ORIGINAL ARTICLE

Antimicrobial Activity of Malaysian *Apis mellifera* Propolis against *Propionibacterium acnes*

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ABSTRACT

Introduction: Acne vulgaris is a common skin disease that affects people all over the world. One of the main pathogenesis of acne is *Propionibacterium acnes* (*P. acnes*) proliferation. Propolis has long been used in folk medicine as a natural remedy. Its antimicrobial properties have all been studied extensively. However, there have been few studies on its use in acne. Thus, the goal of this study was to assess the antimicrobial potential of ethanolic (EEP) and water extracts (WEP) of Malaysian *Apis mellifera* propolis against *P. acnes*. **Methods:** Propolis samples were collected from Acacia mangium apiary from northern and southern regions of Peninsular Malaysia. The propolis extracts were screened for antimicrobial activity against *P. acnes* using an agar well diffusion assay. The minimum inhibitory concentrations (MICs) of the extracts were determined using a resazurin broth microdilution assay. **Results:** The antimicrobial screening demonstrated all extracts had antimicrobial activity against *P. acnes*. The inhibition zones at concentration 20 mg/ml were in the range of 16 mm to 24 mm which was greater than positive control (10% benzoyl peroxide) (15 mm). The EEP from northern region showed the lowest MIC values (0.32 µg/ml), followed by EEP from southern region (0.63 µg/ml), WEP from southern region (625 µg/ml) and WEP from northern region (2500 µg/ml). **Conclusion:** The Malaysian EEP demonstrated promising antimicrobial properties against *P. acnes*. Further study is needed to determine the active constituents and their possible inhibitory mechanisms against *P. acnes*.

Keywords: Antimicrobial, Malaysian propolis, *Apis mellifera*, *Propionibacterium acnes*

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INTRODUCTION

Acne vulgaris (Fig. 1) is a very common skin disease that typically affects areas with high number of sebaceous follicles such as face, trunk and back. It is more pronounced in teenagers and young adults (1) and positively related to androgen spike during puberty which stimulates excessive sebum secretion. Acne is not a life-threatening condition, but it has been linked to anxiety and depression in some people (2).

Hyperkeratinisation, excessive sebum production, microbial hypercolonisation by *P. acnes*, and the release of inflammatory mediators into the skin are all factors in the pathogenesis of acne (3). *P. acnes* is a common commensal bacterium that lives on the surface of the

Figure 1: A patient with moderate acne vulgaris

skin (4). It is an anaerobic Gram-positive bacterium that readily grows in the regions rich in sebaceous glands. *P. acnes* promotes inflammation by activating innate immune system markers like Toll-like receptor 2, which leads to the production of pro-inflammatory cytokines like interleukin (IL) 8 (5). They also interact with other innate immunity by stimulating activity of antimicrobial peptides and inflammasome, a cytoplasmic complex of proteins that controls cytokine activation and secretion (5). Inflammasome activation is accompanied by monocyte activation which resulted in the release of inflammatory mediators. *P. acnes* also regulate the matrix metalloproteinases (MMPs). MMPs play a role in tissue destruction and the formation of scars (5).

Acne vulgaris can be treated with a variety of medications, including topical benzoyl peroxide, retinoids, antibiotics, and hormonal therapies like spironolactone, glucocorticoids, and oral contraceptives (6). However, current treatment options for acne vulagris are associated with adverse effects. The most commonly used acne treatment is topical benzoyl peroxide. Although there are no reports on its bacterial resistance, its adverse effects such as skin irritation, dryness, and occasional peeling (7) can lead to poor adherence. The other commonly used acne treatment is topical retinoids. Similar to topical benzoyl peroxide, topical retinoids are also have several adverse effect include local skin redness, dryness, itching, and stinging (8). Furthermore, use of retinoids are associated with the risk of teratogenicity (9).Therefore, the use of retinoids should be avoided in pregnancy and contraceptive precaution is needed in child bearing women (10).

For years, a combination of topical treatment and oral antibiotics has been the mainstay of acne treatment for moderate to severe acne vulgaris (11). However, long term use of antibiotics has been associated with the emergence of resistant strains (12). Other treatment of severe acne vulgaris is oral retinoids. Isotretinoin is generally effective in treating patient with severe acne vulgaris and helping to prevent extensive scarring (13). The commonly adverse effects of isotretinoin include dry lips, xerosis, and facial erythema (14). One the rare but serious adverse reaction is depression. Therefore, rational treatment for acne vulgaris should be targeted at the known pathogenic factors with optimum efficacy, minimum adverse effects, and fewest complications.

Propolis is a resinous mixture collected by various bee species from a variety of plant sources, primarily flowers and leaf buds, and mixed with their saliva (15). Fig. 2 shows the honeybee (*Apis mellifera*) produces propolis. The chemical compositions of propolis tend to differ according to its geographical origin, plant source, and time of its collection (16, 17). The main chemical compounds of Malaysian *Apis mellifera* (*A. mellifera)* propolis were phenolic acids, fatty acids, terpenoids, and sugars (18).

Figure 2: *Apis mellifera* produces propolis

Propolis has the potential to be used to treat acne vulgaris because of its well-known antimicrobial, anti-inflammatory, and antioxidant properties (19, 20). The antimicrobial properties of propolis ethanolic extract have been extensively investigated, and it has been reported to have antibacterial activity against a wide range of Gram-positive bacteria including *Streptococcus mutans*, *Streptococcus oralis*, and Staphylococcus aureus (21) as well as Gram-negative bacteria like *Pseudomonas sp., Escherichia coli*, and *Yersinia enterocolitica* (17, 22). It was also reported to possesses antimicrobial effects against *P. acnes* (23, 24). However, the studies were limited to ethanolic extracts. Water extract should be considered as an alternative to solvent extract because it is more cost-effective, non-toxic, and easily absorbed. Furthermore, when compared to alcohol or oil-based formulations, water-based formulations are the best for skin.

In view of the troublesome adverse effects of the current standard treatments, positive potential effect of propolis in acne, and differences in chemical constituents of propolis according to its source and origin, a study on the effect of Malaysian propolis against acne is certainly needed before it can be used for therapy. Thus, this study was specifically aimed to evaluate the in-vitro antimicrobial properties of ethanolic and water extracts of Malaysian A. mellifera propolis against *P. acnes*.

MATERIALS AND METHODS

Propolis sample

Department of Agriculture, Johor, Malaysia and MTC Advance Marketing Sdn Bhd, Penang, Malaysia provided the raw *A. mellifera* propolis samples. These samples were taken from Acacia mangium areas and

kept at -20°C until needed.

Preparation of ethanolic and water extract of propolis
Based on the methods described by Ismail et al. (2018), ethanolic extract of propolis (EEP) was prepared. Propolis samples were ground into a fine powder after cooling in the freezer (-20°C). To obtain a 30% (w/w) propolis extract, 50 g of each propolis sample was mixed with 167 mg of 70% ethanol. The mixture was moderately shaken twice a day at room temperature for a week. After that, the extract was filtered twice. To remove the wax, the extract was kept in the refrigerator (2-8°C) before the second filtration. A rotary evaporator operated under vacuum at 35°C was used to remove the ethanol. A freeze dryer was used to remove the remaining water in the extract. The dried extract was kept in a freezer (-20°C) in an amber glass bottle. Water extract of propolis (WEP) was made in the same way as EEP, except for the solvent.

Microorganism
The microorganism used in this study was *P. acnes* (ATCC 11827), and it was obtained from the American Type Culture Collection, USA. The microorganism and media used in this study were purchased from the Oxoid Ltd., UK.

Agar Well Diffusion Assay
To screen for the antimicrobial activity of propolis, EEP powder was dissolved in 70% ethanol at a concentration of 200 mg/ml which was further diluted with distilled water in a ratio of 1:10 to form a final concentration of 20 mg/ml. WEP powder was dissolved in distilled water to a concentration of 20 mg/ml. Ethanol 7% was used as a negative control and 10% benzoyl peroxide was used as a positive control (purchased from OXY®10, USA).

Agar well diffusion assay was performed by using the modified method of Kalogeropoulos et. al (2009) (19). A sterile cotton bud was dipped into *P. acnes* suspension (1 x 10^8 CFU/ml) and was lawned on the surface of Mueller Hinton Blood Agar (MHBA) media plate. Wells were made by using sterile glass pasture pipette with a diameter of 6 mm and labeled accordingly. Each well was filled up with 100 µl of EEP (20 mg/ml), WEP (20 mg/ml), positive, and negative controls. The plates were then incubated at 37°C under anaerobic condition by using anaerobic gas pack for 72 hours. Each test was independently performed in triplicate and antimicrobial activity was expressed as the zone of inhibition in diameters (mm).

Minimum Inhibitory Concentrations (MIC)
The MIC of EEP and WEP from northern and southern regions of Peninsular Malaysia were further tested by using modified resazurin broth microdilution assay (25, 26). The prepared solution of the propolis extracts at concentration of 20 mg/ml was serially diluted two-folds in the sterile well plates containing 100 µl of Cation Adjusted Mueller Hinton Broth to make the concentrations of 10000.00, 5000.00, 2500.00, 1250.00, 625.00, 313.00, 156.00, 78.00, 39.00, 20.00, 10.00, 5.00, 2.50, 1.25, 0.63, 0.32, 0.16, 0.08 and 0.04 µg/ml.

A 10 µl of bacterial suspension (1 x 10^8 CFU/ml) was then added to the test dilutions. Each plate had a set of controls: a column with doxycycline (Pfizer, UK) as a positive control, a column with all solutions except for the propolis extract as a negative control, and a column with all solutions with the exception of the bacterial solution by adding 100 µl of broth to check the sterility of the media. The plates were then incubated at 37°C for 72 hours under anaerobic condition. After the incubation, each well was added with 10 µl of 0.01% resazurin (Sigma Aldrich, US) as indicator solution and incubated for another 2 hours at 37 C under anaerobic condition. The lowest concentration at which the colour changed to pink was considered as the MIC value.

RESULTS

P. acnes was found to be sensitive to all propolis extracts in a screening test using an agar well diffusion assay as shown in Fig. 3. The northern region's EEP had the largest zone of inhibition (29 mm), followed by the southern region's WEP (26 mm), the northern region's WEP (24 mm), and the southern region's EEP (16 mm). Interestingly, when compared to the positive control of 10% benzoyl peroxide, all propolis extracts showed a larger zone of inhibition.

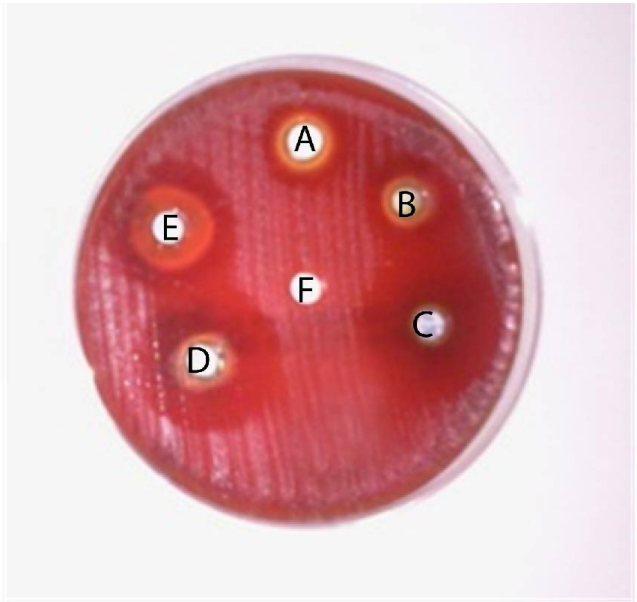

Figure 3: Zones of inhibition of Malaysian *Apis mellifera* propolis against Propionibacterium acnes using agar well diffusion assay. A = Positive control (benzoyl peroxide 10%) (15 mm), B = EEP from southern region (16 mm), C = WEP from southern region (26 mm), D = EEP from northern region (29 mm), E = WEP from northern region (24 mm), F = Negative control (ethanol 7%) (6 mm)

The resazurin broth microdilution assay revealed the MIC values of the propolis extracts against *P. acnes* were 0.32 µg/ml (EEP from northern region), 0.63 µg/ml (EEP from southern region), 625 µg/ml (WEP from southern region), and 2500 µg/ml (WEP from northern region) as shown in Table I.

Table I: Minimum inhibitory concentrations (MICs) of the Malaysian *Apis mellifera* propolis against *Propionibacterium acnes*

Propolis sample	Minimum inhibitory concentration (µg/ml)
Ethanolic extract of propolis from the northern region of peninsular Malaysia	0.32 µg/ml
Ethanolic extract of propolis from the southern region of peninsular Malaysia	0.63 µg/ml
Water extract of propolis from the northern region of peninsular Malaysia	2500 µg/ml
Water extract of propolis from the southern region of peninsular Malaysia	625 µg/ml

Note: Tests were performed in triplicate and the values were median.

DISCUSSION

Acne vulgaris is a very common skin disorder that affects almost all individuals at least once during lifetime. Unfortunately, the drugs commonly used to treat acne vulgaris have a variety of side effects. Natural products are becoming increasingly popular in the treatment of acne, and several studies have demonstrated their efficacy (27, 28). In order to effectively treat the acne lesion, treatments need to address the underlying causative factors (3). A natural product such as propolis has the potential as an alternative therapy for acne vulgaris with its antimicrobial, anti-inflammatory and antioxidant properties.

Propolis ethanolic extracts from various countries such as Korea, America and Brazil have been shown to have antimicrobial effects against *P. acnes* (23, 24). The MIC values range from 1 µg/ml to 5000 µg/ml. Interestingly, our study showed better results. The MIC values of EEP from northern and southern regions against *P. acnes* were 0.32 µg/ml and 0.63 µg/ml, respectively. The results showed that the propolis origin may influence the antimicrobial activities.

To date, there has been no antimicrobial research done on water extract propolis against *P. acnes*. For the first time, antimicrobial activities of WEP against *P. acnes* are studied. Our study found that WEP and EEP from northern and southern regions demonstrated antimicrobial activities against *P. acnes*. Agar well diffusion assay showed all propolis extracts had greater inhibitory zones than 10% benzoyl peroxide. However, this method is not always reliable for determining the antimicrobial activity of natural product, because the polarity of the natural compounds can affect the diffusion of compounds onto the culture medium (29). Compounds with less polarity tend to diffuse slower than more polar ones. Due to these concerns, well diffusion may not be a suitable method to determine the antimicrobial activity of natural compounds. Therefore, it can be used only to screen antimicrobial activity before the MIC study was proceeded to.

Our study showed that EEP from the northern region displayed the greatest zone of inhibition (29 mm), followed by WEP from southern region (26 mm), WEP from northern region (24 mm) and EEP from southern region (16 mm). However, the MIC values were not in line with the zone of inhibition. The MIC values of EEP from the northern region, WEP from southern region, WEP from northern region and EEP from southern region were 0.31 µg/ml, 625 µg/ml, 2500 µg/ml, and 0.63 µg/ml. These results showed that the difference polarity of chemical compound may influence their inhibitory zone.

Lower MIC values of EEP than WEP against *P. acnes* indicate EEP has better antimicrobial activity against this bacterium. These findings were consistent with previous studies showing that the alcoholic extract having the best antimicrobial activities (30, 31). The antimicrobial activity of EEP higher than WEP may be due to the presence of both lipid and water-soluble compounds in the EEP. The extraction method and the type of solvent used influence the propolis properties because different solvents can alter the propolis constituents, thereby affecting their effects.

In the recent study, EEP from northern region showed better antimicrobial activity compared to EEP from southern region. It is possible that this is due to the differences in chemical compounds. The chemical composition of propolis is closely related to the resins of plant sources used to produce it. Although the main plant source of Acacia mangium tree, the shrubs and fruit orchids were different which explains the differences in chemical components and MIC values. The pharmacological effects of propolis and other natural products depend on their active chemical compounds. Fatty acids, polyphenols (phenolic acids, flavonoids), and terpenoids are the main chemical groups found in propolis. Polyphenols and terpenoids are the most active and their biological properties as shown in Table II. A previous study on EEP from Malaysia showed that the main chemical compounds identified were terpenoids (18). The authors found that EEP from northern region had higher terpenoids (11.46%) compared to EEP from southern region (7.19%). The higher terpenoids content in EEP from the northern region could explain its superior antimicrobial activity. Terpenoids are well known to have antioxidant (32), anti-inflammatory (33), analgesic (34), and antimicrobial properties against both Gram-positive and Gram-negative bacteria, as well as fungi (35).

Table II: The main active compounds of propolis and their biological activities

Active compounds	Biological activities	References
Flavonoids		
Chrysin, pinocembrin, apigenin, galangin, kaempferol, quercetin, tectochrysin, and pinostrobin	Antimicrobial, antiproliferative, reduce MMP-9 gene expression and activity in activated macrophages, antioxidant, antidiabetic, antitumour, and anti-inflammatory	(37-42)
Phenolic acids		
Artepillin C, ferulic acid, cinnamic acid, caffeic acid, benzoic acid, salicylic acid, and p-coumaric acid	Antimicrobial, antioxidant, anti-inflammatory, antitumour, antiproliferativ,e and gastroprotective	(37, 43-45)
Terpenoids		
Terpineol, camphor, geraniol, nerol, farnesol, β-caryophyllene, nerolidol, carvacrol, and spathulenol	Antityrosinase, antibacterial, anti-HIV, antioxidant, and antiproliferative	(46-48)

CONCLUSION

The present study demonstrated that the ethanolic extract of Malaysian *A. mellifera* propolis displayed good antimicrobial activities against *P. acnes*. Although the propolis extracts have been shown to have antimicrobial properties against *P. acnes*, the mechanism behind this effect is unknown. More research is needed to determine the active constituents and their potential inhibitory mechanisms against *P. acnes*. On top of that, cosmeceuticals are gaining popularity. The use of natural products like propolis as anti-acne agents in cosmetics is a promising field.

ACKNOWLEDGEMENTS

The authors would like to thank the Universiti Sains Malaysia for providing the financial support under the Short-Term Grant, (304/PPSG/61312066), Mr. Zakbah Mian from the Department of Agriculture, Johor, Malaysia, and Mrs Rohaya Che Abu Bakar from the MTC Advance Marketing Sdn Bhd, Penang, Malaysia, for supplying the raw propolis samples. The authors also would like to acknowledge Mrs Rosmaniza, the Technologist at the Department of Microbiology, School of Medical Sciences, Universiti Sains Malaysia for her assistance in the sample analysis.

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