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DEVELOPMENT OF CREAM FORMULATION CONTAINING *TETRAGONULA PAGDENI* PROPOLIS FROM MANGOSTEEN ORCHARDS AND EVALUATION OF ANTIBACTERIAL ACTIVITY AGAINST *STAPHYLOCOCCUS AUREUS*

Pornanun Chuapaditphun, Bowonlak Kajhonlit, Somchanok Panyakawin, Sumalee Wannachaiyasit*

Faculty of Pharmaceutical Sciences, Burapha University, Chonburi

* Corresponding author: sumaleew@go.buu.ac.th

ABSTRACT

This study investigated antibacterial activity of the propolis extract of Thai stingless bee *Tetragonula pagdeni* (Schwarz) against *Staphylococcus aureus* and formulated a topical antibacterial cream. The stingless bee propolis was collected from mangosteen orchards. The propolis sample was extracted using ethanol as a solvent and partitioned with methanol and hexane. The extraction method provided a yield of 14.15% (w/w). The alpha-mangostin content in the propolis extract was 13.40±0.03% as determined by high performance liquid chromatography. Antibacterial susceptibility was determined by disc diffusion method. The propolis extract showed inhibition zone against *S. aureus*. The MIC and the MBC values of the propolis extract were examined by broth microdilution method and were found to be 3.06 and 6.12 µg/ml, respectively. The propolis cream formulation containing 5% propolis extract was developed. The propolis cream formulation was yellow with good texture and good spreadability with pseudoplastic and thixotropic properties. The 4-fold dilution of propolis cream formulation with culture media showed bactericidal activity against *S. aureus*. This study shows that propolis extract of *Tetragonula pagdeni* (Schwarz) is a promising candidate for the development of topical antibacterial dosage forms.

Keywords: stingless bees, propolis, antibacterial, *Staphylococcus aureus*, cream

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Introduction

Propolis or bee glue is a complex resinous product that bees produce from various sources such as plant buds, leaf buds, pollen, bee secretion and beeswax for construction of their hives.[1-3] Bees use propolis to seal cracks, repair combs and protect their hives from intruders, and it functions as a defense against infections.[4] Propolis has been widely used as a folk medicine for many decades for treatment of wounds, burns, sore throat, gingivitis and stomatitis.[5,6] Many studies have reported its antibacterial[7-9], antiviral[10], antitumor[11], antioxidative[12] and anti-inflammatory activities.[13]

Stingless bees are eusocial insects that play an important role in pollination.[14] There are many species of Thai stingless bees. *Tetragonula pagdeni* (Schwarz) is one of the native Thai stingless bees that bee keepers primarily cultivate for collecting honey. There are limited studies on the biological activity of this stingless bee propolis. To increase the value of this propolis, formulations were developed as antibacterial creams. The main constituents of propolis generally vary with bee species, types of plant sources and geographic regions.[15] The propolis of *Tetragonula pagdeni* (Schwarz) collected from mangosteen orchards in Chantaburi Province contained alpha (α)-mangostin in higher amounts than beta (β)-mangostin, gamma (γ)-mangostin and 3-isomangostin.[16] In this study, α-mangostin was the designated compound for HPLC analysis of the propolis extract that originated from the same source. Raw propolis generally contains around 50% resins and balsam, 30% waxes, 10% aromatic oils, 5% pollen and 5% other substances.[17] Because of the high amounts of resins and waxes in raw propolis, an effective extraction method is required to obtain high α-mangostin content in the extract. The previous report revealed that using 80% ethanol as a solvent in the extraction process provided 2.77±0.08 % α-mangostin.[18] The extraction method using ethyl acetate gave 2.70±0.69% α-mangostin.[16] α-Mangostin is soluble in alcohol and poorly soluble in water.[19,20] In this study the extraction method was modified by using ethanol (absolute ethanol) and, subsequently, using the hexane/methanol partition technique in order to remove waxes and resins resulting in a high content of α-mangostin in the extract.

S. aureus is a human pathogen that causes bacterial skin and soft tissue infections.[21,22] The study of bacterial pathogens in infected wounds isolated from 312 wound swab samples showed that the most common bacteria was *S. aureus* (37%), followed by *Pseudomonas aeruginosa* (17%), *Proteus mirabilis* (10%), *Escherichia coli* (6%) and *Corynebacterium spp.* (5%). Many skin and soft tissue infection models were prepared using *S. aureus*.[23,24] In this study, the antibacterial activity of the propolis extract was investigated using *S. aureus* as a tested microorganism.

This study investigated the antibacterial activity of *Tetragonula pagdeni* propolis extract against *S. aureus* and formulated a topical antibacterial cream. The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of the propolis extract were reported in this study. The propolis cream formulation was also examined for its antibacterial effect against *S. aureus*.

Materials and methods

Materials

Stingless bee propolis of *Tetragonula pagdeni* (Schwarz) was collected from mangosteen orchards in Makham District, Chanthaburi Province, Thailand in 2015. Ethanol (Carlo Erba Reagents, Italy), methanol (Carlo Erba Reagents, Italy), n-hexane (Merck, Germany) were analytical reagent grade. Formic acid was from QRec, New Zealand and methanol (HPLC grade) was from VWR chemicals, France. α-Mangostin (>98% purity) was purchased from Chengdu Biopurify Phytochemicals Ltd., Sichuan, China. Tryptic soya broth (TSB), tryptic soya agar (TSA), Mueller-Hinton agar (MHA),

Mueller-Hinton broth (MHB), oxacillin disc, tetracycline discs and chloramphenicol were from Oxoid, England. *S. aureus* (DMST8840) was supported by the Department of Medical Sciences, Nonthaburi Province, Thailand.

Preparation of the propolis extract

The propolis extract was prepared as in the previous report[18] with modifications. The propolis sample (300 g) was cut into small pieces. Ethanol (1.5 l) was added and the sample was subsequently sonicated for 30 min at 40°C. Brown colored liquid was collected. The process was repeated 3 times. Ethanol extract portions were collected and then centrifuged at 8,000 rpm at 20°C for 10 min. The supernatant was collected and ethanol was removed using a rotary evaporator to obtain a brown crude extract. For further purification, the crude extract was dissolved in methanol and was transferred into a separating funnel. Hexane was added into the separating funnel (hexane/methanol in the ratio 1:1 (v/v)) and the methanol layer was collected. The process was repeated several times. Methanol was removed using a rotary evaporator to obtain the propolis extract. The propolis extract was dissolved in methanol and analyzed by HPLC to determine the amount of α-mangostin in the extract.

HPLC analysis

High performance liquid chromatography (HPLC) analysis was performed on an Agilent instrument with a UV detector (Agilent 1260 Infinity). The analytical column was a reversed phase C-18 (250 x4.6 mm i.d., particle size 5 µm, from Hypersil) connected to a C-18 guard column. HPLC analysis was performed using a mobile phase system as previously reported.[16] The mobile phase system consisted of methanol (Solvent A) and 0.2% (v/v) formic acid in water (Solvent B). A gradient elution program was used. At 0 to 10 min, solvent A increased linearly from 75% to 90%. At 10 to 15 min, solvent A changed linearly from 90% to 100%. At 15 to 25 min, solvent A was fixed at 100%. After 25 min, the mobile phase composition returned to its initial condition for 10 min. The flow rate was fixed at 1 ml/min and the injection volume was 10 µl. The UV detector was set at 245 nm.

Determination of antimicrobial susceptibility of the propolis extract against *S. aureus*

Bacteria samples in nutrient broth were diluted with a sterile saline solution to achieve a concentration of 1 x 10^8 CFU/ml by comparison with 0.5 McFarland standard. The bacteria suspension was spread over the agar surface using a cotton swab. The *α*-mangostin and the propolis extract discs were placed on the surface of MHA. The plate was incubated at 35°C for 18 h. The size of the clear zone (mm) was measured. The negative control was a blank disc. The vehicle control was methanol and the disc with methanol was left to dry before being placed onto the agar surface. The positive controls were oxacillin (1 µg) disc and tetracycline (30 µg) disc (n = 6).

Determination of MIC and MBC of the propolis extract against *S. aureus*

Determination of the MIC and MBC values of the propolis extract against *S. aureus* was performed by broth microdilution method.[25] Bacteria suspensions were added into the serial dilutions of the propolis extracts or *α*-mangostin (as standard) in MHB in 96-well microplates. The final concentrations of *α*-mangostin standard in the wells ranged from 0.38 to 24.00 µg/ml. The final concentrations of the propolis extract ranged from 0.19 to 48.96 µg/ml. *α*-Mangostin and the propolis extract were dissolved with methanol. The vehicle control was methanol (a final concentration of 2.5% (v/v)), and the positive control was chloramphenicol (a final concentration of 16 µg/ml). The plates were incubated at 35°C for 20 h. The lowest concentration of the propolis extract that showed no visible growth was determined as MIC. The assay of MBC was performed by sub-culturing the samples from the wells that did not show bacterial

growth in TSA. The lowest concentration that did not show bacterial growth in TSA was determined as MBC. The experiments were *done in triplicate*.

Preparation of propolis cream formulation and cream base formulation

The formulation was prepared by heating the oil phase to 75°C and the water phase to 72-73°C. The components of the oil phase and the water phase are shown in Table 1. Glyceryl monostearate (GMS) and Tween 20 or Tween 60 were used as emulsifying agents. The water phase was added to the oil phase with a mechanical stirrer until the temperature dropped to room temperature to form semisolid cream. The propolis cream formulations were selected by centrifugation test. The formulation that did not show phase separation was further investigated. The cream base formulation was prepared without adding the propolis extract and was used as a control for antibacterial study.

Evaluation of propolis cream formulation

Spreadability

The cream sample was weighed (1g) and placed between two glass plates. Twenty-five grams of weight were put onto the top plate and left there for one minute.[26] The spreadability factor (*Sf*) was calculated.[27]

$$Sf = A/W$$

Where, *Sf* is spreadability factor, *A* is total area (mm^2) and *W* is total weight (g).

Determination of pH

Five grams of cream formulation were diluted to 10 ml with deionized water in a volumetric flask. The pH was recorded using a pH meter.

Centrifugation test

Five grams of cream formulation were centrifuged at 4,500 rpm, at 20°C for 20 min to determine phase separation.

Table 1 Composition of the propolis cream formulation

Component	Quantity (% w/w)				
	F1	F2	F3	F4	Cream base
Oil phase:					
Stearyl alcohol	5.0	5.0	5.0	5.0	5.0
Petrolatum	5.0	5.0	5.0	5.0	5.0
Spermaceti	4.0	4.0	4.0	4.0	4.0
Cetyl alcohol	3.0	3.0	3.0	3.0	3.0
Jojoba oil	5.0	5.0	5.0	5.0	5.0
Isopropyl myristate	4.0	4.0	4.0	4.0	4.0
Butylated hydroxytoluene	0.1	0.1	0.1	0.1	0.1
Propolis extract	5.0	5.0	5.0	5.0	-
GMS	4.2	2.9	2.2	3.7	3.7
Water phase:					
Propylene glycol	10.0	10.0	10.0	10.0	10.0
Tween 20	2.8	4.1	-	-	-
Tween 60	-	-	4.8	3.3	3.3
Water	51.9	51.9	51.9	51.9	56.9

Viscosity measurement and rheological behavior determination

Viscosity and rheological behavior were analyzed using a rheometer (DSR Malvern-Kinexus Pro, USA). The analysis was carried out at 25°C. Measuring systems were cone and plate combinations (CP4/40). For viscosity measurement, the shear rate was fixed at 5 s^{-1}. Rheological behavior determination was carried out using shear rate of 0.1-10 s^{-1}. Shear rate sweep (up and down) was performed. All measurements were *done in triplicate*.

Evaluation of the antibacterial activity of the propolis cream formulation against *S. aureus*

The propolis cream formulation was investigated for antibacterial activity against *S. aureus* by the pour plate method.[28] The propolis cream formulation and the cream base formulation were diluted with TSA to be 4-fold and 8-fold dilutions. The mixture was transferred onto a petri dish and allowed to solidify. The suspension (0.1 ml) of *S. aureus* was spread over the agar surface using an L-shape glass rod. The plates were incubated at 37°C for 24 h. The growth of bacteria was investigated by using an inoculating loop to streak the surface of the incubated media and transfer it onto new TSA media. The plates were incubated at 37°C for 24 h. The cream base was tested and compared with the propolis cream formulation. The agar media was used as a negative control. The experiments were *done in triplicate*.

Stability study of the propolis cream formulation

The propolis cream formulation F4 was stored at 30 ± 2°C, 75 ± 5% RH and 40 ± 2°C, 75 ± 5% RH for 15, 30 and 45 days. The α-mangostin content in the propolis cream formulation F4 was determined by HPLC.

Statistical analysis

The results were presented as mean ± standard deviation (SD). The statistical analysis of the data was performed using one-way ANOVA (SPSS software version 21). The level of significance was considered as $p < 0.05$.

Results and Discussion

Preparation of the propolis extract

The stingless bees' propolis was extracted with ethanol. After the solvent was removed by a rotary evaporator, a brown crude sample was obtained, which was subsequently dissolved in methanol, and then partitioned using hexane. The methanol layer was collected. Methanol was removed resulting in the propolis extract. The appearance of the extract was a brown sticky mass with an aromatic honey smell. The yield was 14.15% (w/w). The result showed that the extraction method using ethanol as a solvent following by methanol-hexane partition was successful. The propolis extract was used as an active ingredient in the cream formulation; therefore, the amount of α-mangostin was quantified. The amount of α-mangostin in the propolis extract was found to be 13.40±0.03%. The extraction method using ethanol (absolute ethanol) gave the extract with higher content of α-mangostin compared with previously reported methods using 80% ethanol and ethyl acetate.[16,18] The result revealed the extraction method using ethanol as a solvent and the methanol-hexane partition technique was effective.

Antimicrobial susceptibility of the propolis extract against *S. aureus*

The antibacterial activity of the propolis extract against *S. aureus* was determined by disc diffusion method. The result was shown in Figure 1. The positive controls were oxacillin disc (1 µg) and tetracycline disc (30 µg). The inhibition zone of tetracycline and oxacillin were 24.17±0.72 mm and 27.67±0.89 mm, respectively, indicating that the tested bacteria was sensitive to these antibacterial agents.[29] The 62.5, 125 and 250 µg of α-mangostin discs showed a similar size to the inhibition zone ($p > 0.05$), which might have been caused by the limitation of diffability of α-mangostin in agar media. The 35 µg-propolis extract disc showed a

smaller inhibition zone size than 70, 140 and 280 µg-propolis extract discs ($p < 0.05$); whereas, 70 µg and 140 µg discs showed a similar size to the inhibition zone ($p > 0.05$). The 280 µg-propolis extract disc exhibited a significantly wider inhibition zone ($p < 0.05$) compared with the other tested discs. The results revealed that the propolis extract has an antibacterial activity against *S. aureus*.

MIC and MBC of the propolis extract against *S. aureus*

The MIC and MBC against *S. aureus* were investigated using the broth microdilution method. The MIC and MBC values of α-mangostin were 1.5 and 3.0 µg/ml, respectively. The previous report revealed the MIC value of α-mangostin ranged from 1.25-6.25 µg/ml for methicillin-sensitive *S. aureus* and was around 1.57-12.50 µg/ml for methicillin-resistance *S. aureus*.[30] Another study showed that the MIC and MBC values of α-mangostin for methicillin-resistance *S. aureus* were 1.95 and 3.91 µg/ml, respectively.[31] The MIC and MBC values of the propolis extract were 3.06 and 6.12 µg/ml, respectively. The propolis extract's MBC was two-fold greater than the MIC, which was not more than four times the MIC, indicating that the propolis extract exerted a bactericidal effect.[32] α-Mangostin inhibited *S. aureus* growth by disrupting the cytoplasmic membrane and preventing biofilm formation.[33,34] The propolis extract had a potent antibacterial activity against *S. aureus* that may not be only from α-mangostin but from the combination of other compounds in the entire propolis extract exerting the synergistic antibacterial activity.

Preparation of the propolis cream formulation

The propolis cream formulation containing 5% propolis extract was prepared using GMS and Tween 20 or Tween 60 as emulsifying agents (F1-F4). After centrifugation, the propolis cream formulation F1, F2 and F3 showed phase separation; whereas, F4 was homogenous after the centrifugation test (Table 2). The propolis cream formulation had a smooth texture with good appearance; so, it was chosen for further investigation (Table 3 and Figure 2). The cream base formulation with the same ingredients as the propolis cream formulation F4 was prepared without adding the propolis extract with the propolis cream for comparison. The propolis cream formulation F4 was yellow due to the brown color of the stingless bees propolis extract. The propolis cream formulation F4 was less viscous than the cream base formulation ($p < 0.05$). The cream base formulation had a higher spreadability factor indicating that it is easier to apply on the skin than the propolis cream formulation F4.

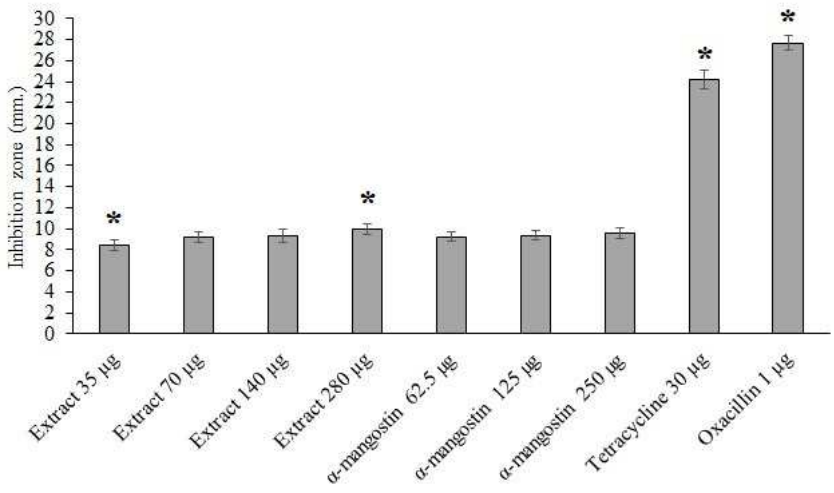

Figure 1 Antibacterial activity (shown as diameter (mm) of inhibition zone) of the propolis extract, α-mangostin standard, oxacillin and tetracycline against *S. aureus*.

Table 2 Physical appearances of the propolis cream formulation F1, F2, F3, F4 and the cream base formulation

	Propolis cream formulation				Cream base
	F1	F2	F3	F4	
Physical appearance	Opaque Yellow	Opaque Yellow	Opaque Yellow	Opaque Yellow	Opaque White
Texture	Not smooth	Not smooth	Not smooth	Smooth	Smooth
Greasiness	Greasy	Greasy	Greasy	Not greasy	Not greasy
Homogeneity	Yes	Yes	Yes	Yes	Yes
Phase separation	Yes	Yes	Yes	No	No

Table 3 Physicochemical properties of the propolis cream formulation F4 and the cream base formulation

	Propolis cream formulation F4	Cream base formulation
pH	7.26 ± 0.17	7.73 ± 0.04 White
Spreadability factor (mm^2/g)	68.86 ± 3.90	85.52 ± 3.82
Viscosity (cPs)	36,948 ± 5,997	55,260 ± 3,624

Figure 2 The cream base formulation (1) and the propolis cream formulation F4 (2)

The rheological behaviors of the propolis cream formulation F4 and the cream base formulation were illustrated in Figure 3. The result showed that both formulations had pseudoplastic and thixotropic properties. The presence of the propolis extract in the formulation resulted in a decrease in size of thixotropic loop (an area between curves) indicating that the structure could rebuild more quickly after the load was removed.

Antibacterial activity of the propolis cream formulation against *S. aureus*

Antibacterial activity of the propolis cream formulation F4 against *S. aureus* was investigated using the pour plate method. The propolis cream formulation was diluted with tryptic soy agar (TSA) media to be 4-fold and 8-fold dilutions. The inoculum of *S. aureus* was spread over the agar surface. The growth of bacteria on the agar media

could not be counted as colony forming units due to the turbidity of the mixture of the media and the cream formulation. Therefore, an inoculating loop was used to streak the surface of the incubated media and transfer it onto new TSA media. The growth of *S. aureus* was illustrated in Figure 3. The results showed that an 8-fold dilution of the propolis cream could not kill bacteria whereas a 4-fold dilution of propolis cream showed bactericidal activity against *S. aureus*.

Stability of the propolis cream formulation

The *α*-mangostin content in the propolis cream formulation F4 was determined by HPLC after the propolis cream formulation F4 was stored at 30 ± 2°C, 75 ± 5%RH and 40 ± 2°C, 75 ± 5%RH for 15, 30 and 45 days. The recovery of *α*-mangostin in the propolis cream formulation was shown in Table 4 indicating that *α*-mangostin in the propolis cream formulation was stable. The propolis cream formulation had a homogeneous texture without phase separation.

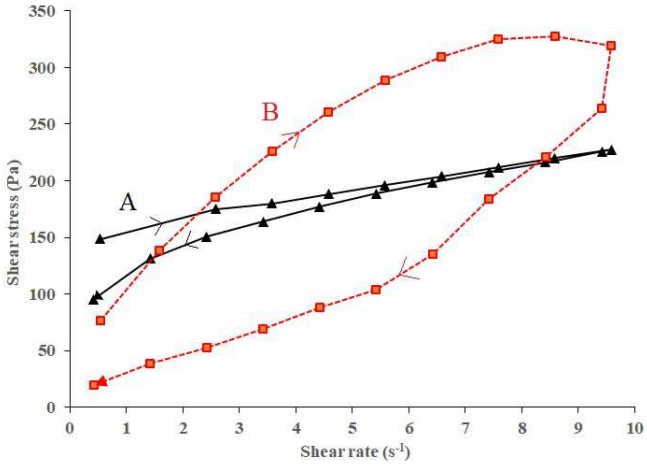

Figure 3 Rheological behaviors of the propolis cream formulation F4 (A) and the cream base formulation (B)

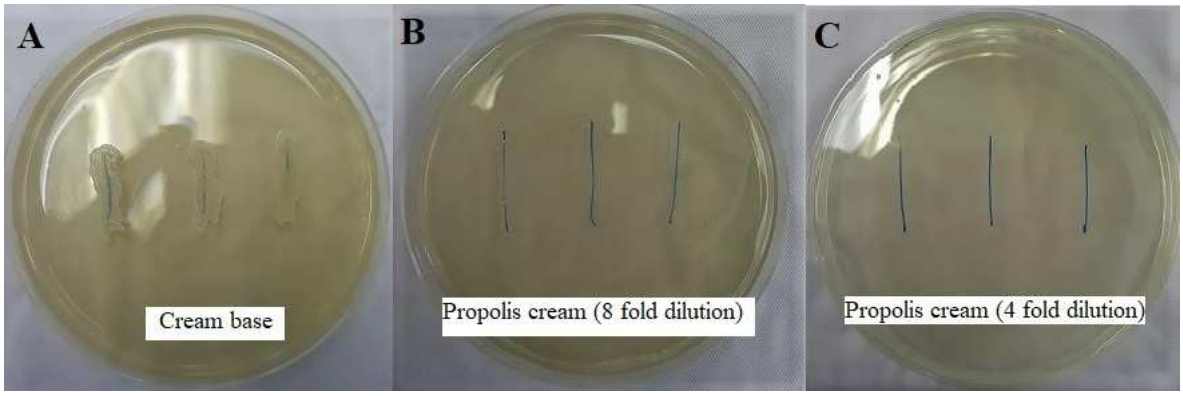

Figure 4 The growth of *S. aureus* on TSA; cream base (A), 8-fold dilution of the propolis cream formulation F4 (B) and 4-fold dilution of the propolis cream formulation (C)

Table 4 Recovery of *α*-mangostin in the propolis cream formulation F4 after the formulation was stored at 30 ± 2°C, 75 ± 5%RH and 40 ± 2°C, 75 ± 5%RH for 15 days, 30 days and 45 days.

Storage condition	Recovery of *α*-mangostin (%) in the propolis cream formulation			
	Day 0	Day 15	Day 30	Day 45
30 ± 2°C, 75 ± 5%RH	99.43 ± 0.87	98.71 ± 0.42	99.01 ± 0.32	99.46 ± 0.96
40 ± 2°C, 75 ± 5%RH	99.43 ± 0.87	99.23 ± 0.66	98.30 ± 1.00	98.82 ± 0.65

Conclusions

The extraction method of the stingless bee propolis using ethanol as a solvent and partitioning it with methanol and hexane provided a high percentage yield and a high content of *α*-mangostin in the extract. The propolis extract showed potent antibacterial activity against *S. aureus* with MIC of 3.06 µg/ml and MBC of 6.12 µg/ml. The 4-fold dilution of propolis cream formulation containing 5% propolis extract showed a bactericidal effect against *S. aureus*. The propolis extract of *Tetragonula pagdeni* (Schwarz) could be a promising candidate for topical antibacterial applications.

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