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Full Length Research Paper

Antibacterial activity of propolis and honey against *Staphylococcus aureus* and *Escherichia coli*

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The disc diffusion method, minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and gradient-plate techniques were implemented to evaluate the antibacterial activity of honey and propolis against *Staphylococcus aureus* and *Escherichia coli*. The growth of *S. aureus* was inhibited by application of propolis and honey at concentrations of 2.74 to 5.48 mg ml^{-1} and 375. 0 at mg ml^{-1}, respectively at both MIC and MBC. The greater inhibition zones (13.0 ± 0.09 to 15.0 ± 0.11 mm) were observed from propolis at concentrations of 2.74 to 5.48 mg ml^{-1} in the disc diffusion method which was closely correlated with the MIC, MBC and gradient-plate technique results. The combined results obtained from the disc diffusion test, MIC, MBC and gradient-plate techniques suggested that propolis at concentrations of 2.74 to 3.5 and 3.5 mg ml^{-1} was effective to inhibit *S. aureus* and *E. coli*, respectively. On the contrary, honey was effective to inhibit *S. aureus* at the concentration of 375.0 mg ml^{-1} but failed to inhibit *E. coli* growth at same concentration. The combined results from all methods indicated that both propolis and honey had antibacterial activity against *S. aureus*. Present findings also suggested that *S. aureus* is more susceptible to the effect of the propolis than its Gram-negative counterpart *E. coli*.

Key words: Antibacterial activity, honey, propolis, MIC, MBC, disc diffusion, *Escherichia coli, Staphylococcus aureus.*

INTRODUCTION

Honey and propolis both are very popular because of their beneficial effect on human health (Dobrowolski et al., 1991) and have been used as folk medicine since long (Ghisalberti, 1979; Bankova, 2005; Molan, 1992). Both substances are bee product (Zumla and Lulat, 1989) and contains phenolic substances includes cinnamic acid derivatives, some flavonoids (Martos et al., 1997; Marcucci et al., 2001) which have been verified as antibacterial applicant (Miorin et al., 2003). Honey contains antioxidants and flavonoids that may function as antibacterial agents. Propolis, a flavonoid-rich product of honey comb, exhibits antibacterial and anti-inflammatory properties (Bosio et al., 2000) which is very powerful natural antibiotic (Miorin et al., 2003) and very useful in fighting upper respiratory infections, such as common cold and influenza viruses (Focht et al., 1993). Propolis contains a variety of potent polyphenols which may enhance the antistaph activity of some pharmaceutical antibiotics including streptomycin (Qiao and Chen, 1991). Honey inhibits the growth of dangerous bacteria such as *Escherichia coli, Staphylococcus aureus, Salmonella, Shigella*, and *Vibrio cholera* (Zumla and Lulat, 1989) and is superior to several well-known antibiotics. Honey inhibits the growth of pathogenic organisms isolated in urine samples of patients with urinary tract infections (Somal et al., 1994).

E. coli are a model organism for bacteria (Peter et al., 1998) and extremely sensitive to antibiotics such as streptomycin or gentamycin but rapidly changing and acquiring drug resistance (Chapman et al., 2002) due to overuse of antibiotics in humans (Johnson et al., 2006).

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Management of *E. coli* infections has been increasingly complicated by the emergence of resistance to most first-line antimicrobial agents including fluoroquinolone (Karlowsky et al., 2001). Thus, they have been relied on for the treatment of *E. coli* infections as emerging resistance has progressively eclipsed the utility of alternative antimicrobial agents (Gupta et al., 2001) . However, the prevalence of fluoroquinolone-resistant *E. coli* has reached alarming levels in many parts of the world, jeopardizing their usefulness (Raz et al., 2002). The use of fluoroquinolones in food animals has been implicated in the development of fluoroquinolone resistance in zoonotic gram- negative bacilli such as *Campylobacter* and *Salmonella* species, with the subsequent occurrence of drug- resistant infections in humans (Smith et al., 1999; Chiu et al., 2002). *E. coli* that are resistant to quinolones and fluoroquinolones contaminate many retail meat products, particularly poultry, corresponding with the use of fluoroquinolones in food animals, particularly chickens and turkeys (Johnson et al., 2003; Johnson et al., 2005). However, whether such drug-resistant organisms pose a threat to human health is unknown.

In particular, the similarity between fluoroquinolone-resistant *E. coli* from retail chicken products and isolates from humans, whether infected or merely colonized, is undefined. Food-borne diseases are of major concern worldwide and *S. aureus* is a leading cause of gastro-enteritis resulting from the consumption of contaminated food (Yves et al., 2003). Studies show that staphylococcal infections in colonized persons are often due to the staphylococcal strain responsible for colonization (Calia et al., 1969; Yu et al., 1986) thus, the infections have an endogenous origin. Antibacterial and antifungal activities of propolis, comparing the susceptibility of microorganisms to different concentrations were demonstrated (Fernandes et al., 1995). Agar diffusion method was also studied for antibacterial activity of beewax and propolis suggested that propolis had inhibitory activities against bacterial pathogens (Azevedo et al., 1963). Fungistatic and fungicidal effects of propolis, especially the susceptibility of yeast to propolis were also investigated (Lilenbaum and Barbosa, 1994).

The chemical composition and antibacterial activity of propolis from bees have been reported by Velikova et al. (2000a, b) and Marcucci et al. (2001). However there are few studies on the honey and proplois under sporadic methods against *S. aureus* and *E. coli.* Natural antibiotics such as propolis and honey may be effective to prevent *S. aureus* and *E. coli* contamination. Therefore, the goal of this study was to investigate the effect of propolis and honey with different concentrations on the antibacterial activity, since there are limited data in the literature concerning combined approach of disc diffusion test, MIC, MBC and gradient plate technique. Thus gram-positive and gram-negative bacteria behavior was analyzed in increasing concentrations of propolis and honey in order to determine the minimal inhibitory concentration for microbial growth.

MATERIALS AND METHODS

A series of experiments were carried out in the laboratory of Biotechnology and Engineering Technology, Centennial College, HP campus, Toronto, Canada. Different methods were used to evaluate the effectiveness of honey and propolis against *S. aureus* and *E. coli*. Honey and propolis was purchased from Natural Medicine Shop, Danforth, Toronto, Canada. Honey was provided by Burkes Honey LTD, OMEMEE, ON, Canada. This liquid honey has been ranked No. 1 in Canada since 1909. The brand name of the propolis is Organika - Canadian Bee Propolis. It is alcohol free and 100% natural and high potency. Tryptic Soy Agar (TSA) was used for the isolation and culture of *S. aureus* and *E. coli*.

Mueller-Hilton broth (MHB brand) was a test medium for antimicrobial suscep-tibility testing. Tyrptic Soy Broth (TSB) was prepared at the rate of 40 gl^{-1} of water, dissolved and boiled until clear solution appeared, then bottled and autoclaved. TSA was poured into sterile petri dish about 15 - 20 ml aseptically then allowed about 10 to15 min to settle. For execution of the studies, *S. aureus* and *E. coli* subculture was done in 5 ml TSB and TSA plate and incubated at 35°C for 24 h. The subculture was done prior to each trial. Gram-staining and biochemical test was done to confirm the supplied microorganisms of *S. aureus* and *E. coli*. Honey and propolis was used at rates of 750.0 and 10.96 mg ml^{-1} and both honey and propolis samples against *S. aureus* and *E. coli* as determined by the method of NCCLS (2000).

Disc diffusion method

Disc diffusion test was used as an alternative measure of susceptibility and a counterpart method of MIC and MBC (NCCLS, 1996). Aseptically TSA plates were swabbed by *S. aureus* and *E. coli*. TSA plate was prepared by dipping sterile swabs into inoculums for antibiotic disc diffusion. Sterile paper discs (3.5 mm) were dipped in different dilution of honey and propolis and placed on swab plates for *S. aureus* and *E. coli* in specific dilutions and placed in agar plate. In each dilution of propolis and honey was replicated thrice under factorial completely randomized design. All agar plates were incubated at 35°C for 48 h. Observed agar plates and measured inhibition's zone from each paper disc in mm. If the test organism grows on the disc it may safely be assumed that the test organism is resistant to that honey or propolis.

Minimum inhibitory concentration and minimum bactericidal concentration

Minimum inhibitory concentration is employed to determine effective concentration of honey and propolis against *S. aureus* and *E. coli*. MHB was employed for the determination of MIC in serial dilution tests tube preparation (NCCLS, 2000). Double strength MHB was prepared at the rate of 42 g l^{-1} (NCCLS, 2000). MHB tubes prepared with diluted antibiotic and inoculated with organism at the rate of 0.1 ml per tube. Then all MHB tubes were placed in an incubator at 35°C for 24 h (120 rpm). Data was recorded to compare with the media control MHB tubes. No turbidity clear tube is negative (-) while turbid cloudy solution indicated growth (+). Where no growth was visible in MIC, the same concentration of MHB tubes included honey and propolis tubes was tested at TSA plates for further verification under MBC. A detailed dilution schedule of MIC and

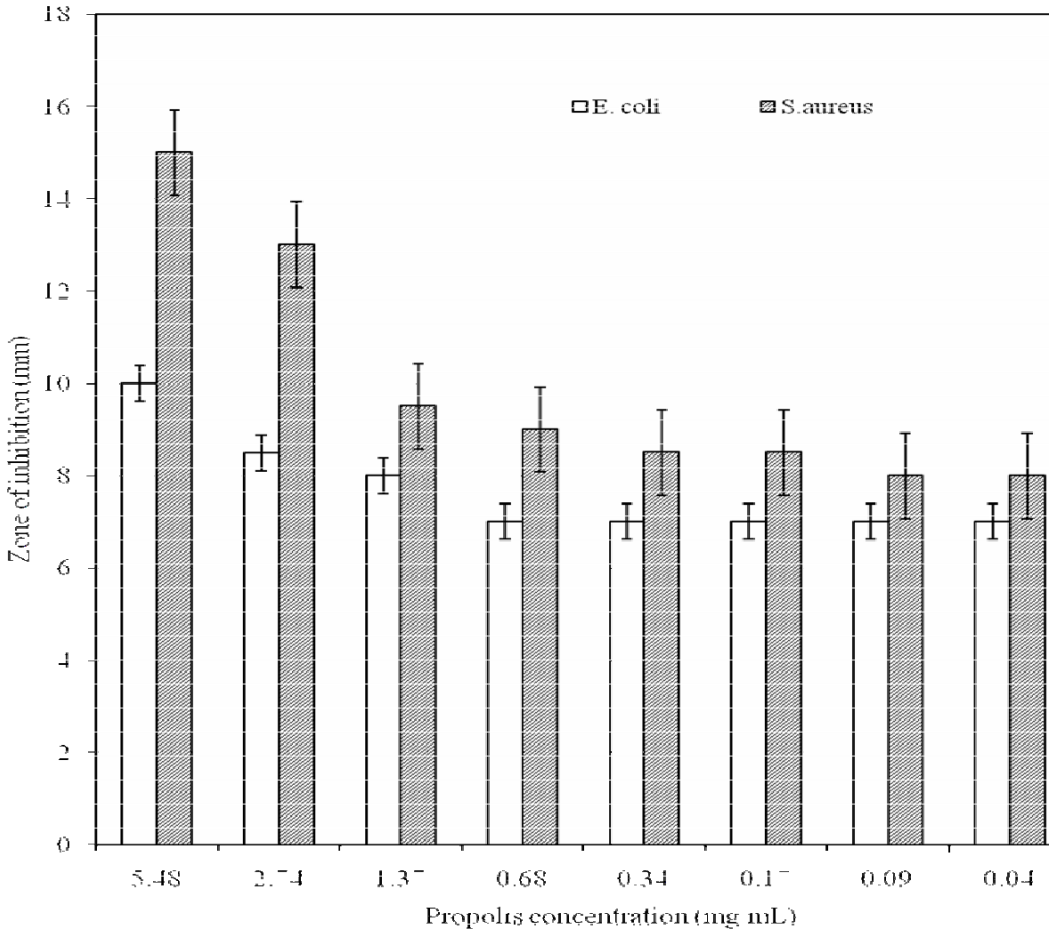

Figure 1. Zone of inhibition by application of propolis against *E.coli* and *S. aureus* by disc diffusion method. Error bar denotes LSD at 0.05.

growth visibility and non-growth tubes were recorded then further proceed with MBC test (NCCLS, 2000).

Gradient plate technique

Gradient plate technique was used to validate the MIC and MBC test result against *S. aureus* and *E. coli*. In this technique propolis and honey were used in a TSA plate. The concentration of propolis and honey was varied infinitely between zero and a given maximum. To perform this test required quantity of propolis and honey was mixed with half of total quantity of TSA media then poured into a sterile Petri dish and allowed set in the form of a wedge. Remain half of the TSA was then poured onto the wedge and allowed to set with the Petri dish flat on the table. The plates were incubated overnight to allow diffusion of the propolis and honey and to dry the surface. The test organism *S. aureus* and *E. coli* were inoculated in a direction running from the highest to the lowest concentration, and placed in the incubator at 35°C for 24 h. Observed the inoculated plates and recorded observation. The length of growth and the total length of the agar surface streaked were measured. Then total length of possible growth is denoted by x cm and total length of actual growth y cm, the inhibitory concentration as determined by this method is: (c x y)/x mg ml^{-1}, where c is the final concentration in mg ml^{-1} of the propolis and honey (Hugo and Denyer, 2004).

RESULTS

The highest inhibition zone (15.0 ± 0.11 mm) was recorded from propolis at the concentration of 5.48 mg ml^{-1} followed by 2.74 mg ml^{-1} (Figure 1) while honey showed slightly lower inhibition zone (12.0 ± 0.09 to 13.0 ± 0.09 mm) than propolis against *S. aureus* in disc diffusion method (Figure 2). On the contrary higher inhibition zone (10.0 mm) was recorded from propolis at the concentration of 5.48 mg ml^{-1} followed by lower to lowest concentrations against *E. coli* (Figure 1). On the contrary honey showed slightly lower inhibition zone (7.0 to 7.5 mm) than propolis against *E. coli* in disc diffusion method (Figure 2). The minimum inhibitory concentration of propolis and honey against *S. aureus* and *E. coli* are presented in Tables 1 and 2. Growth and no growth tubes were identified comparing to the turbidity of the positive control. The negative growth was observed in propolis at concentrations of 2.74 to 5.48 $mgml^{-1}$ while growth was observed at concentrations of 1.37 $mgml^{-1}$ followed by lower concentrations. The negative growth was observed in honey only at the highest concentration up to 375.0 mg

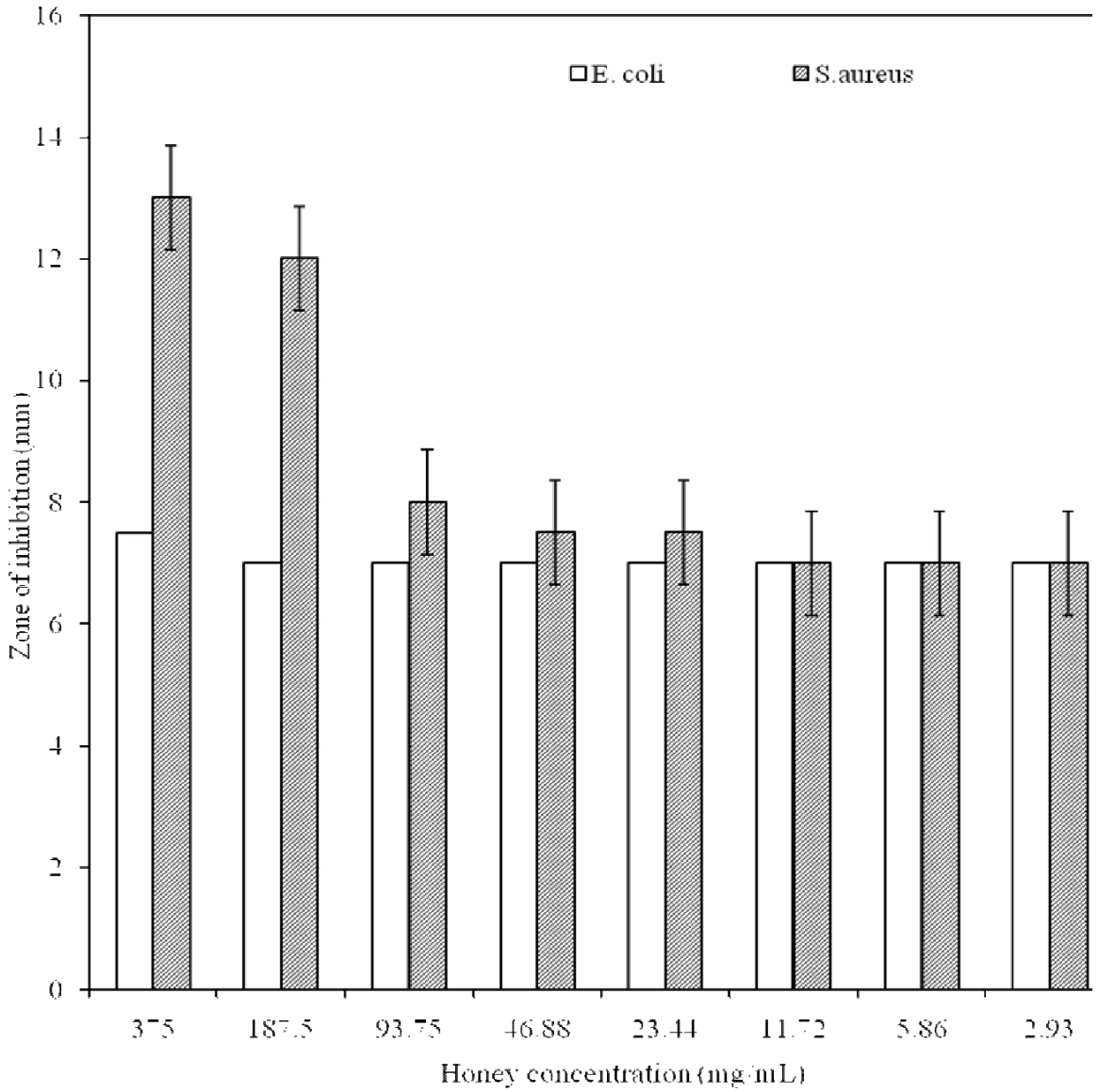

Figure 2. Zone of inhibition by application of honey against *E. coli* and *S. aureus* by disc diffusion method.

Table 1. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of propolis and honey against *S. aureus.*

Dilution	Concentration (mg/ml)		Propolis		Honey	
	Propolis	Honey	MIC	MBC	MIC	MBC
1/2	5.48	375.0	-	-	-	-
1/4	2.74	187.5	-	-	+	+
1/8	1.37	93.75	+	+	+	+
1/16	0.68	46.88	+	+	+	+
1/32	0.34	23.44	+	+	+	+
1/64	0.17	11.72	+	+	+	+
1/128	0.085	5.86	+	+	+	+
1/256	0.043	2.93	+	+	+	+

(-) represents inhibition; (+) represents growth

Table 2. Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of propolis and honey against *E. coli.*

Dilution	Concentration (mg/ml)		Propolis		Honey	
	Propolis	Honey	MIC	MBC	MIC	MBC
1/2	5.48	375.0	-	-	+	+
1/4	2.74	187.5	+	+	+	+
1/8	1.37	93.75	+	+	+	+
1/16	0.68	46.88	+	+	+	+
1/32	0.34	23.44	+	+	+	+
1/64	0.17	11.72	+	+	+	+
1/128	0.085	5.86	+	+	+	+
1/256	0.043	2.93	+	+	+	+

(-) represents inhibition; (+) represents growth

Table 3. Inhibitory concentration of propolis and honey against *S. aureus* and *E. coli* under gradient-plate technique.

Test organism	Growth line length (cm)	Inhibited line length (cm)		Inhibitory concentration (mg ml^{-1})	
		Propolis	Honey	Propolis	Honey
S. aureus	7.50 ± 0.0	2.38 ± 0.66	3.50 ± 2.00	3.50 ± 0.96	350.0 ± 200
E. coli	7.50 ± 0.0	3.70 ± 0.66	7.50 ± 2.00	5.41 ± 0.96	750.0 ± 200

ml^{-1} while growth was observed at lower concentrations against *S. aureus* (Table 1). In case of *E. coli*, negative growth was observed in propolis at the concentration of only 5.48 $mgml^{-1}$ while growth was observed at lower con-entrations. Regardless of concentration honey failed to show any effectiveness against growth of *E. coli* (Table 2).

The MBC of propolis and honey against *S. aureus* and *E. coli* grown in TSA plate are presented in Table 2. TSA plates (spot plate) streaked from no growth (negative growth) tubes showed no colonies while streaked from positive tubes showed colonies of *Staphylococcus aureus* and *E. coli.* The result of MBC value of the propolis and honey against *S. aureus* and *E. coli* was exactly similar compared to MIC value. Gradient plate technique showed both growth and inhibition of growth on streaked line. Total line length in gradient plate was 7.5 cm and inhibited growth line from propolis and honey showed 2.38 and 3.50 cm at concentrations of 3.5 and 350 $mgml^{-1}$, respectively against *S. aureus* (Table 3). The inhibited growth line from propolis and honey showed 3.7 and 7.50 cm at concentrations of 5.41 and 750 $mgml^{-1}$, respectively against *E. coli* (Table 3). This concentration is relatively closer with MIC and MBC concentration of propolis and honey against *S. aureus* and *E. coli.*

DISCUSSION

Both propolis and honey evaluated in this study showed antibacterial activity against *S. aureus* in disc diffusion method, MIC, MBC and gradient plate technique. Significant response was noticed in disk diffusion assay in propolis and honey against *S. aureus* but propolis at higher concentration was only effective to inhibit *E. coli*. The MIC results from propolis of the present study observed that 2.74 $mgml^{-1}$ is effective against *S. aureus*. Similar MIC results from propolis (2.01 to 3·65 $mgml^{-1}$) was obtained by Miorin et al. (2003). Bonvehiet et al., (1994) investigated propolis from *A. mellifera* against *S. aureus* where MIC values ranged from 0.080 to 0.100 $mgml^{-1}$. These MIC results are different than the MICs found to be active in this study because of different methodologies to determine antibacterial activity. Propolis against *S. aureus* was studied by agar dilution method and found average MIC was 22.5 $mgml^{-1}$ (Fernandez et al., 1995). Propolis samples had higher antibacterial activity against *S. aureus* when compared with honey samples.

Similar results were reported by (Miorin et al., 2003). Present study showed that propolis inhibited the gram-positive bacteria better than gram-negative bacteria. Generally, plant extracts are usually more active against gram-positive bacteria than gram-negative bacteria (Lin et al., 1999). Present findings were also supported by other researchers who reported that the crude powder of the galls of *Quercus infectoria* was found to be active against *S. aureus* (Fatima et al., 2001). The range of MIC values for *S. aureus* and *E. coli* correlated well with the results obtained using the gradient plate technique and

correlates with disc diffusion method. Both honey and propolis contain some phenolic substances which include cinnamic acid derivatives and some flavonoids (Marcucci et al., 2001). The main compounds available in propolis are acids such as caffeic, p-Coumaric acid, 3-prenyl-4-hydroxycinnamic, 3,5-diprenyl-4-hydroxycinnamic, 2,2-dimethyl-8-prenyl-2H-1-benzopyran-6-propenoic, a kaempferol derivative (flavonoid) and 2,2-dimethyl-6-carboxyethenyl-2H-1-benzopyran (Marcucci and Bankova, 1999; Bankova et al., 2000; Marcucci et al., 2001). In addition comparartively higher 4-Hydroxybenzoic acid is present in propolis than honey. This may be due to the fact that for asepsis bees cover the comb with a thin layer of propolis before the honey is deposited. The antibacterial activity of propolis may be related to the presence of flavonoids (Bosio et al., 2000). Takaisi-Kikuni and Schilcher (1994) and Nieva Moreno et al. (1999) have shown that propolis extracts have antibacterial properties against some micro-organisms. Antibacterial activity of propolis against *S. aureus* was ovserved mainly because of the presence of most active compounds such as triterpenic acids where communic and imbricatoloic acids and isocupressic methyl ester (Bankova et al., 1996). Kaurenoic acid which is a newly identified compound showed higher inhibitory activity than streptomycin against *S. aureus* (Velikova et al., 2000a). Miorin et al. (2003) suggested that effectiveness of honey or propolis depends on differences in chemical composition, bee species and geographic region.

The MIC and MBC methods are simple and easy for the determination of inhibitory doses of antibiotics or disinfectant for particular bacteria. The effectiveness of antibiotic properties of propolis found that propolis was equal to or slightly more effective than two common antibiotics, erythromycin and amoxicillin, in killing *S. aureus* and *Streptococcus faecalis* bacteria (Zumla and Lulat, 1989). Honey inhibits the growth of dangerous bacteria such as *E. coli*, *S. aureus*, *Salmonella*, *Shigella*, and *V. cholera* (Zumla and Lulat, 1989). The concentration of honey varied 30 to 50% was bactericidal to *S. shigella*, *E. coli* and *v. cholera*, making honey an anti- bacterial agent and superior to several well-known and currently prescribed antibiotics. Honey inhibits the growth of pathogenic organisms isolated in urine samples of patients with urinary tract infections as well (Somal et al., 1994).

Nieva Morneo et al. (1999) reported that ethanolic extracts of propolis showed high antibacterial activity against gram-positive cocci (*S. aureus*), but had a weak activity against gram-negative bacteria (*E. coli* and *Pseudomonas aeruginosa*) and yeast (*Candida albicans*). Propolis is active against Gram-positive bacteria, showing limited activity against Gram-negative ones (Grange and Davey, 1990). From the above results it appears that *E. coli* is more resistant than *S. aureus* against propolis and honey. *E. coli* is considered a particularly dangerous pathogen because of its resistance to many commonly used antibiotics.

Therefore, to prevent contamination from *E. coli* higher concentrated propolis or more effective disinfectant/ antibiotic should be applied. *E. coli* is a gram negative bacteria and it is notorious for its resistance to many antibiotics due to the permeability barrier afforded by its outer membrane (Viveiros et al., 2007).

Conclusion

Disc diffusion method is a very useful tools to evaluate effectiveness of natural antibiotics. Gradient- plate technique is also used as a counterpart method of MIC and MBC. Both MIC and MBC methods are simple and easy for the determination of inhibitory doses of propolis and honey for *S. aureus.* Propolis and honey concentrations at rates of 2.74 - 5.48 and 375.0 $mgml^{-1}$ can be applied to inhibit *S. aureus.*

Collectively, present findings revealed that propolis had higher antibacterial activity against *S. aureus* when compared with honey and more susceptible than *E. coli*. Antimicrobial properties would warrant further studies on the clinical applications of propolis and honey against *S. aureus.* Higher concentration of propolis and honey singly or combined application of both to be further investigated to inhibit *E. coli*.

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