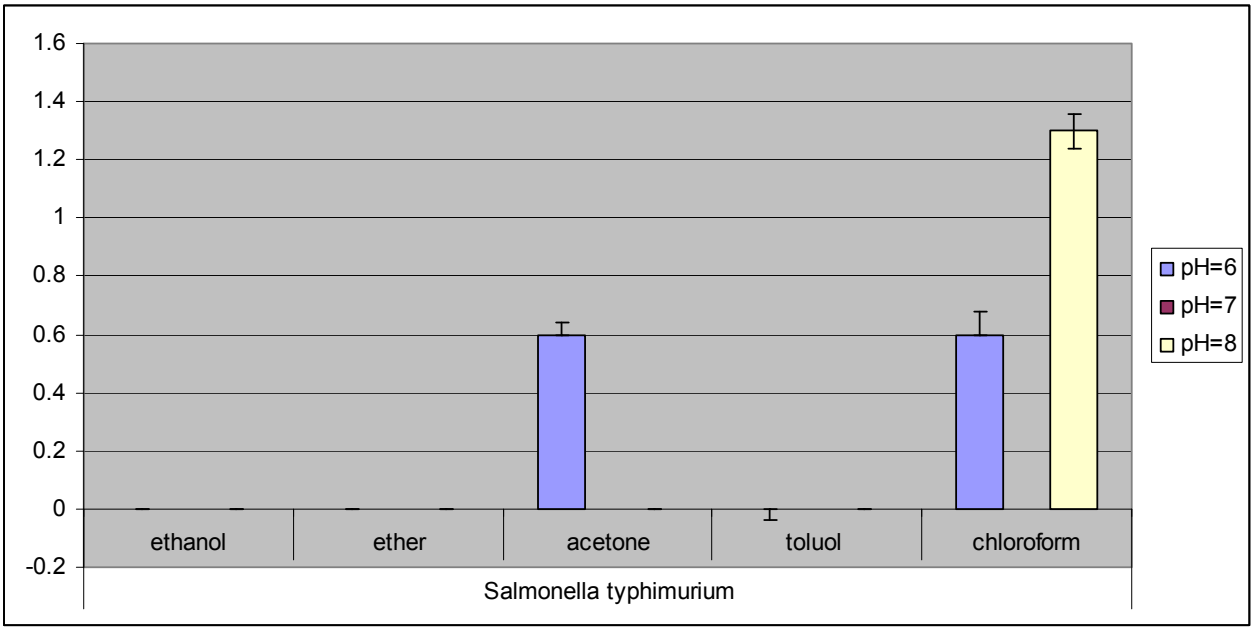

Fig. 7. Activity of the propolis solution aged for 7 days on *Salmonella typhimurium* in slightly acidic (pH=6), neutral (pH=7) and slightly alkaline (pH=8) cultures.

nas hydrophila regardless of the pH value of the medium.

2. Salmonella typhimurium

Table 7 and Fig. 7 show the mean values of the inhibition zones of propolis aged for 7 days on bacterial cultures of *Salmonella typhimurium* in slightly acidic (pH=6), neutral (pH=7) and slightly alkaline (pH=8) cultures.

The results show that the inhibitory effect of propolis extraction on the growth of *Salmonella typhimurium* was minor. Some inhibitory effect was exhibited by propolis extracted by acetone in cultures with a pH value of 6 and propolis extracted by chloroform in cultures with pH values of 6 and 8.

3. Bacillus subtilis

Table 8 and Fig. 8 show mean values of the inhibition zones of propolis aged for 7 days on bacterial cultures of *Bacillus subtilis* in slightly acidic (pH=6), neutral (pH=7) and slightly alkaline (pH=8) cultures.

Table 8. Activity of the propolis solution aged for 7 days on *Bacillus subtilis* in slightly acidic (pH=6), neutral (pH=7) and slightly alkaline (pH=8) cultures.

Bacillus subtilis					
pH=6	pH=6	pH=6	pH=6	pH=6	pH=6
pH=7	pH=7	pH=7	pH=7	pH=7	pH=7
pH=8	pH=8	pH=8	pH=8	pH=8	pH=8
SE pH=6	SE pH=6	SE pH=6	SE pH=6	SE pH=6	SE pH=6
SE pH=7	SE pH=7	SE pH=7	SE pH=7	SE pH=7	SE pH=7

* statistically significant ($p<0.01$)

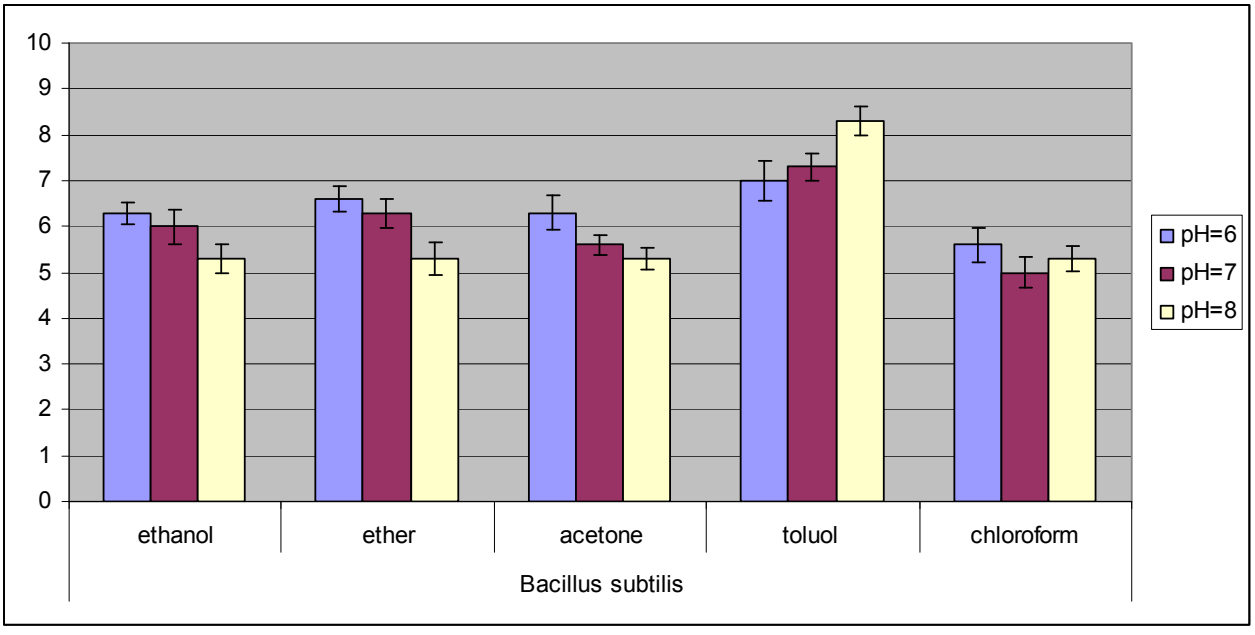

Fig. 8. Activity of the propolis solution aged for 7 days on *Bacillus subtilis* in slightly acidic (pH=6), neutral (pH=7) and slightly alkaline (pH=8) cultures.

Results showed that all types of propolis had a moderate inhibitory activity on the growth of *Bacillus subtilis*. Propolis extracted by toluol had a statistically significantly stronger effect ($p<0.01$) when applied in cultures with pH=6, 7 and 8.

Results of the inhibitory effect of propolis extracted by various types of solvents on *Bacillus subtilis* depending on the pH value of cultures showed that there was a statistically significant ($p<0.05$) stronger effect of propolis extracted by ethanol in cultures with pH=6 than in cultures with a pH value of 8. There was a statistically significant ($p<0.05$) stronger effect of propolis extracted by ether in cultures with pH=6 than in cultures with a pH value of 7 and 8. There was a statistically significant ($p<0.05$) stronger effect of propolis extracted by acetone in cultures with pH=6 than in cultures with a pH value of 8. There was a statistically significant ($p<0.01$) stronger effect of propolis extracted by toluol in cultures with pH=8 than in cultures with a pH value of 6 and 7. Propolis extracted by chloroform did not show any significant inhibitory activity on *Bacillus subtilis* regardless of the pH value of the medium.

Table 9. Activity of the propolis solution aged for 7 days on *Salmonella galinarum* in slightly acidic (pH=6), neutral (pH=7) and slightly alkaline (pH=8) cultures.

Salmonella galinarum					
pH=6	pH=6	pH=6	pH=6	pH=6	pH=6
pH=7	pH=7	pH=7	pH=7	pH=7	pH=7
pH=8	pH=8	pH=8	pH=8	pH=8	pH=8
SE pH=6	SE pH=6	SE pH=6	SE pH=6	SE pH=6	SE pH=6
SE pH=7	SE pH=7	SE pH=7	SE pH=7	SE pH=7	SE pH=7

* statistically significant ($p<0.01$)

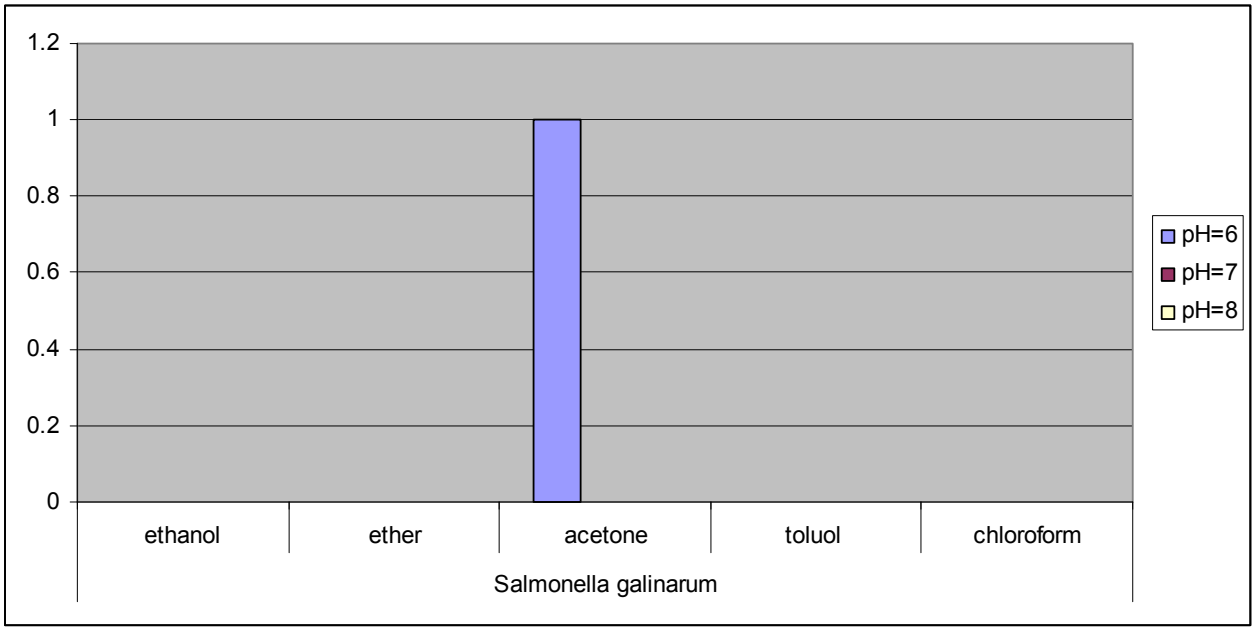

Fig. 9. Activity of the propolis solution aged for 7 days on *Salmonella galinarum* in slightly acidic (pH=6), neutral (pH=7) and slightly alkaline (pH=8) cultures.

Results of the inhibitory effect of 5 preparations of propolis in cultures with a pH value of 6, 7 and 8 on bacteria from group III

1. Salmonella gallinarum

Table 9 and Fig. 9 show mean values of the inhibition zones of propolis aged for 7 days on bacterial cultures of *Salmonella gallinarum* in slightly acidic (pH=6), neutral (pH=7) and slightly alkaline (pH=8) cultures.

These results showed that the inhibitory effect of all propolis preparations on the growth of *Salmonella gallinarum* was minor, in fact it was equal to zero, except for propolis extracted by acetone in a slightly acidic medium (pH=6) where it was 1 mm.

2. Salmonella cholerae

Table 10 and Fig. 10 show mean values of the inhibition zones of propolis for aged 7 days on bacterial cultures of *Salmonella cholerae* in slightly acidic (pH=6), neutral (pH=7) and slightly alkaline (pH=8) cultures.

The results showed that none of the propolis preparations had any effect on the growth of *Salmo-*

Table 10. Activity of the propolis solution aged for 7 days on *Salmonella cholerae* in slightly acidic (pH=6), neutral (pH=7) and slightly alkaline (pH=8) cultures.

Salmonella cholerae					
pH=6	pH=6	pH=6	pH=6	pH=6	pH=6
pH=7	pH=7	pH=7	pH=7	pH=7	pH=7
pH=8	pH=8	pH=8	pH=8	pH=8	pH=8
SE pH=6	SE pH=6	SE pH=6	SE pH=6	SE pH=6	SE pH=6
SE pH=7	SE pH=7	SE pH=7	SE pH=7	SE pH=7	SE pH=7

* statistically significant ($p<0.01$)

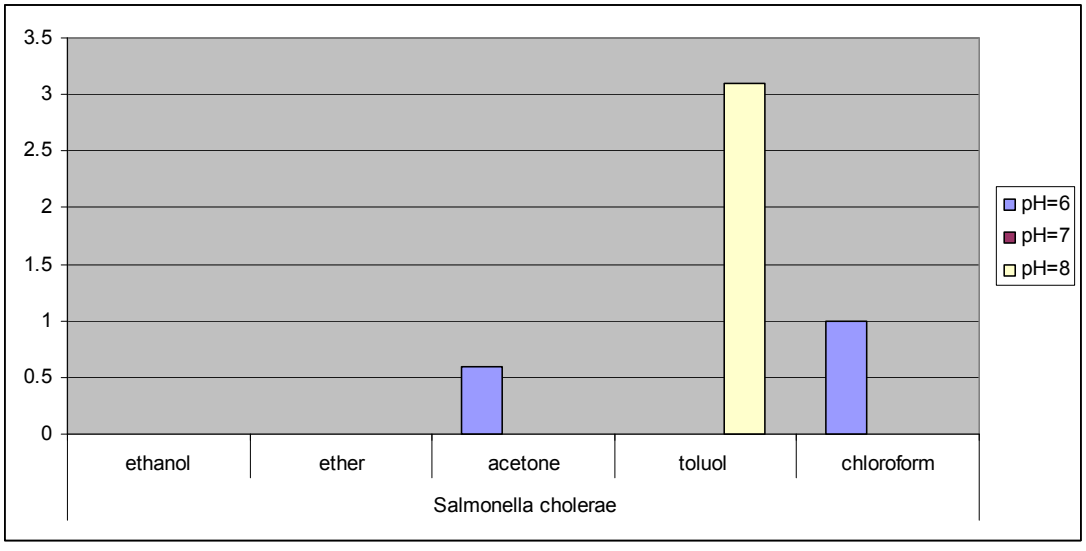

Fig. 10. Activity of the propolis solution aged for 7 days on *Salmonella cholerae* in slightly acidic (pH=6), neutral (pH=7) and slightly alkaline (pH=8) cultures.

nella cholerae. In fact the inhibitory activity may be disregarded, as it was equal to zero, except in the cases of propolis extracted by acetone, in cultures with pH=6, where it was 0.6 mm, propolis extracted by toluol, in cultures with pH=8, where it was 3.1 mm and propolis extracted by chloroform, in cultures with pH=6, where it was 1 mm.

Results of the inhibitory effect of 5 preparations of propolis in cultures with a pH value of 6, 7 and 8 on bacteria from group IV

1. Staphylococcus aureus

Table 11 and Fig. 11 show mean values of the inhibition zones of propolis aged for 7 days on bacterial cultures of *Staphylococcus aureus* in slightly acidic (pH=6), neutral (pH=7) and slightly alkaline (pH=8) cultures.

The results showed that all types of propolis had a uniform, statistically significant ($p<0.01$), inhibitory effect on growth of *Staphylococcus aureus*.

Results of the inhibitory effect of propolis extracted by various types of solvents on *Staphylococcus aureus* depending on the pH value of cultures showed that there was a statistically significant ($p<0.01$) stronger effect of propolis extracted by ethanol in cultures with pH=6 than in cultures with a pH value of 8. There was a statistically significant ($p<0.001$) stronger effect of pro-

Table 11. Activity of the propolis solution aged for 7 days on *Staphylococcus aureus* in slightly acidic (pH=6), neutral (pH=7) and slightly alkaline (pH=8) cultures.

Staphylococcus aureus					
pH=6	pH=6	pH=6	pH=6	pH=6	pH=6
pH=7	pH=7	pH=7	pH=7	pH=7	pH=7
pH=8	pH=8	pH=8	pH=8	pH=8	pH=8
SE pH=6	SE pH=6	SE pH=6	SE pH=6	SE pH=6	SE pH=6
SE pH=7	SE pH=7	SE pH=7	SE pH=7	SE pH=7	SE pH=7

* statistically significant ($p<0.01$)

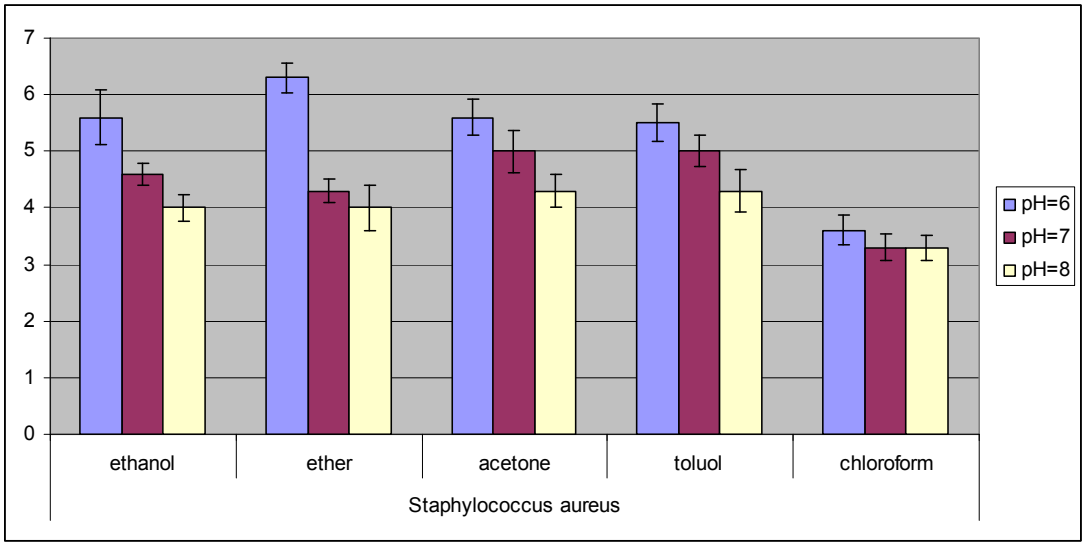

Fig. 11. Activity of the propolis solution aged for 7 days on *Staphylococcus aureus* in slightly acidic (pH=6), neutral (pH=7) and slightly alkaline (pH=8) cultures.

polis extracted by ether in cultures with pH=6 than in cultures with a pH value of 7 and 8. There was a statistically significant ($p<0.01$) stronger effect of propolis extracted by acetone in cultures with pH=6 than in cultures with a pH value of 8. There was a statistically significant ($p<0.01$) stronger effect of propolis extracted by toluol in cultures with pH=6 than in cultures with a pH value of 8. Propolis extracted by chloroform did not show any significant inhibitory activity on *Staphylococcus aureus* regardless of the pH value of the medium.

2. Bacillus cereus

Table 12 and Fig. 12 show mean values of the inhibition zones of propolis aged for 7 days on bacterial cultures of *Bacillus cereus* in slightly acidic (pH=6), neutral (pH=7) and slightly alkaline (pH=8) cultures.

The results showed that all types of propolis had an inhibitory effect on growth of *Bacillus cereus*. Propolis extracted by toluol showed the strongest, statistically significant ($p<0.001$), effect in slightly acidic (pH=6) and neutral (pH=7) environments.

Results of the inhibitory effect of propolis extracted by various types of solvents on *Bacillus cereus* depending on the pH value of cultures showed that there was a statistically significant ($p<0.01$) stronger effect of propolis extracted by ethanol in cultures with pH=6 than in cultures with

Table 12. Activity of the propolis solution aged for 7 days on *Bacillus cereus* in slightly acidic (pH=6), neutral (pH=7) and slightly alkaline (pH=8) cultures.

Bacillus cereus					
pH=6	pH=6	pH=6	pH=6	pH=6	pH=6
pH=7	pH=7	pH=7	pH=7	pH=7	pH=7
pH=8	pH=8	pH=8	pH=8	pH=8	pH=8
SE pH=6	SE pH=6	SE pH=6	SE pH=6	SE pH=6	SE pH=6
SE pH=7	SE pH=7	SE pH=7	SE pH=7	SE pH=7	SE pH=7

* statistically significant ($p<0.01$)

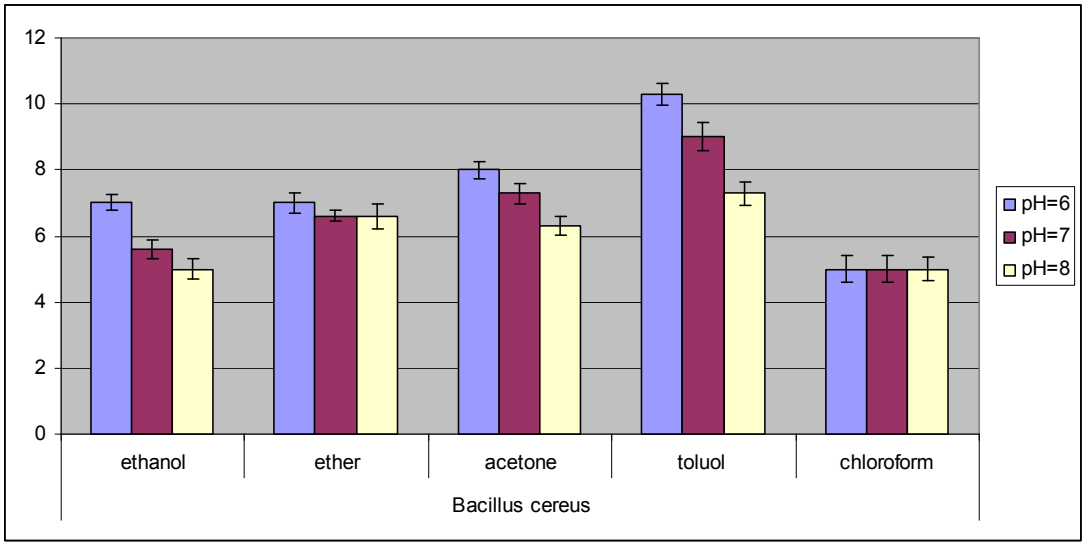

Fig. 12. Activity of the propolis solution aged for 7 days on *Bacillus cereus* in slightly acidic (pH=6), neutral (pH=7) and slightly alkaline (pH=8) cultures.

a pH value of 8. Propolis extracted by ether and chloroform did not show any significant inhibitory activity on *Bacillus cereus* regardless of the pH value of the medium. There was a statistically significant ($p<0.01$) stronger effect of propolis extracted by acetone in cultures with pH=6 than in cultures with a pH value of 8. There was a statistically significant ($p<0.001$) stronger effect of propolis extracted by toluol in cultures with pH=6 than in cultures with a pH value of 7 and 8.

DISCUSSION

This research investigated the effects of propolis extracted by 5 different solvents (ethanol, ether, acetone, toluol and chloroform) and aged for 7 days on twelve species of bacteria classified into four groups according to their pathogenicity in slightly acidic (pH=6), neutral (pH=7) and slightly alkaline (pH=8) environments.

Results of this research showed that propolis had a distinct antibacterial activity and these results were in accordance with the findings of Grange and Davey (1990) which showed that propolis had an anti-bacterial activity, especially on Gram-positive bacteria, while the effect was somewhat less distinct on Gram-negative bacteria.

In our other paper (in preparation) it was shown that propolis had an inhibitory effect on the

growth of bacteria from group I, bacteria banal. This effect was the strongest on *Escherichia coli*, then on *Achromobacter*, and then on *Sarcina lutea* and *Morganella morgani*, while the weakest effect was on *Streptococcus faecalis*.

The inhibitory effect of propolis on the growth of bacterial cultures of *Streptococcus faecalis* should not be disregarded; it was only less effective than on the other species of bacteria from this group. Our findings were in complete agreement with the results of Stepanovic et al. (2003), which showed that propolis had a significant antibacterial activity on Gram-positive bacteria and that *Streptococcus faecalis* was the most resistant Gram-positive bacteria.

The antibacterial activity of propolis was generally investigated on *Escherichia coli*. Our results confirmed a very significant inhibitory activity of propolis on the growth of *Escherichia coli* and were in complete accordance with the results of Sforcin et al. (2000), Drago et al. (2000), Brumfitt et al. (1990) and Simuth et al. (1986). Furthermore, for some bacteria from group I the inhibitory effect of propolis depended not only on the type of the solvent used, but also on the pH of the medium. Propolis extracted by acetone had a statistically significant stronger inhibitory effect on the growth of *Morganella morgani* in cultures with a pH value of 6 compared to the slightly alkaline cultures. Propolis extracted by ethanol, ether, acetone and chloroform had a marked and statistically significant stronger effect on *Streptococcus faecalis* in a slightly acidic environment compared to the neutral and slightly alkaline environments. When extracted by toluol, propolis had a stronger (statistically significant) inhibitory effect on the growth of *Sarcina lutea* in cultures with pH=6 and 7 compared to the cultures with pH=8. Propolis extracted by toluol and chloroform had a significantly stronger effect on *Escherichia coli* in a slightly acidic medium compared to the neutral and slightly alkaline cultures. These findings indicated that these preparations of propolis should be used in cases of inflammatory processes caused by the above-mentioned bacteria from group I.

The analysis of the inhibitory effect of propolis on bacteria from group II, i.e. the group of opportunistic pathogenic bacteria, clearly demonstrated that propolis showed the strongest antibacterial effect on *Bacillus subtilis*, somewhat less on *Aeromonas hydrophila*, and the least on *Salmonella typhimurium*. Very similar results to ours for the antibacterial effect of propolis on *Bacillus subtilis* were published by Brumfitt et al. (1990) and Pepeljnjak et al. (1985). For some bacteria from group II the inhibitory effect of propolis depends not only on the solvent by which it was extracted, but also on the pH of the cultures so that propolis extracted by ethanol, ether, acetone and toluol had a statistically significant stronger inhibitory effect on *Aeromonas hydrophila* in cultures with pH=6 compared to the cultures with pH=8. Propolis extracted by these solvents should be used in inflammatory processes caused by *Aeromonas hydrophila*.

This research showed that the inhibitory effect of propolis on the growth of bacteria from group III, i.e. the group of infectious pathogenic bacteria (*Salmonella gallinarum* and *Salmonella cholera)* was negligible, regardless of the pH of the cultures. These results differed from the findings of Okonenko (1986), who showed that propolis inhibited the activity of free radicals occurring during the oxidation of lipids in salmonelloses. These differences were probably the consequence of the different methods of research that were applied.

Our results very clearly showed that regardless of the solvent or the pH of the cultures, propolis had a very slight, in fact, minor inhibitory effect on the growth of bacteria from the *Salmonella* genus, whether they belonged to the group of opportunistic pathogenic bacteria, e.g. *Salmonella typhimurium,* or to the group of infectious pathogenic bacteria, e.g. *Salmonella gallinarum* and *Salmonella cholerae.*

Results of this investigation have shown that all types of propolis demonstrated a significant antibacterial activity on the growth of bacteria from group IV, i.e. the group of infectious pathogenic

bacteria (*Staphylococcus aureus*, and somewhat less on *Bacillus cereus*). Very similar results for the antibacterial activity of propolis on *Staphylococcus aureus*, were published by Sforcin et al. (2000), Drago et al. (2000), Krol et al. (1993), Brumfitt et al. (1990), Qiao and Chen (1991) and Onlen et al. (2007). Our results showed that for some bacteria from group IV the inhibitory effect of propolis depended both on the solvent used and on the pH of the cultures. Propolis extracted by ether had a statistically significant stronger inhibitory effect on *Staphylococcus aureus* when the pH was 6 compared to its effects in neutral and slightly alkaline environments. Propolis extracted by toluol had a statistically significantly stronger inhibitory effect on the growth of *Bacillus cereus* in cultures with pH=6 compared to the cultures with pH=8.

The results of this extensive research showed that the best inhibitory effects on the growth of all investigated bacteria, except bacteria from the *Salmonella* genus, was exhibited by propolis extracted by ether, acetone, toluol and chloroform. Their inhibitory activity was statistically very significant, since the mean values of the zone of the growth inhibition ranged from 7.6 to 12 mm, which represented almost a complete inhibition of growth of these bacteria.

Our findings show that propolis extracted by toluol and chloroform (where the width of the inhibition zone was greater than 8 mm) had the best inhibitory effect on *Morganella morgani, Achromobacter, Sarcina lutea, Escherichia coli, Bacillus subtilis* and *Bacillus cereus.*

Based on these results it is possible to apply propolis in an adequate solvent in cases of infection by the investigated bacteria with a positive outcome. Thus, the application of propolis would be targeted and subsequently more successful. In cases of hypersensitivity or resistance to certain antibiotics, propolis could be used as replacement therapy.

Solvents used for the extraction of propolis, such as acetone, toluol and chloroform, are toxic substances and may have harmful effects on the human organism even if applied in minimal quantities. Because of this, it is necessary to evaporate the extracted propolis by means of a vacuum evaporator so that this extract is turned into a solid state, i.e. powder, which contains all the active components of propolis extracted by the solvent, while the toxic effects of the solvent itself are eliminated by evaporation. The resulting propolis powder may be used in the pharmaceutical industry for the production of tablets that are administered *per os* or ointments for external application.

Our results have shown that propolis demonstrated its inhibitory effects on the growth of the bacteria in a slightly acidic, neutral and a slightly alkaline environment (pH=6, 7 and 8, respectively) which enables its application in cases of acute and chronic inflammation caused by the tested bacterial species.

Also, in some cases, the inhibitory effect of propolis on the growth of bacteria was the strongest in a slightly acidic environment (pH=6). It is well known that in the center of the inflammatory process there is a slight decrease of pH, which becomes more evident if the inflamed tissue is richer in glycogen, but this decrease rarely falls under the value of pH=6. In cases of an acute inflammatory process caused by bacteria that are sensitive to the inhibitory effects of propolis, it would be most beneficial to use the extract of propolis whose action is most pronounced in a slightly acidic environment.

Based on these results it is possible to go one step further in the application of propolis.

CONCLUSIONS

Propolis demonstrates antibacterial activity on bacteria from group I – bacteria banal (*Morganella morgani, Streptococcus faecalis, Achromobacter, Sarcina lutea* and *Escherichia coli)* when extracted by ethanol, ether, acetone, toluol and chloroform.

Bacteria from group II – opportunistic pathogenic bacteria (*Aeromonas hydrophila* and *Bacillus subtilis*), are significantly sensitive to the activity of propolis extracted in any solvent exami-

ned. Propolis extracted in ether shows most inhibitory activity on *Morganella morgani* and *Aeromonas hydrophila*. Propolis extracted in acetone has the most intensive antibacterial activity on *Streptococcus faecalis, Aeromonas hydrophila* and *Staphylococcus aureus*. Propolis extracted in toluol shows a very significant antibacterial activity on *Morganella morgani, Achromobacter, Sarcina lutea, Escherichia coli, Bacillus subtilis* and *Bacillus cereus*. Propolis extracted in chloroform shows a significant antibacterial activity on *Escherichia coli*. Propolis extracted by acetone has a statistically significantly stronger inhibitory effect on the growth of *Morganella morgani* in cultures with pH=6 than with pH=8. Propolis extracted by ethanol, ether, acetone and chloroform has a significantly stronger effect on the growth of *Streptococcus faecalis* in cultures with pH=6 than with pH=7 and 8. Propolis extracted by toluol has a statistically significantly stronger inhibitory effect on the growth of *Sarcina lutea* in cultures with pH=6 and 7 than with pH=8. Propolis extracted by toluol and chloroform has a statistically significantly stronger effect on the growth of the bacterium *Escherichia coli* in cultures with pH=6 compared to the cultures with pH=7 and 8. Propolis extracted by ethanol, ether, acetone and toluol has a statistically significantly stronger inhibitory effect on the growth of *Aeromonas hydrophila* in cultures with pH=6 than with pH=8.

Bacteria from group III – infectious pathogenic bacteria (*Salmonella gallinarum* and *Salmonella cholerae)* are relatively insensitive to the activity of propolis.

Propolis extracted in any type of solvent demonstrates a significant antibacterial activity on bacteria from group IV – exotoxic pathogenic bacteria (*Staphylococcus aureus* and *Bacillus cereus*). Propolis extracted by ether has a statistically significantly stronger inhibitory effect on the growth of *Staphylococcus aureus* in cultures with pH=6 than with pH=7 and 8. Propolis extracted by toluol has a statistically significantly stronger inhibitory effect on the growth of *Bacillus cereus* in cultures with pH=6 than with pH=8. By taking into account the results of this research the therapeutic application of propolis could be improved making it more effective, whether it is used as the medicament of choice or together with an adequate antibiotic, depending on the seriousness of the disease.

REFERENCES

Brumfitt W., Hamilton-Miller J. M., and *I. Franklin* (1990). Antibiotic activity of natural products: 1. Propolis. *Microbios*. **62 (250)**, 19 – 22.

Drago L. et al. (2000). In vitro antimicrobial activity of propolis dry extract. *J. Chemother*. **12 (5)**, 390 – 395.

Ghisalberti E. L. (1979). Propolis, a review. *Bee World*. **60**, 59-84.

Grange J. M. and *R. W. Davey* (1990). Antibacterial properties of propolis (bee glue). *J. Roy. Soc. Med.* **83**, 159-160.

Hegazi A.G. and *F. Abd El Hady* (2001). Egyptian propolis; 1- Antimicrobial activity and chemical composition of Upper Egypt propolis. *Z. Naturforsch.* **56c**, 82-88.

Hegazi A.G. and *F. Abd El Hady* (2002a). Egyptian propolis; 2 – Chemical composition, antiviral and antimicrobial activity of East Nile Delta propolis. *Z. Naturforsch*. **57c**, 386-391.

Hegazi A.G. and *F. Abd El Hady* (2002b). Egyptian propolis; 3 – Antioxidant, antimicrobial activities and chemical composition of propolis from reclaimed lands. *Z. Naturforsch*. **57c**, 395-402.

Janković A. (1968). *Pčelinji proizvodi kao hrana i lek*. Beograd.

Krol W. et al. (1993). Synergistic effect of ethanolic extract of propolis and antibiotics the growth of *staphylococcus aureus. Arzneimittelforschung*. **43 (5)**, 607 – 609.

Okonenko L. B. (1986). Propolis as an inhibitor of free radical lipid oxidation in salmonellosis. *Vopr. Med. Khim*. **32 (3)**, 45 – 48.

Onlen Y. et al. (2007). Antibacterial activity of propolis against MRSA and synergism with topical mupirocin. *J. Altern. Complement. Med.* **13**, 713 – 718.

Pepeljnjak S., Jelsenjak I., and *D. Maysinger* (1985). Flavonoid content in propolis extracts and growth inhibition of *Bacillus subtilis. Pharmazie*. **40 (2)**, 122-123.

Popova M. et al. (2004). Antibacterial activity of Turkish propolis and its qualitative and quantitative chemical composition. *Phytomedicine*. **61**, 602-604.

Popravko S.A. Sokolov I.V. and *I. V. Torgov* (1983). New natural phenolic triglycerides. *Chem. Natural. Compounds.* **18**, 153-157.

Qiao Z. and *R. Chen* (1991). Isolation and identification of antibiotic constituents of propolis from Henan. *Chung Kuo Chung Yao Tsa Chin*. **16 (8)**, 481 – 482.

Sforcin J.M.et al. (2000). Seasonal effect on Brazilian propolis antibacterial activity. *Apitherapy*. **23 (5)**, 314 - 316.

Simuth J., Trnovsky J., and J. Jelokova (1986). Inhibition of bacterial DNA – dependent RNA polymerases and restriction endonuclease by UV – absorbing components from propolis. *Pharmazie*. **41 (2)**, 131 – 132.

Steel R. G. D. et al. (1960). Principles and procedures of statistics, Mc Grow-Hill, New York.

Stepanović S. et al. (2003). Protective action of propolis on cartilage. *Microb.* **158 (4)**, 353 – 357

Tortora J.G., Funke R.B., and *L. C. Case* (2004). Microbiology, an Introduction, 8th edition. 1-25. Pearson, Benjamin Cummings San Francisco.