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Cytotoxicity and Antimicrobial Activity of Propolis from *Trigona itama* Stingless Bees against *Staphylococcus aureus* and *Escherichia coli*

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Abstract

Trigona itama is a stingless bees that produce honey which has been used widely as a traditional medicine. Due to limited research and knowledge on *T. itama* propolis, it becomes less popular in industrial production than honeybee propolis. The aim of this study was to identify the antibacterial activity and toxicity level in hexane, ethyl acetate and methanol extracts of *T.itama* propolis. *T.itama* propolis crude extract was tested for antibacterial activity by using disk diffusion method. The antibacterial activities were assessed according to the inhibitory zone of agar medium with sample concentration of 1000 µg/mL, 750 µg/mL and 500 µg/mL. The result from this study revealed that all *T. itama* propolis crude extract shows presence of inhibition zone against *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922. The brine shrimp lethality test was used to monitor the toxicity level of *T. itama* propolis where methanol, ethyl acetate and hexane fraction showed low activity of toxicity where all crude extract LC_{50} showed above 250 µg/mL. It could be concluded from this study that extract from *T. itama* propolis possessed antimicrobial activity and low level of toxicity.

Keywords: *Escherichia coli*, propolis, stingless bees, *Staphylococcus aureus, Trigona itama*

Sitotoksisitas dan Aktivitas Antimikroba Propolis dari *Trigona itama* Lebah Tanpa Sengat terhadap *Staphylococcus aureus* dan *Escherichia coli*

Abstrak

Trigona itama merupakan jenis lebah tidak menyengat yang menghasilkan madu yang telah digunakan secara luas sebagai obat tradisional. Terbatasnya penelitian dan pengetahuan tentang *T. itama* propolis, ia menjadi kurang populer dalam produksi industri daripada propolis lebah madu. Tujuan penelitian ini adalah untuk mengetahui tingkat aktivitas antibakteri dan toksisitas ekstrak heksana, etil asetat dan metanol dari propolis *T.itama*. Ekstrak kasar *T.itama* diuji coba untuk aktivitas antibakteri dengan menggunakan metode disk diffusion. Aktivitas antibakteri ditentukan berdasarkan zona penghambatan media agar dengan konsentrasi sampel 1000 µg/mL, 750 µg mL dan 500 µg/mL. Hasil dari penelitian ini menunjukkan bahwa semua ekstrak kasar *T. itama* propolis menunjukkan adanya zona penghambatan terhadap *Staphylococcus aureus* ATCC 25923 dan *Escherichia coli* ATCC 25922. Uji letalitas artemia digunakan untuk memantau tingkat toksisitas propolis *T. itama* dimana fraksi metanol, etil asetat dan heksana menunjukkan aktivitas toksisitas rendah dimana semua ekstrak kasar LC_{50} menunjukkan di atas 250 µg/mL. Dari penelitian ini dapat disimpulkan bahwa ekstrak dari *T. itama* propolis memiliki aktivitas antimikroba dan tingkat toksisitas rendah.

Kata kunci: *Escherichia coli*, lebah tanpa sengat, propolis, *Staphylococcus aureus, Trigona itama*

1. Introduction

Drug resistance have become a major problem in developed country due to rapid usage of main drugs to treat infection diseases and unavailability of alternate medicine to overcome antimicrobial resistance. Previous studies showed that the rates of antimicrobial resistance have increased during the past decade and give negative impact to the society, hospital patient and pharmaceutical industry.[1] Demand for safe, new and effective antimicrobial is the major challenges in pharmaceutical and agriculture industry due to increase number of drug resistant microorganism.

Over 500 species of stingless bees are discovered and most of stingless bees can be located in the tropical and subtropical region.[2] *Trigona itama* is a stingless bees that categorized under the tribe of Meliponini and belong to *Trigona* genus. *T. itama* is also known as "lebah kelulut" by Malaysian community. Propolis was used by stingless bees as a building structure, defense system and sealing agent for the extra space surrounding the hexagon-shaped nest combs.[3] Propolis helped in preservation of stingless bees honey from microbial spoilage.[4] Antimicrobial properties of propolis showed that propolis have high level of flavonoid.[5] Stingless bees propolis have been used as a traditional medicine to treat skin wounds, burn and infection.[6] Previous research showed that propolis consisted of versatile phytochemical constituent that brought many therapeutic benefits to human, such as antiviral, antitumor, antiulcer and antibacterial.[7] This study was performed to investigate antimicrobial and cytotoxicity activity of *T. itama* propolis.

2. Materials and Methods

2.1. Source of microorganism

Staphylococcus aureus (ATCC 25923) and *Escherichia coli* (ATCC 25922) bacteria was collected from UNIMAS Microbiology Laboratory. All the microorganism was preserved and stored in selected agar.

2.2. Bacteria media

Bacterial media Mueller Hinton was used in antibacterial assay. Mueller Hinton media was mixed with distilled water and sterilized in autoclave at 15 lb pressure for 15 minutes. The sterilized media was poured into petri dishes and allow for solidification.

2.3. Plant material and extraction

T. itama propolis was collected from the local stingless bee keeper (Mohammad Faizol Bin Rupni) in Kuching, Sarawak. *T. itama* propolis was weighted and grounded using industrial blender. 786 gram of grounded propolis was soaked with 1000 mL of hexane and left for 72 hours in an orbital shaker at 20 shakes per minute. After 72 hours, the extracts was filtered using filter paper (0.040-0.063mm) and concentrated using a rotary evaporator at 65^{O}C to obtain brown colour crude extract. After hexane crude extract was obtained, the remaining propolis was extracted in the same manners using ethyl acetate. Black colour ethyl acetate extract was obtained. Remaining propolis was extracted with the same previous procedure with methanol and brown *T. itama* propolis extract was obtained. Each crude extract was weighed, its character recorded, wrapped in aluminum foil, stored at -2^{O}C and the percentage of yield was calculated.

2.4. Kirby Bauer Test

T. itama propolis crude extract was tested for antibacterial activity by using disk diffusion method (Bauer, 1966)[8] to determine the presence of antimicrobial activity in *T. itama* propolis. *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 strains were cultured on the blood agar plate and incubated for 18 to 24 hours at 37^{O}C. A single colony was then cultured in 10 mL of Mueller Hinton Broth for 24 hours at 37^{O}C in incubator. Density of bacteria was standardized to 0.5 McFarland standard, which is equivalent to 1×10^8 CFU/mL by adjusting the absorbance value within range from 0.08 to 0.10 at 625nm measured using spectrophotometer.[9] Cotton swab was dipped into bacterial suspension and rotated against the side of the tube with firm pressure to remove excess fluid. The swab was streaked over the entire plate for

three times and each time the MHA plate was rotated 90^{O} to ensure constant distribution of bacterial suspension on the MHA surface.[10] The plates were dried for 15 minutes and then proceed with sensitivity test. Sample was dissolve in extract solvent for preparation of different concentration ranging from 500, 750 and 1000 µg/mL. 20 µL sample of concentration 1000, 750, and 500 µg/mL were treated on the blank disc. Impregnated disc with series of propolis extract were placed on the Mueller Hinton agar surface using sterile forceps. Each MHA plate consists of 5 disc. 1 positive control, 1 negative control and 3 treated disc. 10 µg benzylpenicillin disk was used as a positive control for *S. aureus* ATCC 25923 and 1 IU of imipenem was used as a positive control for *E. coli* ATCC 25922. Extract solvent was used as negative control. The plate was incubated at 37^{O}C temperature for 24 hours for bacterial growth activity. After the incubation, the plates were examined for inhibition zone. The zone of inhibition was determined by measuring the diameter of clear zones around the disk where inhibited the growth of bacteria. Three replicates were performed in this test to ensure reliability of test.

2.5. Brine shrimp lethality

Brine shrimp lethality,[11] was used in this study to determine the toxic potentiality of the different fraction of the *T.itama* propolis. Brine shrimp (*Artemia salina*) eggs were hatched in a beaker filled with saltwater and constant oxygen supply for 48 hours. After 24 hours of hatching, shrimp larvae was used as a test organisms. 6 mg of each of the extracts was dissolved in 6mL of methanol solvent. From this solution, (10 µg/mL, 20 µg/mL, 100 µg/mL, 200 µg/mL, 600 µg/mL, 1000 µg/mL) were transferred into each well. Methanol solvent was used as a control. Six graded concentration methanol solvent was transferred into each of multi well. Well was left for evaporation for 24 hours inside the fume hood. 50 µl dimethyl sulfoxide, 1 mL of salt water and 10 brine shrimp were transferred to each well using pipette. After 24 hours of incubation, shrimp larvae were examined using magnifying glass and the number of survived shrimp was counted as a data. LC_{50} of the sample was obtained by plotting percentage of the dead shrimp against the logarithm of the sample concentration.

2.6. Data and statistical analyses

Toxicity of *T. itama* crude extract was analyzed by plotting graph of mortality percentage versus log concentration using GraphPad 6.0 Prism (Sigmodal mode) to determine LC_{50} for *T. itama* crude extract. Concentration curves of inhibition was calculated using GraphPad Prism 6.0 (linear regression).

3. Result

3.1. Characteristic and yield of the obtained propolis crude extract

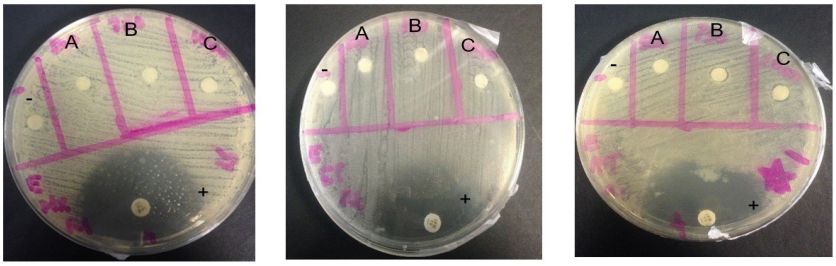
Table 1 shows the characteristic, weight and yield percentage of the obtained propolis crude extract. Hexane crude extract showed light brown colour, weight of crude extract was 62.12 g and yeild percentage was 7.89%. Ethyl acetate crude extract black colour could be observed, weight of crude extract 47.54 g and yeild percentage was 6.04%. Methanol crude extract dark brown colour could be observed, weight of crude extract 56.15g and yeild percentage was 7.14%.

3.2. Antibacterial test

Figure 1 and Table 2 shows the inhibition zone of *T. propolis* extract (500, 750, and 1000 µg/mL) on *E.coli* plates. 10 µg imipenem was used as positive control.

Tabel 1. Characteristic and yield of propolis crude extracts

Crude extract	Character	Weight	Yield%
Hexane	Light brown and sticky solid	62.12g	7.89
Ethyl acetate	Black and sticky solid	47.54g	6.04
Methanol	Dark brown and sticky solid	56.15g	7.14



(i) Hexane extract (ii) Ethyl acetate extract (iii) Methanol extract

Figure 1. Inhibition zone of *T. itama* propolis extract i, ii and iii on *E.coli* plates. (A) 500 µg/mL, (B) 750 µg/mL, (C) 1000 µg/mL, (+) Positive control and, (-) Negative control.

All *T. itama* propolis crude extract showed presence of inhibition zone against *E.coli* and no inhibition zone could be observed at concetration of 500 µg/mL. *T. itama* propolis. The mean of inhibition zone for *E.coli* by *T. itama* propolis crude was compared with inhibition zone of standard drug and negative control. The mean of inhibition was recorded as data and was analyzed using GraphPad 6.0 Nonlinear regression mode (Figure 3A).

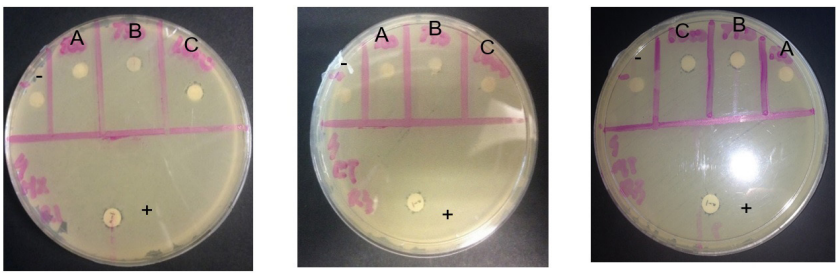
Figure 2 and Table 3 show the inhibition zone of *T. itama* propolis extract (500 µg/mL, 750 µg/mL and 1000 µg/mL) on *S. aureus*. 10 µg imipenem was used as positive control. All *T. itama* propolis crude extract showed presence of inhibition zone against *S. aureus* and no inhibition zone can be observed at concentration of 500 µg/mL *T. itama* propolis. The mean of inhibition zone for *S. aureus* by *T. itama* propolis crude was compared with inhibition zone of standard drug and negative control. The mean of inhibition was recorded as data and was analyzed using GraphPad 6.0 Nonlinear regression mode (Figure 3B).

3.3. Brine shrimp lethality

Table 4 and Figure 4 show toxicity percentage of *T. itama* propolis extract. Based on Figure 4 and Table 4, methanol crude extract show high level of toxicity compared to other type of *T. itama* crude extracts and ethyl acetate crude extract showed the lowest level of toxicity level. Table 4 shows toxicity percentage of *T. itama* propolis extracts by plotting percentage of the dead shrimp against the logarithm of the sample concentration.

4. Discussion

Disk diffusion method was performed to identify the antimicrobial activity of *T. itama* propolis against *S. aureus* ATCC 25923 and *E. coli* ATCC 25922. Based on Table 2 and Figure 1, *T. itama* propolis crude extract showed inhibitory activity against *E. coli* ATCC 25922 at concentration of 750 µg/mL and 1000 µg/mL. This indicated that crude extract from *T. itama* were able to inhibit the growth of *E.coli* in MHA plate. Methanol crude extract produced higher rate of inhibition in concentration of 750 µg/mL and 1000 µg/mL compared to other types of crude extract from propolis. Methanol extract of 1000 µg/mL was able to inhibit *E.coli*



(iv) Hexane extract (v) Ethyl acetate extract (vi) Methanol extract

Figure 2. Inhibition zone of T. *itama* propolis extract iv, v and vi on *S. aureus* plates. (A) 500 µg/mL (B) 750 µg/mL (C) 1000 µg/mL (+) Positive control and (-) Negative control.

Tabel 2. The mean of inhibition zone for *E.coli* by *T. itama* propolis crude extracts, standard drug and negative control in disk diffusion test.

Crude extract	Mean zone of Inhibition (MM)				
	Concentration of T. itama propolis extract			Standard antibiotic	Negative control
	500 µg/mL	750 µg/mL	1000 µg/mL		
Hexane	-	4	8	40	-
Ethyl acetate	-	-	5	40	-
Methanol	-	6	10	40	-

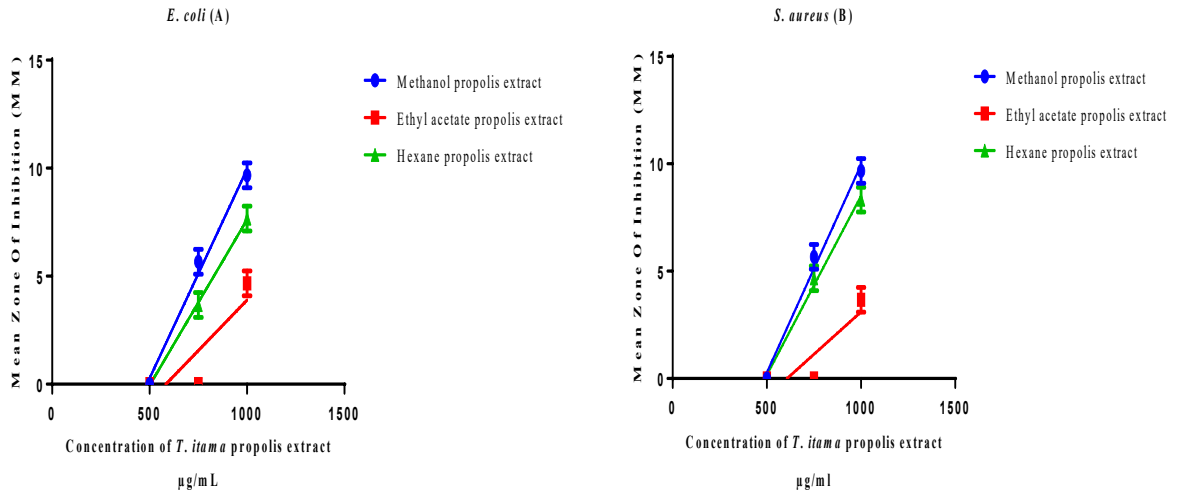
growth by 10 mm of mean zone diameter. Ethyl acetate propolis crude extract showed the lowest zone of inhibition compared to methanol and hexane propolis crude extract for 1000 µg/mL and no inhibition on 750µg/mL. The size of inhibition zone for imipenem 10 µg was 40 mm and this indicate that *E. coli* was not resistant to imipenem antibiotic.

The inhibition zone of the *T. itama* crude extracts, standard drug (antibiotic) and negative control are shown in Figure 2 and Table 3. All *T. itama* propolis crude extract showed presence of inhibition zone against *S. aureus* ATCC 25923. This indicated that *T. itama* propolis extract able to inhibit the growth of *S. aureus*. In 500 µg/mL of all *T. itama* propolis extract showed no inhibition zone. 750 µg/mL and 1000 µg/mL of *T. itama* propolis shows inhibition zone against *S. aureus* ATCC 25923. Methanol crude extract showed the highest inhibition rate in concentration of 750 µg/mL (6 mm) and 1000 µg/mL (10 mm) compared to hexane and ethyl acetate crude extract. On the opposite side, ethyl acetate showed the lowest value of inhibition zone at 1000 µg/mL and no inhibition on 750 µg/mL of *T. itama* propolis extract. Benzylpenicilin showed presence of inhibition zone against *S. aureus* ATCC 25923.

This study showed that *T. itama* propolis crude extracts were able to stop the growth of gram positive bacteria and gram negative bacteria. Campos et al., (2014) discovered that propolis from Brazil had broad spectrum antimicrobial properties.[12] Based on Choudhari et al., 2012 research, ethanolic extract propolis from *Trigona sp* showed potent antimicrobial activity against gram positive and gram negative bacteria as well as other microorganism such as yeast.[13] Previous research showed that propolis from *Tetragonula iridipennis* stingless bees (Indian Stingless bees) was able to inhibit the growth of fungus such as *Aspergillus niger, Candida albicans* and *Trichophyton rubrum* but unable to inhibit the growth of *S. aureus* and *E. coli* at concentration level of 500, 750 and 1000 µg/mL.[14] Based on previous research, it was reported that propolis collected from different regions and different solvents extraction gave different antimicrobial action.[14] The different results were due to different botanical origin. Other product such as honey from *Trigon sp* was also able to inhibit the growth of bacteria. Based on previous research by Boorn (2010), *Trigona sp* honey was able to inhibit variety of bacteria species such as *Salmonella typhimurium, Enterococcus faecalis* and *Staphylococcus epidermidis*.[4] According

Tabel 3. The mean of inhibition zone for *S. aureus* by *T. itama* propolis crude extracts, standard drug and negative control in disk diffusion test.

Crude extract	Mean zone of Inhibition (MM)				
	Concentration of T. itama propolis extract			Standard antibiotic	Negative control
	500 µg/mL	750 µg/mL	1000 µg/mL		
Hexane	-	5	8	7	-
Ethyl acetate	-	-	4	7	-
Methanol	-	6	10	7	-



(A) (B)

Figure 3. Statistical analysis of data was analyzed using GraphPad 6.0 Nonlinear regression mode. Figure 3 (A) and (B) shows that all *T. itama* propolis crude extract able to produce zone of inhibition for *E.coli* and *S. aureus*. 750 and 1000 µg/mL of *T. itama* propolis extract produce inhibition zone. Methanol crude extract produced higher mean of inhibition compared to hexane and ethyl acetate crude extract.

to Boorn (2010), the size of inhibition zone against *S. aureus* NCTC 6571 was 23.3 mm.[4]

Antimicrobial activity of propolis has been linked to the contents of flavonoids and sesquiterpenes.[15] Phytochemical compound such as flavanones give anti-staphylococcal activity of propolis and other natural product compounds such as abietic acid and phenolics might influence antimicrobial effect of propolis.[16] Antimicrobial compound in *T. itama* propolis played an important part by maintaining low level of bacteria and fungi in the hive.[17] Further research needs to be performed to determine the compound that gives antimicrobial effect and mechanisms of antimicrobial activity from the compound.

Brine shrimp lethality assay was used in this study as the primary screening of 3 types of *T. itama* propolis crude extracts to monitor toxicity level towards the brine shrimp. The brine shrimps lethality supported the antibacterial activities of *T. itama* propolis on tested organisms observed in this study. The results from this test indicated the ability of the propolis extract to kill 50 percent of brine shrimp larvae. Based on Figure 4 and

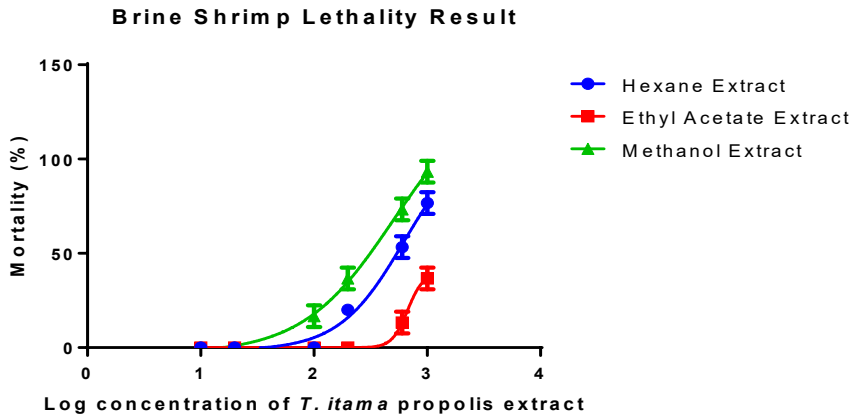


Figure 4. Toxicity of *T. itama* crude extract was analyzed by plotting graph of mortality percentage versus log concentration using GraphPad Prism 6 (Sigmodal mode).

Table 4. Toxicity percentage of *T. itama* propolis extracts

Sample	Average death of Artemia salina (%)						LC_{50} µg/mL
Crude extract	Concentration (ppm)						
	10	20	100	200	600	1000	
Hexane	0	0	0	20.00	53.33	73.3	623.7
Ethyl acetate	0	0	0	0	13.3	37.3	670.8
Methanol	0	0	16.67	36.67	70.33	93.33	501.2

Table 4, methanol crude extract showed high level of toxicity compared monitor to other type of *T. itama* crude extracts. At 1000 ppm, 93% of average death of brine shrimp was observed and LC_{50} for methanol crude extract is 501.2 ppm. Hexane crude extract shows intermediate toxicity effect compare to other *T. itama* propolis extract, 50% percent death of brine shrimp in concentration of 600 ppm and at 1000 ppm more than 70% percent of brine shrimp could not survive. This result indicated that LC_{50} for hexane is 623.7 ppm. Ethyl acetate crude showed the lowest level of toxicity compared to *T. itama* propolis extract, where at 1000 ppm concentration was only observed 37.3% average death of brine shrimp. *T. itama* propolis crude extract showed low level of toxicity because based on previous studies sample with LC_{50} values lower than 250 were considered significantly active for toxicity effect.[18] The variation of toxicity result may due to the difference amount of phytochemical compounds such as flavonoid in *T. itama* propolis extract that might be able to produce toxicity effect to the brine shrimp.

5. Conclusion

This study was performed to investigate antimicrobial activity and toxicity level of *T. itama* propolis crude extracts. The results of this study showed that all *T. itama* propolis crude extracts were able to produce zone of inhibition against *E.coli* and *S. aureus*. Concentration of 750 µg/mL and 1000 µg/mL of *T. itama* propolis extract produced inhibition zone and no inhibition zone at concentration of 500 µg/mL. The brine shrimp lethality test was used to monitor the toxicity level of *T. itama* propolis where methanol, ethyl acetate and hexane fraction showed low activity of toxicity where all crude extract LC_{50} shows above 250 ppm.

6. Acknowledgement

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References

1. National Nosocomial Infections Surveillance (NNIS). System report, data summary from January 1992-June 2011, Am J Infect Control 2011, vol. 29 (pg. 404-21)
2. Rasmussen C, Cameron SA. A molecular phylogeny of the Old World stingless bees (Hymenoptera: Apidae: Meliponini) and the non-monophyly of the large genus Trigona. Systematic Entomology. 2007 Jan 1;32(1):26-39.
3. Bankova V. Chemical diversity of propolis makes it a valuable source of new biologically active compounds. Journal of ApiProduct and ApiMedical Science. 2009;1(2):23-8.
4. Boorn KL, Khor YY, Sweetman E, Tan F, Heard TA, Hammer KA. Antimicrobial activity of honey from the stingless bee Trigona carbonaria determined by agar diffusion, agar dilution, broth microdilution and time-kill methodology. Journal of applied microbiology. 2010 May 1;108(5):1534-43.
5. Salomão K, Dantas AP, Borba CM, Campos LC, Machado DG, Aquino Neto FR, Castro SL. Chemical composition and microbicidal activity of extracts from Brazilian and Bulgarian propolis. Letters in Applied Microbiology. 2004 Jan 1;38(2):87-92.
6. Barroso PR, Lopes-Rocha R, Pereira EM,

Marinho SA, de Miranda JL. Effect of propolis on mast cells in wound healing. Inflammopharmacol. 2012. 20: 289-294. PubMed: 2219947.

7. Machado BA, Silva RP, de Abreu Barreto G, Costa SS, da Silva DF, Brandão HN, da Rocha JL, Dellagostin OA, Henriques JA, Umsza-Guez MA, Padilha FF. Chemical composition and biological activity of extracts obtained by supercritical extraction and ethanolic extraction of brown, green and red propolis derived from different geographic regions in Brazil. PloS one. 2016 Jan 8;11(1):e0145954.
8. Bauer AW, Kirby WM, Sherris JC, Turck M. Antibiotic susceptibility testing by a standardized single disk method. American journal of clinical pathology. 1966 Apr;45(4):493.
9. Hudzicki J. Kirby-Bauer Disk Diffusion Susceptibility Test Protocol. ASM Microbe Library. American Society for Microbiology 2009
10. Wayne PA. Clinical and laboratory standards institute. Performance standards for antimicrobial susceptibility testing. 2007;17.
11. Meyer BN, Ferrigni NR, Putnam JE, Jacobsen LB, Nichols DJ, McLaughlin JL. Brine shrimp: a convenient general bioassay for active plant constituents. Planta medica. 1982 May;45(05):314.
12. Campos JF, dos Santos UP, Macorini LF, de Melo AM, Balestieri JB, Paredes-Gamero EJ, Cardoso CA, de Picoli Souza K, dos Santos EL. Antimicrobial, antioxidant and cytotoxic activities of propolis from Melipona orbignyi (Hymenoptera, Apidae). Food and Chemical Toxicology. 2014 Mar 31;65:374-80.
13. Choudhari, M.K., Punekar, S.A., Ranade, R.V., Paknikar, K.M. Antimicrobial 409 activity of stingless bee (Trigona sp.) propolis used in the folk medicine of 410 Western Maharashtra, India. J. Ethnopharmacol. 2012. 141, 363–367.
14. Kothai S, Jayanthi B. Anticancer activity of silver nano particles biosynthesized using stingless bee propolis (Tetragonula iridipennis) of Tamilnadu. Asian Journal of Biomedical and Pharmaceutical Sciences. 2015 Jan 28;4(40).
15. Petrova A, Popova M, Kuzmanova C, Tsvetkova I, Naydenski H, Muli E, Bankova V. New biologically active compounds from Kenyan propolis. Fitoterapia. 2010 Sep 30;81(6):509-14.
16. Häberlein H, Tschiersch KP. On the occurrence of methylated and methoxylated flavonoids in Leptospermum scoparium. Biochemical Systematics and Ecology. 1998 Jan 15;26(1):97-103.
17. Popova M, Reyes M, Le Conte Y. Propolis chemical composition and honeybee resistance against Varroa destructor. Nat Prod Res. 2014. 1–7.
18. Rieser MJ, Gu ZM, Fang XP, Zeng L, Wood KV, McLaughlin JL. Five novel mono-tetrahydrofuran ring acetogenins from the seeds of Annona muricata. Journal of Natural Products. 1996. 22;59(2):100-8.