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Antioxidant and Antibacterial Activity of Propolis Extract from Stingless Bee in Malaysia

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Abstract: Propolis is a resinous natural product by honeybees, rich in biologically active compound. Biochemical composition and activities of propolis critically depend on the geographical region and plant source. Therefore, the research was conducted to extract and characterize "propolis" from Malaysian stingless bee. Based on these observations, aims of the research were to extract the components in propolis, analyze the components and functional groups, and evaluate the anti-oxidant and antibacterial activity of propolis. Propolis was extracted using ethanol as solvent. The yield has sweet balsamic aroma and gold in color. The ethanolic extraction of propolis was analyzed using FTIR. The results of FTIR shows that propolis saturated with various phenolics, flavanols and aromatic compounds. Anti-bacterial test was carried out using agar well diffusion method against *Escherichia coli*, *Staphylococcus aureus*, *Salmonella enterika*, and *Bacillus cereus*. Propolis extract showed highest inhibition towards *S. aureus* and followed by *E. coli*, *B. subtilis*, and *S. enterika* in decreasing order. Anti-bacterial test results show that Malaysian stingless bee propolis can be a very good substitute for Amoxicillin antibiotic. Finally, the anti-oxidant test was conducted using DPPH free radical method, where propolis exhibited an antioxidant property. The antioxidant and antibacterial test results were correlated with compounds found using FTIR. SEM micrograph shows that raw propolis has rugged surfaces covered by layers of wax, extractives, vegetal constituents, resinous substances from plants. Since most results favors to the local stingless bee propolis, therefore antioxidant and antibacterial compounds found in propolis are highly recommended for further application in food or other industries.

Keywords: Propolis, Stingless bee/*Trigona itama*, Antibacterial, Antioxidant

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1. Introduction

Recent investigations indicated that the interest in natural substitutes for chemicals has increased. The demands of healthy alternatives are increasing all over the world, including Malaysia (World Health Organisation, 2001) [1]. This research is focused to one of the most extra-ordinary and abundant natural compounds from Malaysian stingless bee, named propolis. The use of stingless bee (known as "kelulut") and its products is strongly encouraged in Malaysia by Malaysian Agricultural Research and Development Institute (MARDI) [2].

Propolis is a resinous sticky plant substance collected by honeybees, which may include different types of secretions or exudates [3]. Bees use it mainly to cover the hive interior and the breeding cells as well as to repair cracks and fissures. Propolis has been reported to improve human health and to prevent diseases such as inflammation, heart disease, diabetes and even cancer by possessing various biological activities, namely anticancer, antioxidant, anti-inflammatory, antibiotic, antifungal and antihepatotoxic [4].

Propolis is rich in bioactive compounds, made up of flavonoids and phenolic acids as well as their derivatives. However, the biochemical compounds and activities of propolis are critically depend on geographical region of collection and plant source. Although numerous researchers revealed the biological activities of propolis, its composition and contributions to health, information about Malaysian propolis are still limited [4].

Based on the limited information, this study attempts to investigate the components in ethanolic extract of propolis (EEP). The to evaluate the antioxidant and antibacterial activity of EEP against *Staphylococcus aureus (S.aureus)*, *Bacillus cereus (B.cereus)*, *Salmonella enterika (S.enterika)* and *Escherichia coli (E.coli)*.

2. Materials and Methods

2.1 Extraction of propolis

Propolis of Trigona Itama was obtained from MAEPS Kelulut Garden Serdang Selangor, Malaysia. Propolis (20 g) was ground and homogenized with 80% ethanol (80 mL) for 24 hours at room temperature with continuous agitation. After homogenization, sample was filtrated using Whatman paper 1 to obtain 60 mL of clear solution. After the solvent evaporation via rotary evaporator, the yield was 5 mL. The yield was in liquid state, gold color, and released pleasant and balsamic aroma. Next, the solvent was evaporated using a rotary evaporator and propolis extract was used for characterization.

2.2 Scanning electron microscopy (SEM)

Phenom ProX Desktop SEM from Phenom World Eindhoven, Netherlands was used to capture the propolis prior to extraction. A thin film of the sample was prepared on a carbon coated grid by just dropping a very small amount of the sample on the grid, extra solution was removed using a blotting paper and then the film on the SEM grid was allowed to dry by placing it under mercury lamp for 5 mins.

2.3 FTIR Spectrophotometry

The FTIR spectra were recorded using Perkin Elmer Spectrophotometer Version 10.4.3 re-equipped with a full-sized sample compartment and a kinematic base plate. Since the extracted propolis was in liquid form, one drop of sample is placed between two plates where the drop formed a thin film between the plates. The spectroscopic range scanned for each sample was 4000–400 cm^{-1} at room temperature and generally 3 scans were collected per sample.

2.4 Antibacterial activity of propolis

The test was carried out by using a gram-positive (*S. aureus* and *B. subtilis*) and gram-negative (*E. coli* and *S. enterika*) bacteria. The cultures were grown overnight before the test. The antibacterial activities of EEP and raw propolis were tested and compared using agar-well diffusion method based on Venom (2006) [5].

20 µL of suspensions of each bacteria type was spread into respective isolation plates. After 5 minutes, 7 mm diameter well were dug. Every petri dish has 4 test samples which are antibiotic (positive control), raw propolis, one-time diluted EEP and pure EEP. A positive control was prepared from Amoxilin (as Trihydrate), a 500 mg tablet. 75 g of Amoxilin powder was dropped into one well in every petri dish. Plates were sealed, labeled, incubated at 37 °C and diameter of the inhibition zones were measured in millimeters after 24 hours. The antibacterial activity index (%) was then calculated using the following equation (Eq.1):

$$\text{The antibacterial activity index (\%)} = \frac{\textit{Activity of sample} \times 100}{\textit{Activity of antibiotics}} \qquad \text{Eq. 1}$$

2.4 Antioxidant activity of propolis

Antioxidant test was adapted from Moţ, Silaghi-Dumitrescu, & Sârbu (2011) [6]. UV–vis spectra and the absorption time-courses for 2, 2-diphenyl-1-picrylhydrazyl (DPPH) bleaching were recorded on a UV–vis spectrophotometer (Agilent 8453, Waldbronn, Germany) equipped with a diode array detector. The methanol used was of spectrophotometric grade (990 g/L) from Analyticals Carlo Erba (Milano, Italy), (DPPH' 950 g/kg) were collected on propolis extract samples which were diluted 100-times in methanol.

A stock solution of 3.8 mM DPPH in methanol was prepared for each sample. 20 µL of this stock solution was added into 950 µL methanol found in a quartz UV–vis cuvette. After approximately 150 s, 30 µL of 100-times diluted methanol extract of propolis was added to the DPPH solution. The bleaching of DPPH by propolis was monitored spectrophotometrically at λ=520 nm every minute for 1 hour. The anti-oxidant activity was valued in terms of the percentage of reduction of the DPPH, Q,

$$Q = \left(\frac{A_0 - A_c}{A_0}\right) \times 100 \qquad \text{Eq. 2}$$

A_0 is the initial absorbance and A_c is the absorbance with antioxidant sample reactions which were compared at different time.

3. Results and Discussion

3.1 FTIR Spectrum Analysis

The FT-IR spectrum of the EEP (Figure 1), shows significant peaks at 3272 cm^{-1} and 2930cm^{-1} which correspond to the hydrogen bond of O-H and N-H in alcohols, phenols and amides. In addition, the peak at 2856 cm^{-1} is attributed to C-H stretching vibrations in alkyl chain [6]. The peak at 1033 cm^{-1} corresponds to symmetric stretching vibration of C-H interaction bond. The peak 1595 cm^{-1} corresponds to asymmetric C=O frequency and to the overtone frequency of N-H bonds. The peak at 1450 cm^{-1} corresponds to asymmetric C=O and C-N of amines. The peak at 1337 cm^{-1} indicates the presence of symmetric C=O, symmetric C-H and N=O bonds. The peak at 1033 cm^{-1} corresponds to fundamental C-C bond peaks. The early conclusion is that almost all functional groups matched amino acids, lipid and flavonoids.

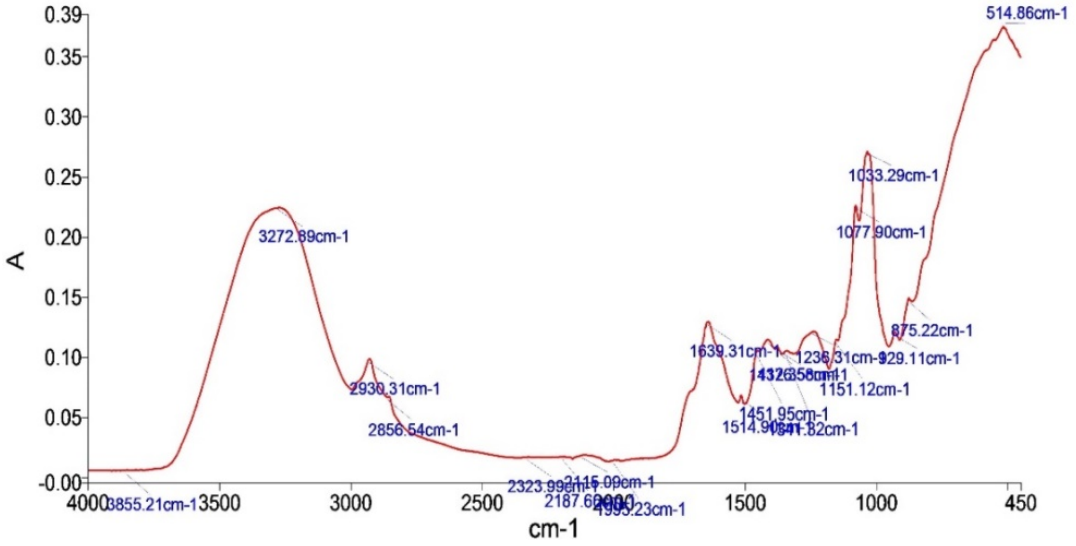

Figure 1: FTIR Spectrum for extracted propolis sample

The antioxidant capacity of the samples is well sync at 1630 cm^{-1} signal, where the possible compounds will be aromatic compounds and flavanols. However, the intensity of other bands has poor correlation to the measured antioxidant activity for instance band 1515 cm^{-1}. The 1515 cm^{-1} band corresponds to an aromatic vibrational mode, but has little relationship to the antioxidant behavior, while the 1630 cm^{-1} band correlates to some antioxidant compounds. It shows the antioxidant activity does not only depend on the amount of compounds in solution, but also depends on their chemical structures [6].

3.2 Antibacterial activity

The antibacterial test of ethanolic extract of propolis showed positive results. The inhibition range radius (mm) of each test samples against each type of bacteria are shown in Table 1. The antibacterial activity indices were calculated by comparing the results of the antibacterial activity of EEP with the antibacterial activity of the standard antibiotic (Figure 2).

Table 1: The inhibition radius (mm) of test samples

	Radius of inhibition (mm)			
Bacteria	**Positive Ampicillin**	**EEP**	**Diluted EEP**	**Raw Propolis**
Esterichia Coli	13	15	10	1
Salmonella enterika	30	11	10	n.a.
Bacillus subtilis	15	13	1	n.a.
Staphylococcus aureus	25	25	10	8

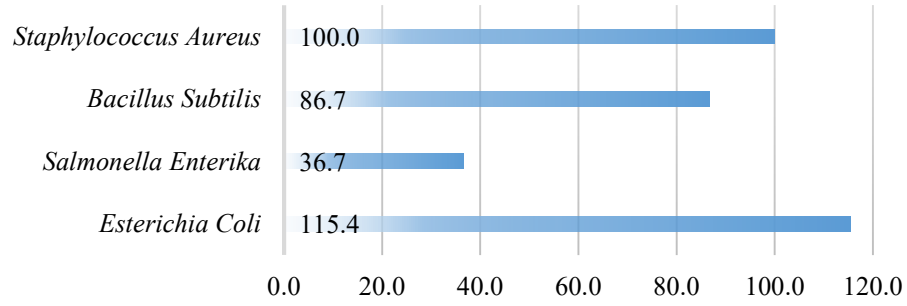

Figure 2: The Antibacterial Activity Index (%)

Based on Fig.2, the maximum inhibition against *E. coli* was higher than antibiotics. For *S. aureus*, the inhibition is the same as antibiotics. The inhibition of *B. subtilis* was 86.7% of antibiotics. The least inhibition was for *Salmonella* which is 36.7% of antibiotics. Unusual from other regions of world, propolis sample from Batu Pahat, Malaysia showed relatively good inhibition against gram-negative compared to gram positive [7]. As the components and active compounds of propolis differs by various reasons especially geographical conditions, it is proven that Malaysian propolis might have different components responsible for antibacterial different from propolis of other parts of world. For the gram-positive bacteria, *S. aureus* and *B. subtilis*, the results were positive. These results sync with other findings against gram positive bacteria.

Propolis activity is not dependent on a single compound but the combination of many compounds [8]. Thus, the interesting combination of compounds in Malaysian propolis which inhibit gram-negative bacteria recommends future research on the compounds responsible for the difference. Also, extreme results of inhibition are obtained for both gram-negative bacteria samples which are *Salmonella enterika* (lowest) and *E. coli* (highest). There is no apparent explanation for this finding. It is interesting that it is very extreme for both gram-negative bacteria. In fact, gram-negative bacteria have a cell wall chemically more complex and a higher fat content [9], but in this case, the possibilities are due to different mechanism of propolis compound to certain bacteria. The anti-bacterial mechanism of certain drug is depending on the target compounds in the bacteria [10].

Based on comparison between results of EEP and diluted EEP, it is concluded that, the higher the concentration of propolis, the higher the anti-bacterial activity against pathogens. From the anti-bacterial test, it is found that similarity between both propolis and propolis extract is the antibacterial property. However, if compared, propolis extract will have higher antibacterial property than propolis. The extraction process has haul out the important biological and chemical compound out of the waxy coating of propolis. The longer the time of extraction, the higher the total active compound extracted from propolis. There will be less compounds available for reactions for raw propolis compared to EEP. Thus, higher antibacterial property is observed in extracted propolis compared to raw propolis.

3.3. Antioxidant activity

The antioxidant activities were determined using DPPH as a free radical method [11]. The reaction kinetics was plotted in Fig. 3 using the absorbance for every min for 60 mins.

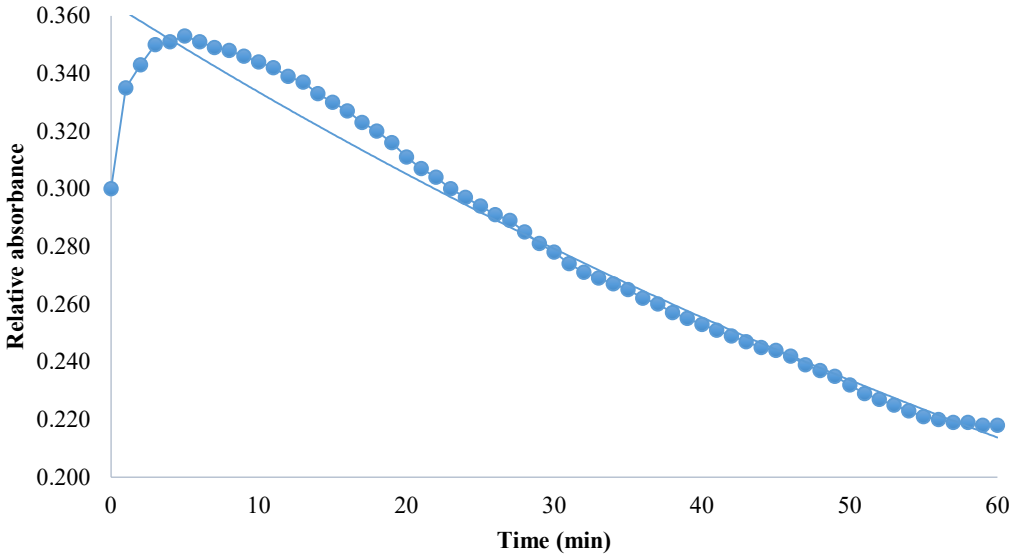

Figure 3: Relative Absorbance of DPPH versus Time

The antioxidant assay results showed that the ethanolic extract of stingless bee propolis sample is potential to have antioxidant property. The analysis of the entire DPPH anti-oxidant bleaching kinetic profile brings useful information about types of antioxidants as well as measures the total antioxidant capacity of a sample.

Three processes occurred in the kinetic profiles associated with DPPH bleaching. First phase, a negative result was obtained. This reaction occurs during the first stage, which also means the negative result is due to the light that accidentally reacted during the transfer into the UV-Vis cuvette. Since DPPH is extremely reactive to light [12], the reading could be interrupted by the transferring processing. The second stage was a positive fast process followed by the third stage where the positive reaction occurred slightly slower at longer reaction time.

3.4. Scanning electron microscope (SEM) analysis

Fig. 4 (a-d) showed photomicrographs of SEM. The SEM images (a) and (c) showed rugged surfaces covered by layers of wax and extractives, while (d) revealed the presence of vegetal constituents, probably non-glandular trichrome and/or glandular and resinous substances from plants such as pollen (b).

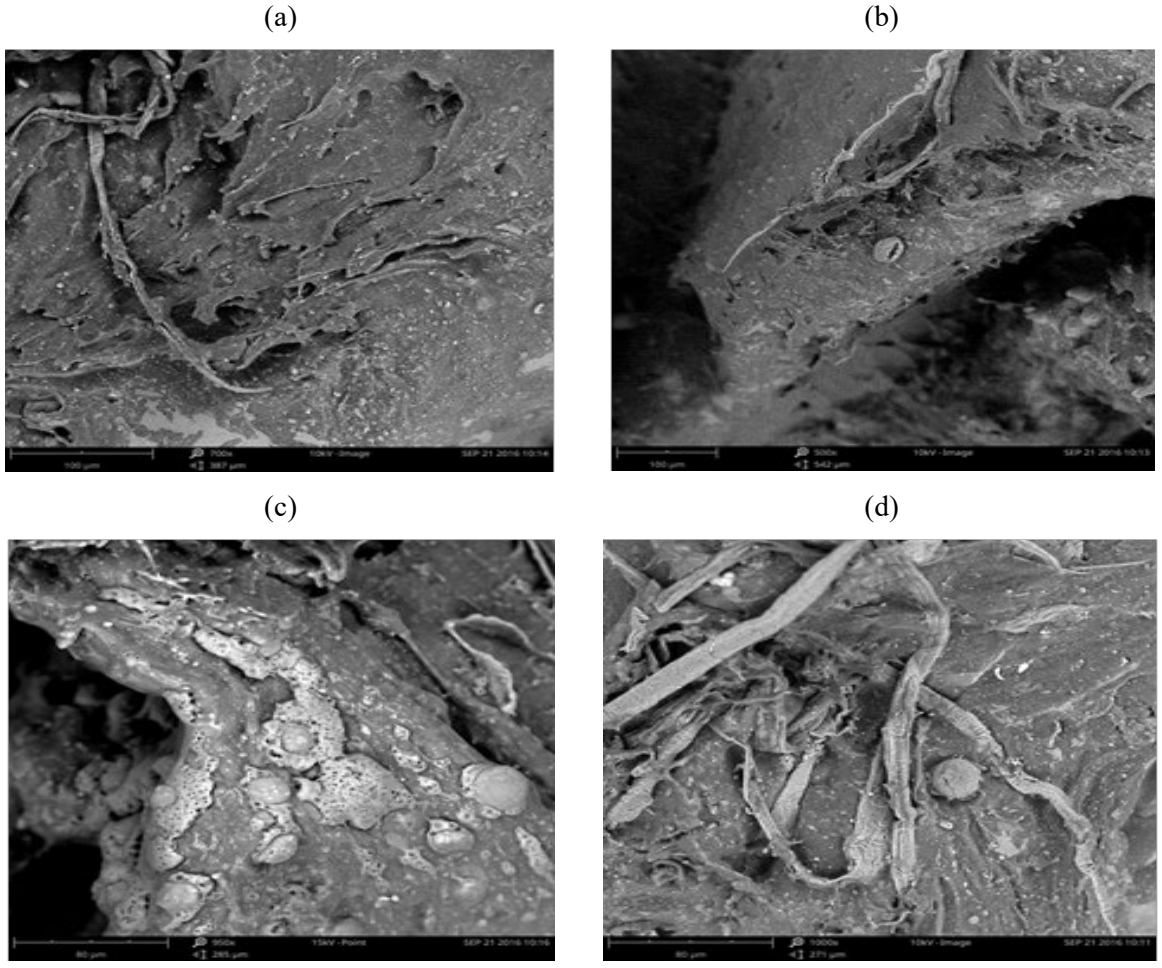

Figure 4: SEM micrographs of propolis before extraction.

4. Conclusion

In conclusion, the extracted compounds present in propolis are responsible for many biological and chemical mechanism of propolis, especially towards antioxidant and antibacterial activity. Propolis showed a positive result for inhibition against gram-negative and gram-positive bacteria. Unusual from propolis from most regions of world, Malaysian propolis manage to inhibit gram-negative bacteria. In overall, propolis is found almost same or higher anti-bacterial when compared to anti-biotic in market. Propolis, with further applications, can be used as a natural substitute for antibiotics in the market. The results of the antioxidant assay, showed that the ethanolic extract of stingless bee propolis sample has an antioxidant potential. Thus, in the future the stingless bee propolis extract is a potential natural antioxidant source and can be used to treat diseases associated with oxidative stress. Finally, according to SEM micrograph before extraction, it is observed that propolis has rugged surfaces covered by layers of wax, extractives, vegetal constituents and resinous substances from plants. The achieved objectives contribute to the knowledge especially regarding the Malaysian propolis antibacterial and antioxidant activity. As an advantage of Malaysia's wide tropical biodiversity, Malaysian propolis can economically contribute to the Malaysian agriculture.

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