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Antioxidative and Antibacterial Activities of Indonesian Propolis Extracts against Methicillin-Resistant *Staphylococcus aureus* (MRSA) *in Vitro*

Endonezya'da ki Propolis Ekstratlarının Metisiline Dirençli *Staphylococcus aureus*'lara Karşı Antibakteriyal ve Antioksidatif Aktiviteleri

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ABSTRACT

Aim: This study aims at determining the antioxidant and antibacterial activities of Indonesian propolis extracts against Methicillin-Resistant Staphylococcus aureus (MRSA).

Material and Methods: The antioxidant activity was assessed using DPPH radical and H2O2 scavenging method. Antibacterial activity of propolis extracts against MRSA (Methicillin-Resistant Staphylococcus aureus) was tested using the Kirby Bauer agar diffusion method.

Results: At concentration of 100%-15.525%, the DPPH radical scavenging activity of vitamin C is higher than that of the ethanol, n-hexane, and ethyl acetate extracts. At a concentration of 7.8125%, the DPPH radical scavenging activity of the ethanol extract is 37.170 %, n-hexane at 38,310% and ethyl acetate at 36.807%. H2O2 scavenging activities of the three propolis extracts are higher than that of vitamin C (86.642%), in which the scavenging activity for a fraction of 7.813 ug/mL n-hexane is 94.925 %, ethanol extract at 94.617 % and ethyl acetate fraction at 87.608%. Ethanol extract of 500 ug/mL propolis has the highest total phenol level at 59.1250%. Ethanol and ethyl acetate fractions are capable of inhibiting all three types of bacteria. Ethanol fraction is only able to inhibit the growth of S. *aureus* and B.*subtilis*. N-hexane fraction cannot inhibit all three types of bacteria. Minimum inhibitory concentration (MIC) values for ethanol and ethyl acetate extracts occur at a concentration of 4% (40 mg/mL) with 2-mm inhibition zone.

Conclusion: Propolis extract contains phenols and can scavenge free radicals and H2O2 and thus potentially inhibit oxidative stress. Ethyl acetate fraction of propolis has antibacterial activity which is greater than the ethanol and n-hexane fractions. In addition, propolis extract, ethanol fraction and ethyl acetate fraction are antibacterial against MRSA.

Key Words: free radicals, oxidants, Gram-positive bacteria, Gram-negative bacteria, spore-producing bacteria.

ÖZET

Amaç: Bu çalışma Endonezyada ki propolis ekstratlarının, metisiline Dirençli *Staphylococcus aureus'* lara (MRSA) yönelik antibakteriyal ve antioksidatif aktivitelerini tespit etmeyi amaçlamaktadır.

Materyal ve Metod: Antioksidan aktivitesi DPPH radikali ve H2O2 giderme metodu kullanılarak değerlendirilmiştir. MRSA'ya yönelik antibakteriyel aktivite ise Kirby Bauer agar diffusion yöntemi kullanılarak test edilmiştir.

Bulgular: Konsantrasyon %100-%15.525 iken Vitamin C'nin DPPH radikalini giderme aktivitesi; etanolün, n-hexane ve etil asetat ekstratlarının aktivitesinden daha yüksektir. Konsantrasyon %7.8125 iken etanol ekstraktının DPPH radikalini giderme aktivitesi %37.170, n-hexane'ın aktivitesi %38,310, etil asetatın aktivitesi %36.807'dir. H2O2 giderme aktivitesi

üç propolis ekstratında, Vitamin C ye nazaran daha yüksektir (%86.642), öyle ki 7,813 ug / mL 'lik bir n-hexane fraksiyonunun giderme aktivitesi %94.925, etanol ekstraktının ki % 94.617 ve etil asetat fraksiyonunun ki %87.608'dir. Etanol ekstraktının oranı 500 ug/mL olan propolis, maksimum total fenol seviyesine sahiptir (%59.1250). Etanol ve etil aseteat fraksiyonları üç tip bakterinin hepsinide inhibe edebilirler. Etanol fraksiyonu S*. aureus* ve B*. subtilis'* in sadece gelişmesini inhibe edebilir. N-hexane üç tip bakterinin hiçbirini inhibe edemez. Etanol ve etil asetat için minimum inhibitor konsantrasyonu (MIC) değerleri, 2 mm lik inhibisyon bölgesinde %4 (40 mg/mL) olarak tespit edilmiştir.

Sonuç: Propolis ekstraktı fenolleri içerir, serbest radikalleri ve H2O2 yi giderebilir, böylece oksidatif stresi engeller. Propolis içinde ki etil asetat fraksiyonu ise etanol ve n-hexane fraksiyonlarından daha fazla olarak antibakteriyel aktiviteye sahiptir. Buna ek olarak propolis ekstraktı, etanol ve etil asetat fraksiyonları MRSA ya karşı antibakteriyeldir.

Anahtar Kelimeler: serbest radikaller, oksidan, Gram pozitif bakteriler, Gram negatif bakteriler, spor üreten bakteriler.

BACKGROUND

Staphylococcus aureus is a major human pathogen as the cause of the syndrome of life-threatening diseases, such as endocarditis, meningitis, and pneumonia[1]. *S. aureus* can trigger and develop infection in highly efficient manner due to the ability of dozens of virulence factors on the one hand, and on the other hand due to the development of antibiotic resistance[2]. *S. aureus* already resistant to methicillin is called Methicillin-Resistant Staphylococcus aureus (MRSA). In Asia, the prevalence of MRSA infections reached 70%. While in Indonesia in 2006 the prevalence reached 23.5%[3]. In addition there is a potential antibiotic resistance, an oxidative stress is found to be involved in endocarditis, meningitis, and pneumonia[4-5]. This indicates the need for management of treatment strategy that can work in antibiotic resistance condition and can reduce oxidative stress.

Propolis is a natural resin product collected by honey bees and derived from a variety of plant sources. Various biological activities of propolis have been revealed, as an antiseptic, antimycotic, bacteriostatic, astringent, spasmolytic, anti-inflammatory, anesthetic, and antioxidants. The chemical composition of propolis (polyphenols, terpenoids, steroids, and amino acids) are very varied, depending on the location of the plant and the source[6]. Propolis from Europe contains flavonoids and phenolic acid esters of about 10-15% as its main components. Meanwhile, propolis from Brazil has major components of terpenoids and prenylated p-coumaric acids. Levels of flavonoids and phenolic acid ester are only about <4% [7-10]. Antimicrobial activity of propolis is determined by the high proportion of fatty acids (oleic, palmitic, linoleic and stearic acids). Although the benefits of propolis have been reported in Europe, Brazil, Egypt, China and other countries, information about propolis from Indonesia is very limited. This study aimed at determining the antioxidative and antibacterial activities of *Apis mellifera honey* bees-produced propolis extracts of Indonesia against some selected bacteria and Methicillin-Resistant Staphylococcus aureus (MRSA).

MATERIAL and METHOD

Material used in this study is the *Apis mellifera* species honey bees-produced propolis. After a propolis extract was obtained, treatment was continued with the process of concentration.

DPPH radical scavenging activity analysis

50 mL of sample was added to the 200 mL of 0.077 mmol DPPH in methanol (on microplate). The mixture was incubated at room temperature for 30 minutes and then absorbance value was measured at a wavelength of 517 nm with the use of microplate reader. 250 mL of DPPH was used as negative control, while 250 mL of absolute methanol was used as blank solution. Antioxidant effect of propolis samples was proportional to the intensity of 1,1 diphenyl-2-picrylhydrazyl (DPPH). Antioxidant activity percentage (%) can be determined by comparison of the solution

absorbance containing the sample with control solution without the sample (blank). Vitamin C is used as a positive control[11,12].

Antioxidant activity (%) with DPPH method:

$$1 - \frac{\text{sample absorbance}}{\text{negative control absorbance}} \times 100$$

H_2O_2 scavenging activity analysis

0.6 mL of H_2O_2 (2 mM/L dissolved in PBS/phosphate buffer saline at pH 7.4) was added to 1 mL sample. The mixture was reacted for 10 minutes. Then the absorbance value was measured at a wavelength of 230 nm using spectrophotometer. Use about 1.6 mL of pure H_2O_2 (without PBS) as negative control, and use the 1.6 mL of PBS or 1.6 mL of phosphate buffer as blank solution.

H_2O_2 scavenging activity (%):

$$1 - \frac{\text{sample absorbance}}{\text{negative control absorbance}} \times 100$$

Total phenol content analysis

Total phenol content was analyzed according to the method used in previous studies[13]. 100 mL of sample or standard using EGCG (Epigallocatechin Gallate) was reacted with 75 mL of 10% Folin-Ciocalteu reagent and 60 mL of 7.5% Na2CO3 on the microplate. The mixture was incubated at a temperature of 45°C - 50°C (using oven) for 10 minutes. Subsequently, absorbance value was measured at a wavelength of 750 nm using a microplate reader. Based on the standard absorbance value of EGCG, then the regression equation and slope values were obtained (a) =

$$\frac{\sum xy}{\sum x^2}$$

Phenol level (mg) of EGCG = absorbance / slope

Antibacterial activity test

All fractions (ethanol, n-hexane and ethyl acetate) were firstly diluted with a concentration of 1%, 5% and 10%. Antimicrobial sensitivity screening of the three kinds of fractions extracted used three test bacteria of Escherichia coli, Bacillus subtilis and Staphylococcus aureus.

Antibacterial activity of propolis extracts against MRSA (*Methicillin-Resistant Staphylococcus aureus*) was tested using the Kirby Bauer agar diffusion method. This cylinder was then filled with the sample solution and control using 50 mL, and then put the Petri dish in an incubator at 37°C for 24 - 48 hours. The testing results were stated qualitatively by a clear zone around the cylinder. Diameter of each inhibition zone of bacterial growth was measured using vernier caliper/ruler.

RESULTS

DPPH radical scavenging activities of the three extracts compared with vitamin C were shown in Figure 1. At concentrations ranging from 100% to 15.525%, the DPPH radical scavenging activity of vitamin C is higher than that of the three extracts. At a concentration of 7.8125%, the DPPH radical scavenging activity of the ethanol extract was 37.170%, n-hexane extract at 38.310% and

ethyl acetate at 36.807%. As a control, vitamin C has the DPPH radical scavenging activity at 35.562%, lower than that of the extracts. This was also found in concentration of 3.90625%. Ethanol extract had IC50 of 29.87 ± 0.98 μg/ml. Meanwhile, hexane and ethyl acetate fractions had IC50 of 124.47 ± 6.52 μg/ml.

As presented in Figure 2, the H_2O_2 scavenging activities of the three propolis extract fractions were higher than vitamin C. The n-hexane fraction of 7.813 μg/mL has the highest scavenging activity of 94.925% compared with vitamin C (86.642 %), while the ethanol extract of 94.617 % and ethyl acetate fraction of 87.608%. At concentration of 3,906 μg/mL, the activities of the three propolis extracts decreased compared with the previous concentration. Ethanol extract had IC50 of 52.01 ± 0.16 μg/ml. Meanwhile, hexane and ethyl acetate fractions had IC50 of 43.94 ± 0.24 μg/ml and 53.15 ± 0.36 μg/ml.

Ethanol extract fraction of propolis at concentration of 500 μg/mL had the highest total phenol levels of 59.1250%. At the same concentration, ethyl acetate fraction had total phenol content of 46.5491%, while the n-hexane fraction of 38.1081%. This data can be seen in Figure 3.

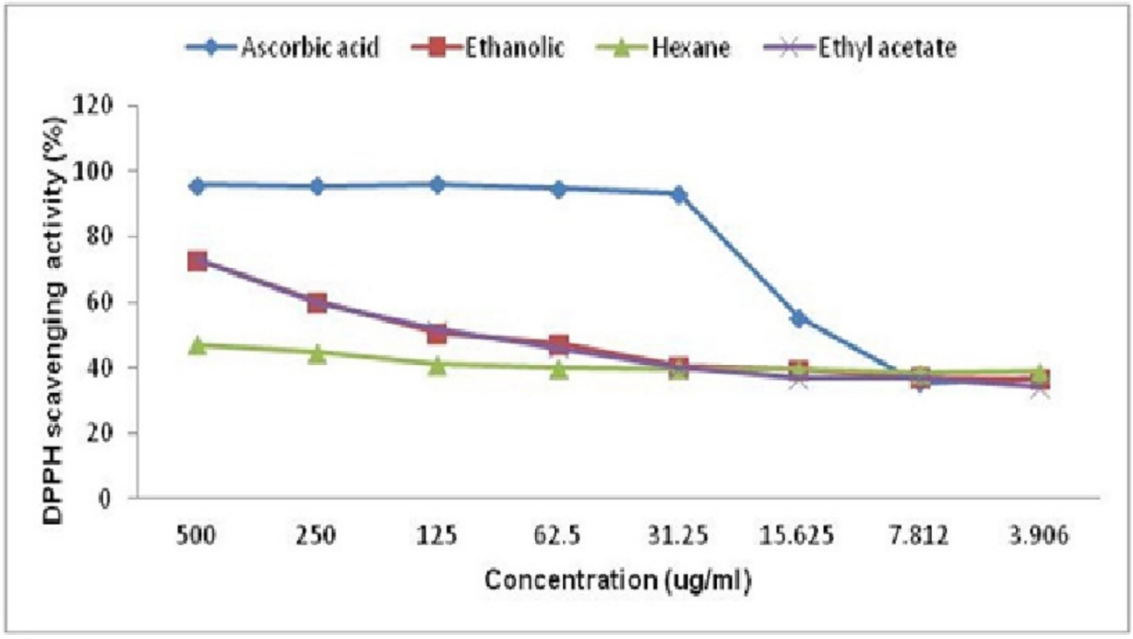

Figure 1. DPPH free radical scavenging activity of propolis extracts. At concentrations ranging from 100% to 15.525%, the DPPH radical scavenging activity of vitamin C is higher than that of the three extracts. At a concentration of 7.8125%, the DPPH radical scavenging activity of the ethanol extract is 37.170 %, n-hexane at 38,310% and ethyl acetate at 36.807%.

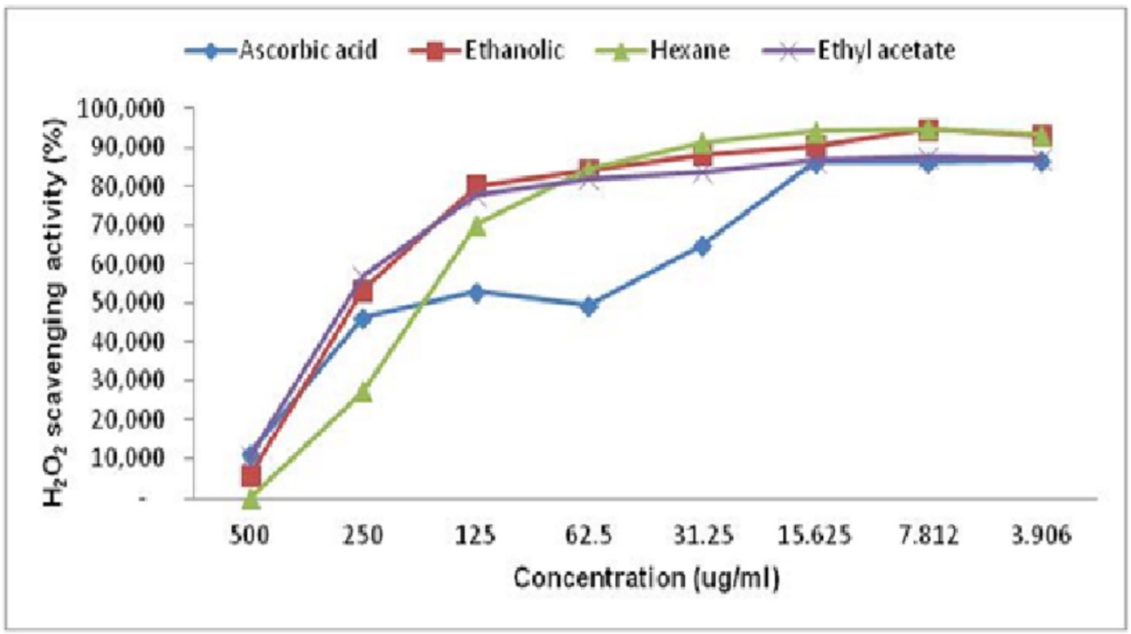

Figure 2. The H_2O_2 scavenging activity of propolis extracts. The H_2O_2 scavenging activities of the three propolis extract fractions are higher than vitamin C. The 7.813 ug/ml of n-hexane fraction has the highest scavenging activity of 94.925% compared with vitamin C (86.642%), while the ethanol extract of 94.617 % and ethyl acetate fraction of 87.608%. At concentration of 3,906 μg/mL, the activities of the three propolis extracts decreased compared with the previous concentration.

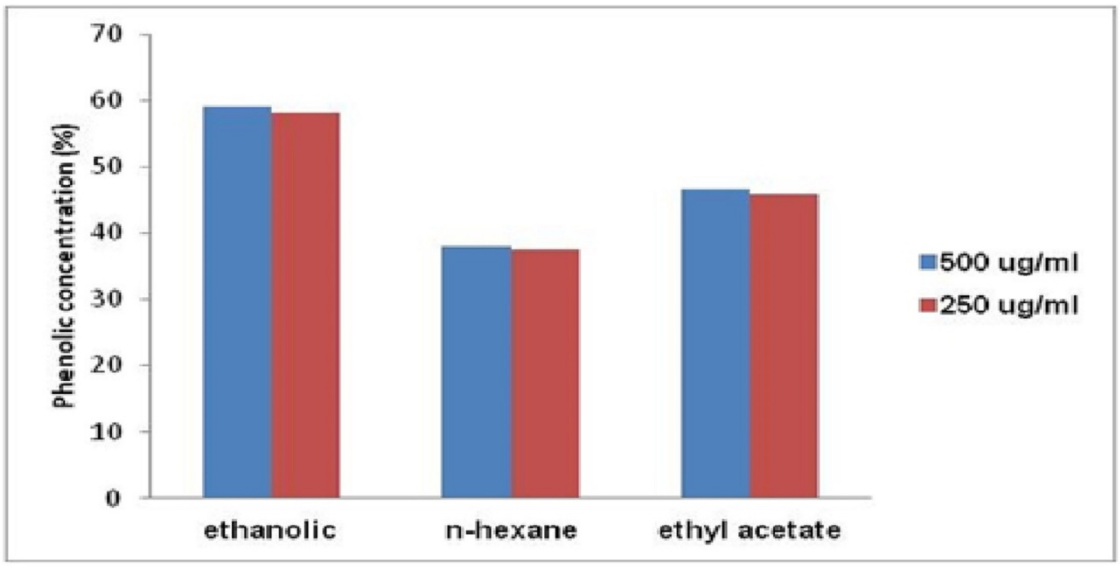

Figure 3. Total phenol content in various fractions of propolis extract. Ethanol extract fraction of propolis at concentration of 500 ug/mL had the highest total phenol levels of 59.1250%. Meanwhile, ethyl acetate fraction has total phenol content of 46.5491% and the n-hexane fraction of 38.1081%.

Antimicrobial sensitivity screening test showed two fractions capable of inhibiting all three types of the test bacteria namely ethanol and ethyl acetate fraction. Ethanol fraction is able to inhibit the growth of S. aureus and B. Subtilis but not able to inhibit E. coli. The n-hexane fraction cannot inhibit the three types of test bacteria, as shown in Table 1.

Propolis extract having antimicrobial activity against MRSA is found in ethanol and ethyl acetate fractions. Analysis of antimicrobial sensitivity against the growth of MRSA showed that at a concentration of 1%, there was no inhibition zone, but at concentrations of 5% and 10%, there was considerable inhibition zone as shown in Table 2. Furthermore, dilutions were made at 2%, 4%, 6%, 8% and 10% to determine Minimum Inhibitory Concentration (MIC) of the propolis extracts which can inhibit the growth of MRSA. As showed in Table 3, the MIC values for ethanol and ethyl acetate extracts occurred at concentration of 4% (40 mg/mL) having a inhibition zone of 2 mm.

Table 1. Inhibition zones of the three different fractions of propolis extracts against three kinds of bacteria.

Bacteria	Ethanol extract		Ethyl acetate extract				N-hexane extract		
	1%	5%	10%	1%	5%	10%	1%	5%	10%
E. coli	-	5	6	-	5	6	-	-	-
S. aureus	-	4	6	-	4	5	-	-	-
B. subtilis	-	5	6	-	4	5	-	-	-

Table 2. Preliminary antimicrobial sensitivity tests of ethanol and ethyl acetate fractions of propolis against the growth of MRSA

Bacteria	Ethanol extract (polar)			Ethyl acetate extract (semipolar)		
	1%	5%	10%	1%	5%	10%
MRSA	-	4	8	-	5	8

Table 3. Minimum Inhibitory Concentration (MIC) of ethanol and ethyl acetate fractions of propolis against the growth of MRSA

Bacteria	Ethanol extract (polar)					Ethyl acetate extract (semipolar)				
	2%	4%	6%	8%	10%	2%	4%	6%	8%	10%
MRSA	-	2	6	7	7.7	-	2	7	7	7.7

DISCUSSION

The chemical composition of propolis depends on the geographical location. Propolis from Bulgaria, Turkey, Greece and Algeria usually contain most of the flavonoids, caffeic acid ester and ferulic acid ester[14]. Ethanol fraction of propolis extract 500 μg/mL has the highest total phenol level (59.1250%) followed by ethyl acetate (46.5491%) and n - hexane fractions (38.1081%). Phenol contains -OH group binding to the benzene ring[15]. Phenol group has an ideal chemical structure for scavenging free radicals through the mechanism of hydrogen donor and an electron donor[16,17]. Phenolic antioxidants occur by breaking free radical chain reaction to form a stable phenoxyl radical product. Phenoxyl radical stability is caused by electron delocalization on the aromatic ring[18].

At concentration of 100%-15.525%, the DPPH radical scavenging activity of vitamin C is higher than that of the ethanol, n-hexane of 38.310%, and ethyl acetate extracts. At a concentration of 7.8125%, the DPPH radical scavenging activity of the ethanol extract is 37.170%, n-hexane extract at 38.310% and ethyl acetate at 36.807%. As a control, vitamin C has the DPPH radical scavenging activity at 35.562%. Previous research has shown anti-radical activity of ethanol extract of propolis[19]. When compared with other geographical locations, Indonesian propolis extracts have lower DPPH scavenging activity compared with Andalusia (49.12 ± 16.02%), Argentina (46.6 - 89.6 %), Sonoran (86%) , and Japan[20-23]. Various ethanol extracts of propolis from Taiwan have DPPH radical scavenging activity with IC50 in range of 17.90 - 108.05 μg/ml[24]. In this study, ethanol extract has IC50 of 29.87 ± 0.98 μg/ml. Meanwhile, hexane and ethyl acetate fractions have IC50 of 124.47 ± 6.52 μg/ml.

Phenolic compounds can also scavenge H_2O_2[25,26]. The H_2O_2 scavenging activities of the three propolis extract fractions are higher than vitamin C. The 7.813 ug/ml of n-hexane fraction has the highest scavenging activity of 94.925% compared with vitamin C (86.642%), while the ethanol extract of 94.617 % and ethyl acetate fraction of 87.608%. At lower concentrations, the H_2O_2 scavenging activity of the three propolis extracts will decrease. Ethanol extract has IC50 of 52.01 ± 0.16 μg/ml. Meanwhile, hexane and ethyl acetate fractions have IC50 of 43.94 ± 0.24 μg/ml and 53.15 ± 0.36 μg/ml.

The three types of bacteria represent Gram-negative bacteria, Gram-positive bacteria and spore -producing bacteria. These highly variable antimicrobial activities of propolis are due to the composition of propolis used. Propolis is found to have antibacterial activity against cocci bacteria and Gram positive bacilli, but it is weak in inhibiting the growth of Gram-negative bacteria[27,28]. Mechanism of antimicrobial activity of propolis is complex and represents a good synergy between flavonoids, hydroxyacids, and sesquiterpenes[29].

Ethyl acetate fraction of propolis has the largest antibacterial activity than ethanol and n-hexane fractions. Inhibition zone of ethyl acetate fraction at concentration of 10% against the test E. coli bacteria is larger than the inhibition zone against the test B. *subtilis* and *S. aureus* bacteria. These results prove that there is difference in resistance to antimicrobial compounds of the positive Gram and negative Gram bacteria due to differences in the composition of the cell wall constituents. In addition, difference in activity between the factions is also due to the synergistic effects of various compounds[30].

The results of the current research proved that propolis is able to inhibit the growth of MRSA consistent with the previous studies[27]. Propolis inhibits bacterial growth by inhibiting cell division, resulting in a formation that resembles a multicellular streptococcus. Propolis can disrupt cytoplasmic membrane and cell wall permeabilities, thereby leading to bacteriolysis. Propolis also inhibits protein synthesis[31]. Another mechanism works through cytoplasmic membrane

transduction energy breakdown and inhibition of bacterial motility. Bioenergetic effect on membrane causes propolis to have antimicrobial activity. This works synergistically with antibiotic action[32]. In addition, propolis possesses bacteriostatic activity against different bacterial genera and may be bactericidal in high concentrations[33].

CONCLUSION

Propolis extract contains phenols and can scavenge free radicals and H_2O_2 and thus potentially inhibit oxidative stress. Ethyl acetate fraction of propolis has antibacterial activity which is greater than the ethanol and n-hexane fractions. In addition, propolis extract, ethanol fraction and ethyl acetate fraction are antibacterial against MRSA.

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