

DOI 10.7251/VETJEN2001173K

UDK 615.281:[615.324:638.16/.17

Original Scientific Paper

ANTIBACTERIAL ACTION OF PROPOLIS ON SELECTED BACTERIAL REFERENCE STRAINS

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Abstract: Propolis alcoholic tincture is most commonly used propolis product on the market for the treatment of minor infections in the oral cavity; angina, some skin diseases etc. As propolis is still an unofficial drug in pharmacies, we tested its antimicrobial activity using a disk-diffusion test on six reference bacterial species-Salmonella Enterica WDCM 00030, Salmonella typhimurium WDCM 00031, Listeria monocytogenes WDCM 00020, Staphylococcus aureus WDCM 00032, Escherichia coli WDCM 00013 and Pseudomonas aeruginosa WDCM 00024.

The aim of this paper was to examine the antibacterial action of alcoholic solution of propolis on reference bacterial cultures and to determine the type of action. Based on the obtained results, it can be concluded that the bacterial strains of *Salmonella Enteritidis* WDCM 00030, *Salmonella Typhimurium* WDCM 00031, *Listeria monocytogenes* WDCM 00020, *Staphylococcus aureus* WDCM 00032 and *Pseudomonas aeruginosa* WDCM 00024 are highly sensitive to the action of propolis alcoholic tincture.

Key words: propolis, bacterial reference strains, antimicrobial activity

INTRODUCTION

Propolis is a bee product of resinous consistency, yellow to dark brown in color, with a faint odor, which honey bees collect from the buds and bark of trees. The word propolis is of Greek origin, derived from two words “*pro*” - in front and “*polis*” – city, which means “in front of the city” and perfectly describes the role of propolis in hives, which is closing hive openings and having protective role of bee colony (Ghisaberti, 1979; Marcucci, 1995; Torres et al., 2008; Boonsai et al., 2014). Propolis is known since ancient times and was used as an adhesive in Egypt. The Greek philosopher Aristotle wrote about the resinous substrate that bees smeared at the entrance to their hives, and was used as a remedy for bruises and ulcers (Crane, 1999). Greeks used propolis as the main ingredient in making perfumes, while the ancient Jews considered it a natural remedy. The Roman scientist

Plinius (23-79 BC) was convinced that propolis originated from various buds of various trees such as willow, poplar, elm, reed and other plants (Fearnley, 2001). Hippocrates (460-377 BC) was the first to use propolis for treating ulcers, which is considered to be the first use of propolis as a drug to be recorded (Najafi, 2007).

Propolis is considered a universal drug with many clinically proven therapeutic properties. The anti-inflammatory action of propolis enables epithelisation of wounds and has a positive effect on the body's immunity protection. Propolis is irreplaceable in acute and chronic inflammations such as: rhinitis, sinusitis, bronchitis, laryngitis etc. Experimental studies have shown anti-inflammatory effects in rats with arthritis, since alcohol tincture of propolis had anti-inflammatory effects in both acute and chronic inflammation (Park and Kahng, 1999; Boonsai et al., 2014). The chemical composition of propolis has not been fully investigated, but it is known that the main components of propolis are natural flavonoids (galangin, quercetin, chrysin, kaempferol, apigenin, pinocembrin, pinobaksin), that are responsible for medicinal properties of propolis. There is plenty of evidence that propolis has antibacterial activity, i.e., that it slows down the growth of bacteria (Kalogeropoulos et al., 2009; Petrova et al., 2010; Boonsai et al., 2014).

The antibacterial activity of propolis has been investigated on a wide range of aerobic and anaerobic Gram-positive and Gram-negative bacteria. Propolis has been shown to be more active against Gram-positive bacteria, but it also shows inhibitory properties against some Gram-negative bacteria (*Klebsiella pneumoniae*) (Meresta, 1997; Park et al., 2005; Boonsai et al., 2014; Bogdanov, 2017). Mostly, the antimicrobial activity of crude propolis decreases with its prolonged storing and longer storage. In contrast, it has been confirmed that ethanol extract of propolis, stored for 10 to 15 years, shows increased antibacterial activity (Meresta, 1997). Stepanović et al. (2003) examined the antimicrobial activity of propolis extracts obtained from the territory of the Republic of Serbia against selected microorganisms, as well as synergistic effect of propolis and selected antibiotics. The obtained results of this study showed a strong antimicrobial effect of ethanol extract of propolis against Gram-positive bacteria, individually and in combination with commercial antibiotics.

Propolis has a strong bactericidal effect, destroys certain bacteria and is the strongest natural disinfectant. Due to the content of many pharmacologically active substances, propolis has a local anesthetic, hepatoprotective, anti-cancer and immunostimulating effect (Burdock, 1998; Ito J et al., 2001; Toreti, 2013).

Propolis acts as bacteriostatic on bacteria by damaging their cytoplasm, cell membrane, causing partial bacteriolysis and inhibiting protein synthesis. Antibacterial substances in propolis are thermostable, do not damage the normal intestinal flora, are non-toxic and do not cause resistance in body. If propolis is used in combination with standard antimicrobial drugs (streptomycin, ampicilin, gentamicin, tetracycline, cephalosporin), it can intensify their effect up to 100 times. Propolis moderately increases the antibacterial activity of chloramphenicol, ceftriaxone and vankomycin, while it has no effect on erythromycin.

The fungicidal action of propolis has been proven against factors that cause skin diseases of the feet such as psoriasis, alopecia, neurodermatitis, etc., which means that propolis can help on treating process of these diseases (Ota et al., 2001; Gekker et al., 2005; Dota et al., 2011).

Some studies have shown that consumption of propolis not only prevents cancer formation, but also stops cancer cell growth and metastasis (breast, colon, kidney, liver, uterus, stomach, lung, skin, blood cancers) (Orsolić et al., 2004; Kalogeropoulos et al., 2009; Valente et al., 2011).

Due to its biological characteristics, propolis is considered a highly functional ingredient that is important for health, and therefore is added to food and cosmetics, and is also used for medical purposes with the aim of health improvement and various diseases prevention (IFIC, 2009).

The antiviral effect of propolis is direct on some viruses (*Herpes simplex*, *Influenza*, *Herpes genitalis*, *Herpes zoster* etc.), since it prevent their replication, i.e., reproduction within the cell and reduces DNA synthesis, leading to reduction in viral activity. In some oncogenic viruses, it prevents the transformation of healthy into cancer cells by breaking down their DNA and at the same time triggering the mechanism of apoptosis. Test results (*in vitro*) of infections caused by HIV 1 virus with 66.6 µg/ml ethanolic tincture of propolis showed suppression of several types of HIV1 virus infected with CD4 lymphocytes and microglia cells (suppression of 85-98%) (Gekker et al., 2005; Diaz-Carballo et al., 2010).

Despite the differences on chemical composition between different types of propolis due to different botanical and geographical origins, propolis exhibits a strong antimicrobial effect. Tests of chemical and microbiological properties of propolis are important in terms of the use of propolis as a natural preservative in the food industry.

The aim of this study was to determine the antimicrobial activity of alcoholic tincture of propolis on selected bacterial reference strains.

MATERIALS AND METHODS

The research was done with 20% alcoholic tincture of propolis, which was previously made with 200 g of propolis dissolved in 1000 ml of 96% ethyl alcohol. After propolis was poured with ethyl alcohol, propolis was kept in dark bottle, with occasional shaking, for at least three weeks. After that, the propolis was filtered through filter paper to obtain alcoholic tincture of propolis which was ready for further use.

Reference strains *Salmonella* Enteritidis WDCM 00030, *Salmonella* Typhimurium WDCM 00031, *Listeria monocytogenes* WDCM 00020, *Staphylococcus aureus* WDCM 00032, *Escherichia coli* WDCM 00013, *Pseudomonas aeruginosa* WDCM 00024 (BCCM™/LMG BACTERIA COLLECTION, Belgium) were used to test the antimicrobial activity of alcoholic tincture of propolis. Cultures were seeded in nutrient broth and incubated at 37°C for 18h. Petri dishes with a suitable medium (Müller-Hinton

agar) were seeded with 0.1 ml of bacterial suspension, the concentration of which was 10^7 cfu/ml.

To investigate the effect of alcoholic tincture of propolis on the growth of bacterial reference strains *Salmonella Enteritidis* WDCM 00030, *Salmonella Typhimurium* WDCM 00031, *Listeria monocytogenes* WDCM 00020, *Staphylococcus aureus* WDCM 00032, *Escherichia coli* WDCM 00013 and *Pseudomonas aeruginosa* WDCM 00024, the agar diffusion method on a solid sterile nutrient medium (Müller-Hinton agar) was used. Metal cylinders with a diameter of 9 mm were placed on the surface of a solid nutrient medium on which a certain pure bacterial culture was previously sown. 10 µl of alcoholic tincture of propolis was instilled into cylinders with a micropipette. As a control, 10 µl of 96% alcohol was added to the cylinder.

The principle of this method is based on the fact that the antimicrobial agent diffuses into the substrate and spreads radially. If the bacterium is sensitive to the action of the tested antimicrobial agent, it will not grow in the zone of its action. Therefore, after incubation, around the cylinder, zones of absence of growth are observed, the so-called zones of inhibition. Zones of growth inhibition were measured with a millimetre ruler, on the basis of which the sensitivity of the bacterial strain to the tested alcoholic tincture of propolis was determined.

Petri dishes were incubated for 24h at 37°C. Three replicates were performed for each bacterial culture and the mean value for each bacterial culture was calculated.

In addition to above, the type of action of the alcoholic tincture of propolis was also determined. To see if propolis had bactericidal or bacteriostatic activity, a small piece of agar was taken from the inhibition zones and added to the nutrient broth. Incubation was performed at 37°C for 24h. If the broth became cloudy after incubation, it is considered that the propolis had a bacteriostatic effect, i.e. if the broth remained clear after incubation, the effect of propolis is bactericidal.

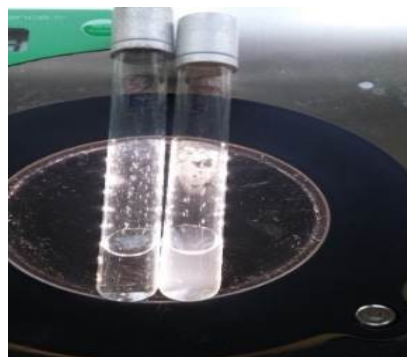


Figure 1. Disk-diffusion method; **Figure 2.** Bactericidal and bacteriostatic effects of propolis

RESULTS AND DISCUSSION

The antibacterial effect of the alcoholic extract of propolis was investigated on the reference strains *Salmonella Enteritidis* WDCM 00030, *Salmonella Typhimurium* WDCM 00031, *Listeria monocytogenes* WDCM 00020, *Staphylococcus aureus* WDCM 00032, *Escherichia coli* WDCM 00013 and *Pseudomonas aeruginosa* WDCM 00024. The obtained results are shown in figure 3.

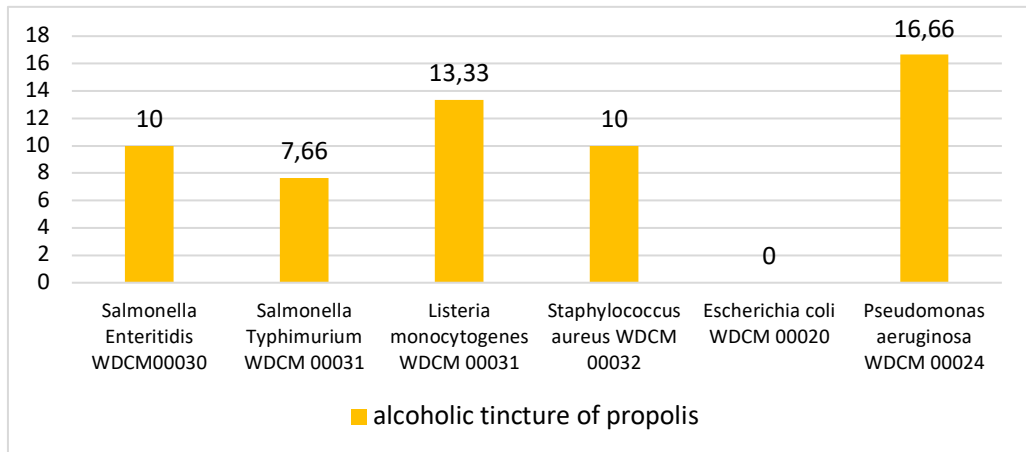


Figure 3. Antibacterial action of alcoholic tincture of propolis on selected reference strains (zones of inhibition are given in mm)

Based on the results shown in figure 3., it can be seen that bacteria *Pseudomonas aeruginosa* WDCM 00024, *Listeria monocytogenes* WDCM 00020, *Staphylococcus aureus* WDCM 00032 and *Salmonella Enteritidis* WDCM 00030 showed the greatest sensitivity to the antimicrobial effect of alcoholic tincture of propolis. The greatest antimicrobial effect of the tested sample was observed in the strains *Pseudomonas aeruginosa* WDCM 00024, with an inhibition zone of 16.66 mm and *Listeria monocytogenes* WDCM 00020, with an inhibition zone of 13.33 mm. They are followed by *Staphylococcus aureus* WDCM 00032 (11.33mm), *Salmonella Enteritidis* WDCM 00030 (10.33mm) and *Salmonella Typhimurium* WDCM 00031 (7.66 mm). Alcoholic tincture of propolis did not show an inhibitory effect on growth of *Escherichia coli* WDCM 00013.

There are a large number of publications related to antimicrobial activity of propolis on *E. coli*. Studies performed on alcoholic tinctures of propolis from different regions of Turkey showed that the zone of inhibition of *Escherichia coli* was >12 mm, while *Pseudomonas aeruginosa* and *Proteus mirabilis* showed much lower sensitivity (Katircioglu et al., 2006). Popova et al. (2005) believe that phenolic glycerides and diterpens could be responsible for

activity against *Escherichia coli*. Stepanović et al. (2003) examined the antimicrobial activities of thirteen tinctures of propolis and showed the largest zone of inhibition for *Staphylococcus aureus* (9-12 mm), *Listeria monocytogenes* (10-13 mm) and *Bacillus subtilis* (9-13 mm), while for strains of *Escherichia coli*, *Pseudomonas aeruginosa* and *Serratia marcescens* obtained zones of inhibition were from 0 to 2 mm.

Variations in the antibacterial activity of alcoholic tincture of propolis are in line with the results published in the literature and probably depend on geographical area from which the bees collected honey (Stepanović et al., 2003; Fernandes Junior et al., 2006; Adewumi et al., 2011; Boonsai et al., 2014; Wolska et al., 2016). Many studies have shown that flavonoid and polyphenyl content affect the antimicrobial activity of propolis (Pepeljnjak et al., 2004; Mercen et al., 2006; Boonsai et al., 2014). Also, the activity of propolis is influenced by the method of storage, production time, proportion of ingredients and method of application (Haynes and Callaghan, 2011; Tasleem et al., 2011; Boonsai et al., 2014; Wolska et al., 2016).

The results of this study are in line with the results of other researchers who have dealt with this topic (Gatto et al., 2002; Stepanović et al., 2003; Kosalec et al., 2005; Park et al., 2005; Rushdi et al., 2013; Wojtyczka et al., 2013; Boonsai et al., 2014; Kalaba et al., 2019).

Differences in susceptibility to antimicrobial agents observed between Gram-positive and Gram-negative bacteria most likely originate from differences in the structure and chemical composition of the bacterial wall. The cell of Gram-negative bacteria is multi-layered and the presence of a two-layer outer membrane represents a natural barrier for the penetration of antibiotics and other foreign substances into the cell interior. On the other hand, Gram-positive bacteria have a single-walled cell wall and are more sensitive to the action of antimicrobial agent (Gatto et al. 2002; Pepeljnjak et al., 2004). According to other authors, the outer membrane can only slow down the entry of lipophilic substances into the bacterial cell. Pumps in the cell membrane that prevent the entry of propolis constituents or actively expel them from the cell are responsible for resistance to propolis (Gatto et al. 2002). The studies of some researchers have shown that propolis prevents the growth of microorganisms, whether they are pure or mixed cultures. Also, the use of an alcoholic solution of propolis, alone or in combination with other antibacterial agents, can be used in the development of alternative products for the treatment of methicillin-resistant staphylococci (MRSA). Studies report synergistic activity between alcoholic solution of propolis and antibacterial drugs against methicillin-resistant staphylococci (MRSA) (Noori et al., 2012; Wojtyczka, 2013; Ali Saddiq and Abouward, 2016).

To see if propolis had a bactericidal or bacteriostatic effect, a small piece of agar was taken from the inhibition zones and added to the nutrient broth. After incubation for 24 h at 37⁰C, the broth became cloudy, i.e. the alcoholic solution of propolis had a bacteriostatic effect on all tested strains. The obtained results are shown in figure 4.

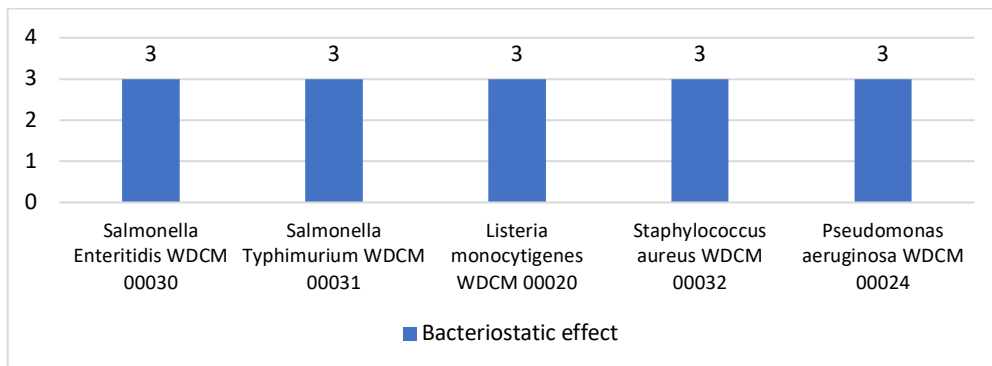


Figure 4. Graphical representation of the effect of alcoholic propolis solution on selected reference strains.

The results of this study confirm the bacteriostatic effect of alcoholic tincture of propolis, which is in line with studies conducted by other researchers (Bonvehi et al., 1994; Salas et al., 2014).

CONCLUSION

Based on obtained results, it can be concluded that the bacterial reference strains of *Pseudomonas aeruginosa* WDCM 00024, *Listeria monocytogenes* WDCM 00020, *Staphylococcus aureus* WDCM 00032, *Salmonella* Enteritidis WDCM 00030 and *Salmonella* Typhimurium WDCM 00031 are sensitive to the action of alcoholic tincture of propolis.

Alcoholic tincture of propolis did not show an inhibitory effect on the growth of *Escherichia coli* WDCM 00013.

Due to occurrence of an increasing number of resistant microorganisms to certain conventional microbial drugs, this study is an introduction to future tests and an incentive to return the use of herbal preparations in the treatment of various diseases in humans and animals.

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Paper received: 30.12.2019.

Paper accepted: 16.04.2020.
