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Preliminary studies on extraction of propolis using vitamin E d-α-Tocopheryl polyethylene glycol succinate (vitamin E TPGS) and compare their antimicrobial activities

Yee Tong Kong[1], Viviana Abdullah[1], Siti Umairah Mokhtar[1] and Rajaletchumy Veloo Kutty[1,2]*

[1]Faculty of Chemical and Process Engineering Technology, College of Engineering Technology, University Malaysia Pahang, 26300, Kuantan, Pahang, Malaysia.
[2]Center of Excellence for Advanced Research in Fluid Flow, University Malaysia Pahang, 26300, Kuantan, Pahang, Malaysia.
Email: vrajaletchumy@ump.edu.my

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ABSTRACT

Aims: Phenolic compounds with various biological activities such as antimicrobial, anti-inflammatory and antioxidative activity are considered as key compounds in propolis. In this study, propolis was obtained in Kuantan, Pahang and is known to be collected from stingless honey bee *Trigona thoracica*. The objective of this study is to extract propolis using surfactant vitamin E d-α-Tocopheryl polyethylene glycol 1000 succinate (Vitamin E TPGS) and evaluate its antimicrobial activity compared to water and ethanolic propolis extracts.
Methodology and results: Quantitative determinations of phenolic acid and flavonoid such as caffeic acid and quercetin, respectively in propolis extracts were conducted by using High Performance Liquid Chromatography (HPLC). As a result, 70% ethanol extracted propolis (EEP), water extracted propolis (WEP) and 0.02% vitamin E TPGS extracted propolis successfully demonstrate the presence of hydrophilic caffeic acid, while only 70% EEP and 0.02% vitamin E TPGS extracted propolis show the presence of hydrophobic quercetin. Lastly, antimicrobial testing was conducted towards *Staphylococcus aureus* by using all three different propolis extracts.
Conclusion, significance and impact of study: The results showed EEP and vitamin E TPGS propolis extracts exhibit higher antimicrobial activity compared to the WEP.

Keywords: flavonoids, surfactant, quercetin, caffeic acid, antimicrobial activity, propolis

INTRODUCTION

Propolis is a complex mixture that is synthesized by honeybee from the resin of a plant. It is rich in phenolic compounds, exhibiting properties that are essential for pharmaceutical development such as antibacterial, antifungal, antioxidants, anti-inflammatory, antidiabetic and antitumoral properties (Al Hariri, 2011). The chemical composition of propolis is quite complex and over 150 components have been identified in the propolis (Chang *et al.*, 2002). Raw propolis is mainly composed of plant resins (50%-70%), oil and wax (30%-50%), pollen essential (5%-10%) and other chemical compounds including amino acids, minerals, sugars, vitamin B, C and E, flavonoids, phenol and aromatic compound (Ahangari *et al.*, 2018).

Although extraction of propolis using ethanol is a simple and effective method, it has disadvantages such as strong residual flavour. Besides, it has limitations of application in cosmetics and pharmaceutical industry. For example, it is not suitable for treatment of some diseases in ophthalmology, otorhinolaryngology, paediatrics, or in cases of alcohol intolerance. Therefore, it is desirable to develop non-ethanolic propolis preparations method (Kubiliene *et al.*, 2015). However, most of the biologically active substances in propolis have low solubility in water, and the amount of phenolic compounds in water extracts is 10-fold lower than that in ethanolic extracts. Therefore, it is important to find an effective extraction method which increase the solubility of these substances in water (Kubiliene *et al.*, 2015).

In this study, we employed vitamin E d-α-Tocopheryl polyethylene glycol 1000 succinate (Vitamin E TPGS) to effectively extract both hydrophobic and hydrophilic compound from propolis in water. Vitamin E TPGS is a novel biocompatible non-ionic surfactant. It shows amphipathic properties due to its hydrophilic head and lipophilic tail where the hydrophile-lipophile balance (HLB) is 13. The HLB is the system invented for non-ionic surfactant to indicate its hydrophilicity. Compounds with

***Corresponding author**

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HLB greater than 12 are considered as hydrophilic or water soluble. In contrast, those with a very low HLB values (below 9) are hydrophobic or water insoluble (Tzia and Giannou, 2015). Thus, we hypothesized that the presence of Vitamin E TPGS during extraction of propolis in water will able to extract out both hydrophobic and hydrophilic compounds. We further evaluate the antimicrobial activities of the extracted propolis using *Staphylococcus aureus* and compare with the propolis extracted using 70% ethanol and water alone.

MATERIALS AND METHODS

Materials

Raw propolis was obtained from Q'Lulut Global in Kuantan, while commercial propolis extract was purchased from H&B Honey in Kajang, Malaysia. Vitamin E TPGS, ethanol, distilled water, quercetin, caffeic acid, phosphoric acid, methanol, 1× phosphate buffered saline were all purchased from Sigma Aldrich.

Raw material preparation

Raw propolis was directly collected from hives of stingless bees (*Trigona thoracica*) in Q'Lulut Global, Kuantan, Malaysia. Raw propolis was grounded into fine powder and kept in freezer under -20 °C for 2 days before freeze drying. Next, it was freeze dried to allow frozen water in propolis to sublime directly from solid phase into gas phase.

Extraction of propolis

All ingredients were weighed and measured accurately according to the compositions shown in Table 1 by using weighing balance and measuring cylinder. The extraction methods applied in this research were ethanol extracted propolis (EEP), water extracted propolis (WEP) and Vitamin E TPGS extracted propolis. The ratio of solvent and propolis was 10:1 (v/w). In this study, maceration method was used because it is the easiest and simplest method (Azwanida, 2015). First of all, 10 g of propolis was mixed vigorously with 100 mL of extraction solvent for 3 min at room temperature, 25 °C. The mixture was left overnight at room temperature in the incubator shaker (Infors HT Ecotron, Switzerland) with rotation speed of 120 rpm for 24 h.

Purification of propolis extracts

The suspension was filtered through Whatman No. 1 filter paper. The filtrate obtained should be a clear liquid and free of particles. The colour of the filtrate should be dark brown or slightly reddish. Then, the filtrate was kept in clean and dark airtight bottles.

The filtrate for EEP was then concentrated using a rotary evaporator at 78 °C with rotation speed of 120 rpm for 3 h. Next, the EEP extract was freeze dried under 5 mmHg pressure at -80 °C using VirTis Benchtop Pro freeze dryer (SP Scientific, New York, United States). The water content in the filtrate of EEP, WEP and Vitamin E TPGS propolis extract was removed by freeze drying.

Table 1: Ingredient compositions for propolis extraction.

Ingredient	70% EEP	WEP	0.02% Vitamin E TPGS extracted propolis
Propolis	10 g	10 g	10 g
70% Ethanol	100 mL	-	-
Distilled water	-	100 mL	98 mL
Surfactant	-	-	2 mL of Vitamin E TPGS

Calculation of percentage yield of propolis extract

The percentage yield for 70% EEP, WEP and 0.02% Vitamin E TPGS extracted propolis were calculated by using the formula as shown in Equation 1. The yield percentage of each propolis extract were then compared to each other and tabulated.

Yield percentage (%)=
Mass of propolis extract after purification/ Mass of raw propolis × 100.....(Equation 1)

Determination of quercetin and caffeic acid content by High Performance Liquid Chromatography (HPLC)

Instrumentation and conditions

Chromatographic separation was performed using an Eclipse Plus C18 column (250 mm × 4.6 mm, 5 µm) (Agilent, United State) by isocratic elution. The temperature of column was set at 25 °C and the injection volume was 20 µL. The mobile phase was a mixture of 1× phosphate buffered saline (pH = 4.5) and methanol (40:60, v/v) used in isocratic mode. Before the HPLC analysis started, mobile phase was filtered through a 0.45 µm membrane filter. After that, all the samples, standard solutions and mobile phase were sonicated for 30 min before conducting HPLC analysis to quantify the quercetin and caffeic content in the propolis extracts. The flow rate was set at 0.8 mL/min and the effluent was monitored using a diode array detector set at 260 nm for caffeic acid quantification and 325 nm quercetin content determination (Yang *et al.*, 2013).

Standard solution preparation

The stock solution was prepared by dissolving 32 mg of caffeic acid in 10 mL of methanol. Then, 200, 400, 800, 1600 and 3200 µg/mL of standard solutions were diluted from the stock solution by using mobile phase. The standard solutions were filtered through 0.45 µm nylon syringe filter before inserting into the vials. These steps were repeated for quercetin.

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Sample preparation

Analysis of propolis extract was performed by dissolving 0.1 g of propolis extracts in 10 mL of mobile phase. Before inserting into the vials, the propolis samples were filtered through 0.45 µm nylon syringe filter.

Preparation of bacterial suspension

The *Staphylococcus aureus* isolates were streaked onto tryptone soya agar (TSA) plates to obtain single colonies. The plates were incubated for 24 h at 38 °C. After 24 h, five morphologically similar colonies from the fresh agar plates were selected and transferred into a sterile metal capped containing nutrient broth by using an inoculating loop. The suspension was then mixed with a vortex mixer for 1 min. The turbidity of the suspension was adjusted to 0.063 absorbance when measured at OD_{600}, by adding nutrient broth if the turbidity was too high or by adding more bacterial colonies if the turbidity was too low. After the turbidity adjustment, the bacterial suspension was used within 30 min to avoid any changes in cells number.

Antimicrobial susceptibility testing

Broth macrodilution method was used to evaluate the antimicrobial activity of the propolis extracts. A total of 4.32 mL of nutrient broth and 7.68 mL of the propolis stock solution were dispensed in the glass tubes accordingly to obtain a 0.064 g/mL of propolis concentration. A 6 mL of the nutrient broth were added into six other tubes. A two-fold serial dilution was then performed by transferring 6 mL of the mixtures from the first tube to the second tube. The procedure was repeated to obtain 0.032 g/mL, 0.016 g/mL, 0.008 g/mL, 0.004 g/mL, 0.002 g/mL until the last tube where the propolis concentration became 0.001 g/mL. Another tube was prepared by dispensing 6 mL of nutrient broth in it as a control of growth where no propolis was added in. After that, 50 µL of inoculum was then transferred into each tubes. From each tube, 3 mL of the mixture were transferred into a cuvette to determine the initial turbidity by using the UV-Vis spectrophotometer. All samples were incubated for 24 h at 38 °C. After incubation, the samples were taken for the turbidity reading. The changes in OD_{600} were then recorded.

RESULTS AND DISCUSSION

Percentage yield of propolis extracts

The purified propolis extracts were obtained in powder form after freeze drying. The yield percentage of each propolis extract was calculated and the results shown in Table 2.

Based on Table 2, 70% EEP extracted propolis demonstrates the highest yield percentage among the three propolis extracts. This is followed by 0.02% Vitamin E TPGS extracted propolis (22.18%) and WEP shows the lowest yield percentage which is 8.50%. These differences probably depend on the compounds solubility at different solvents (Usman *et al.*, 2016). The yield percentage of the propolis extracts can also be explained with the colour of filtrate gained during filtration process as shown in Figure 1.

Table 2: Yield of propolis extract.

Propolis extract	Yield (%)
70% EEP	30.28
WEP	8.50
0.02% Vitamin E TPGS extracted propolis	22.18

The colour of WEP and Vitamin E TPGS extracted propolis are lighter than EEP. These three propolis extracts present a clear filtrate after being filtered by using Whatman filter no. 1 with pores size of 11 µm as resin and impurities are removed from the extracts. Filtrate of Vitamin E TPGS propolis extract exhibits a clear dark brownish filtrate. This is because Vitamin E TPGS is a hydrophilic surfactant which has HLB value of 13 (Zhang *et al.*, 2012). It has both amphiphilic structures and therefore, it is able to dissolve both polar and non-polar component in the propolis. Thus, the filtrate of Vitamin E TPGS extracted propolis consists of both hydrophilic and hydrophobic components which make its yield a higher mass compared to WEP.

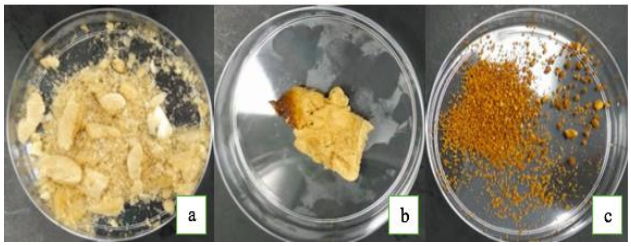

Figure 1: Propolis extracts after purification includes (a) WEP, (b) Vitamin E TPGS extracted propolis and (c) EEP.

Quantification of quercetin in propolis extract

The standard calibration curve of quercetin with correlation coefficient (r^2) of 0.9553 was successfully plotted for concentration in range of 0 µg/mL to 3500 µg/mL (Supplementary data 1). The retention time of quercetin is at 4.668 min (Figure 2). The concentration of quercetin in each propolis extract was calculated based on the calibration equation of y = 0.0417x + 4.3067 generated by Microsoft Excel and the results are summarized in Table 3.

In this study, commercial propolis was used as reference. From HPLC analysis, the commercial propolis contains quercetin concentration of 20753.07 ug/mL, which is the higher than the three extracted propolis in this study. The retention time of quercetin in commercial propolis is 4.617 min which is almost similar with the

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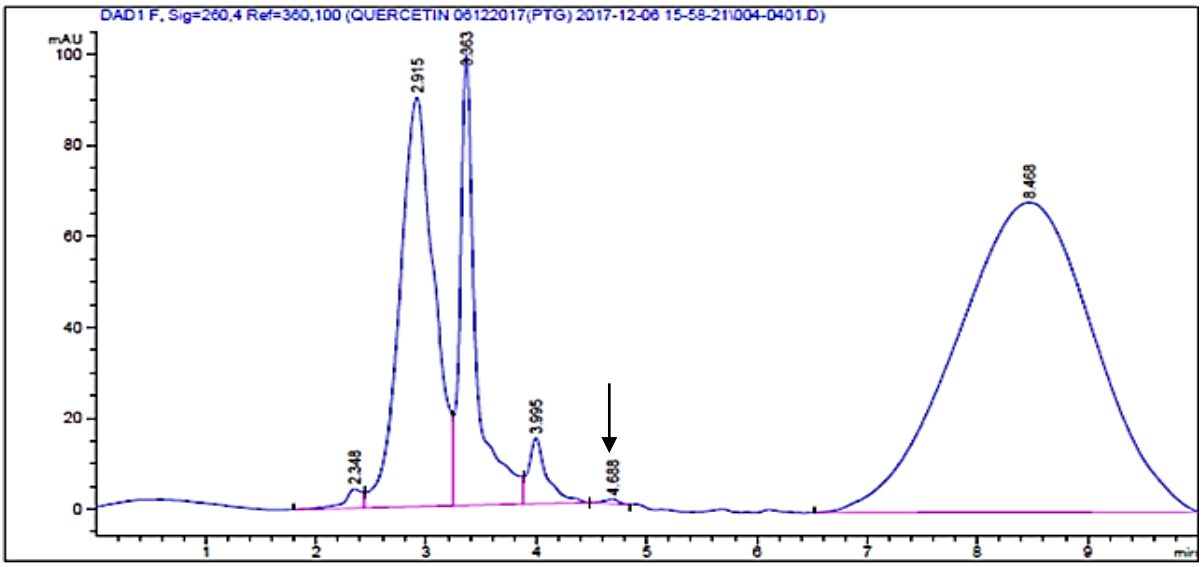

Figure 2: Chromatogram of quercetin (indicated by arrow at 4.688 min) from 0.02% Vitamin E TPGS extracted propolis.

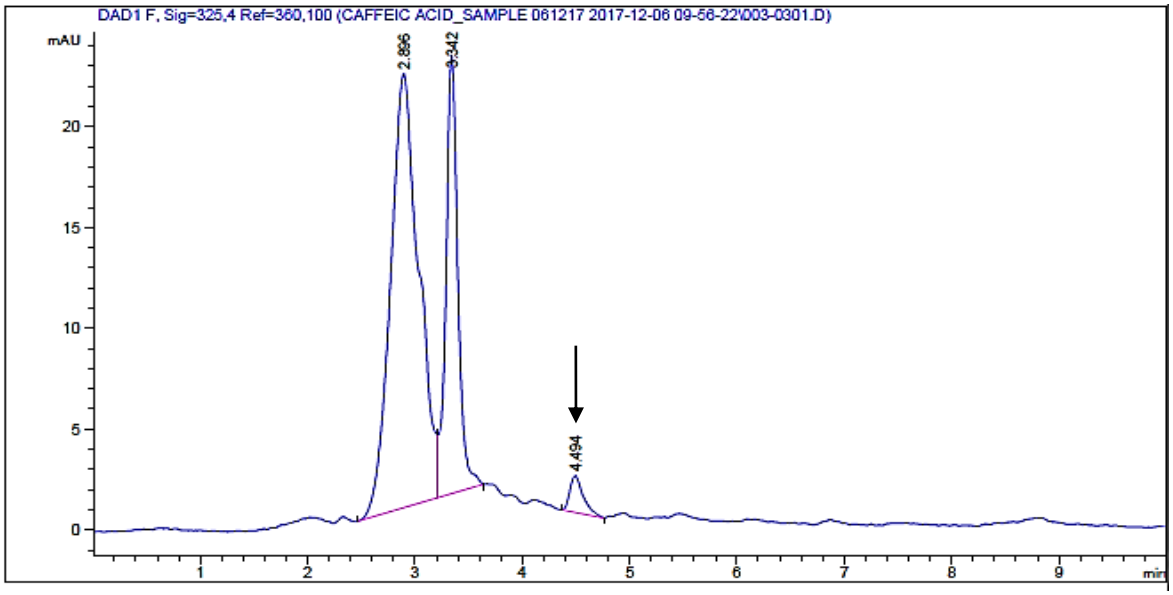

Figure 3: Chromatogram of caffeic acid (indicated by arrow at 4.494 min) from 0.02% Vitamin E TPGS extracted propolis.

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standard (4.668 min). This is because the commercial propolis is highly concentrated and viscous as compared to all the propolis extracts in this study. According to Table 3, quercetin concentration in 70% EEP and 0.02% Vitamin E TPGS extracted propolis are 1699.58 µg/mL and 109.84 µg/mL, respectively. In contrast, chromatogram of WEP does not show any quercetin content.

Table 3: Quercetin concentration in the propolis extracts.

Propolis extract	Quercetin concentration (µg/mL)
70% EEP	1699.58
WEP	ND
0.02% Vitamin E TPGS extracted propolis	109.83

ND = Not detected

Based on the Table 3, 70% EEP demonstrated higher quercetin concentration as compared to WEP and 0.02% Vitamin E TPGS extracted propolis. This is because 70% EEP is a mixed extraction solvent where it consists of 70% ethanol and 30% water. Besides, ethanol is an intermediate polarity molecule (Hanson, 2005). The presence of polar and non-polar molecules in 70% EEP allows both hydrophilic and hydrophobic quercetin derivatives to be extracted in the extraction solvent. Furthermore, 70% EEP exhibits higher amount of quercetin as compared to 0.02% Vitamin E TPGS extracted propolis. This result is probably caused by low concentration of Vitamin E TPGS used in this propolis extraction may lead to lower quercetin yield in 0.02% Vitamin E TPGS extracted propolis. The reason why WEP does not extract hydrophobic quercetin is because there are only polar molecules present in the extraction solvent. Thus, water is impossible to extract the hydrophobic quercetin. Materska mentioned that, quercetin molecule has a lipophilic character despite the presence of five hydroxyl groups (Materska, 2008).

Quantification of caffeic acid in propolis extracts

The standard of caffeic acid shows a good resolution between matrix and analyte peaks. The standard calibration curve with correlation coefficient (r^2) of 0.9729 was successfully plotted by using Microsoft Excel (Supplementary data 2). It shows an acceptable linearity between the concentrations in range of 0 µg/mL to 3500 µg/mL. The retention time of caffeic acid is 4.494 min as shown in Figure 3. The concentration of caffeic acid in the propolis extracts was determined by using the calibration equation of y = 0.041x + 9.312 (Table 4). As a control in this research, commercial propolis exhibits caffeic acid concentration of 23618.99 µg/mL. This is followed by WEP (721.90 µg/mL), 70% EEP (176.84 µg/mL) and 0.02% Vitamin E TPGS extracted propolis (158.33 µg/mL). From this result, it can be seen that vitamin E TPGS extracted propolis contains hydrophilic compound nearly similar to 70% EEP.

Jacobsen *et al*. (2013) mentioned that caffeic acid is a hydrophilic antioxidant. In this research, WEP successfully demonstrates the highest amount of caffeic acid among the others because there is large number of polar molecules exist in water. Hence, WEP is able to extract hydrophilic caffeic acid from the propolis. Apart from that, 0.02% Vitamin E TPGS extracted propolis supposed to show a higher caffeic acid concentration than 70% EEP because it consists of higher water content in extraction solvent. However, the caffeic acid content in 0.02% Vitamin E TPGS extracted propolis (158.33 µg/mL) is still comparable to 70% EEP because the caffeic acid concentration value does not deviate much with 70% EEP (176.84 µg/mL).

Table 4: Caffeic acid concentration in the propolis extracts.

Propolis extracts	Caffeic acid concentration (µg/mL)
70% EEP	176.84
WEP	721.90
0.02% Vitamin E TPGS extracted propolis	158.33

Antimicrobial susceptibility testing

Staphylococcus aureus is Gram-negative bacteria. According to Ahuja and Ahuja, ethanolic extracts of propolis were more effective against Gram-positive bacteria and showed limited effect against Gram-negative bacteria (Ahuja and Ahuja, 2011). Since this study aims to compare the antimicrobial activity between ethanolic and Vitamin E TPGS propolis extracts, Gram-positive bacteria was chosen in antimicrobial susceptibility testing. From the Figure 4, a clear comparison can be seen where EEP, WEP and propolis extracted using Vitamin E TPGS show a positive antimicrobial activity towards *S. aureus* which can be explained by the reduction of bacterial optical density. Comparing the EEP, WEP and propolis extracted with Vitamin E TPGS, WEP actually has a very low antimicrobial effect towards the *S. aureus* while EEP and propolis extracted with Vitamin E TPGS have better killing effect. This is because most of the biologically active substances contained in the propolis have low solubility in water where the amount of phenolic compounds in water extracts is 10-fold lower than the ethanolic extract (Krasavage and Terhaar, 1977).

From Figure 4, propolis extracted with Vitamin E TPGS displays a similar antimicrobial trend with the EEP. Vitamin E TPGS propolis extract contains hydrophobic and hydrophilic regions which facilitate the extraction of non-polar molecules in the extraction solvent. Besides, 0.02% Vitamin E TPGS extraction solvent consists of

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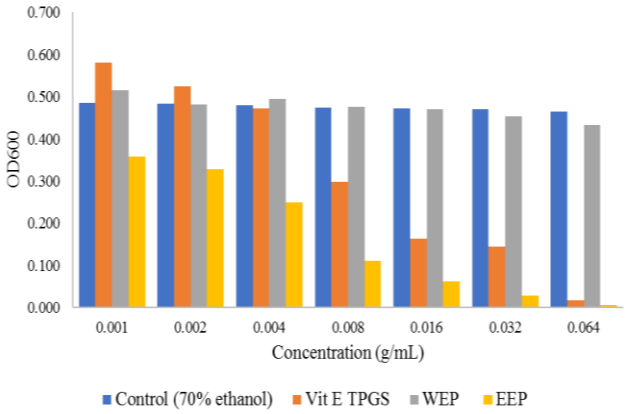

Figure 4: Comparison of the antimicrobial activity of three propolis extracts toward *S. aureus*.

99.98% of water, thus it has the ability to extract the hydrophilic phenolic components in propolis which is responsible for the antimicrobial properties of the propolis.

CONCLUSION

In short, propolis was extracted by using three different solvents such as 70% ethanol, water and 0.02% Vitamin E TPGS. Generally, EEP demonstrates the highest yield percentage (30.28%) and followed by Vitamin E TPGS extracted propolis (22.18%) and WEP (8.50%). The HPLC results successfully showed the presence of hydrophobic quercetin in EEP (1699.58 µg/mL) and Vitamin E TPGS extracted propolis (109.83 µg/mL). Moreover, all the propolis extracts successfully demonstrated the presence of hydrophilic caffeic acid at different concentration. The caffeic acid content in WEP, EEP and Vitamin E TPGS are 721.90 µg/mL, 176.84 µg/mL and 158.33 µg/mL, respectively. For antimicrobial susceptibility testing, a lower antimicrobial activity is exhibited by WEP whereas Vitamin E TPGS and EEP show similar trend for bacteria killing. Therefore, Vitamin E TPGS is suggested to be used as solvent to extract propolis for pharmaceutical formulation. However, the concentration of Vitamin E TPGS should be optimized to increase the yield of propolis extract.

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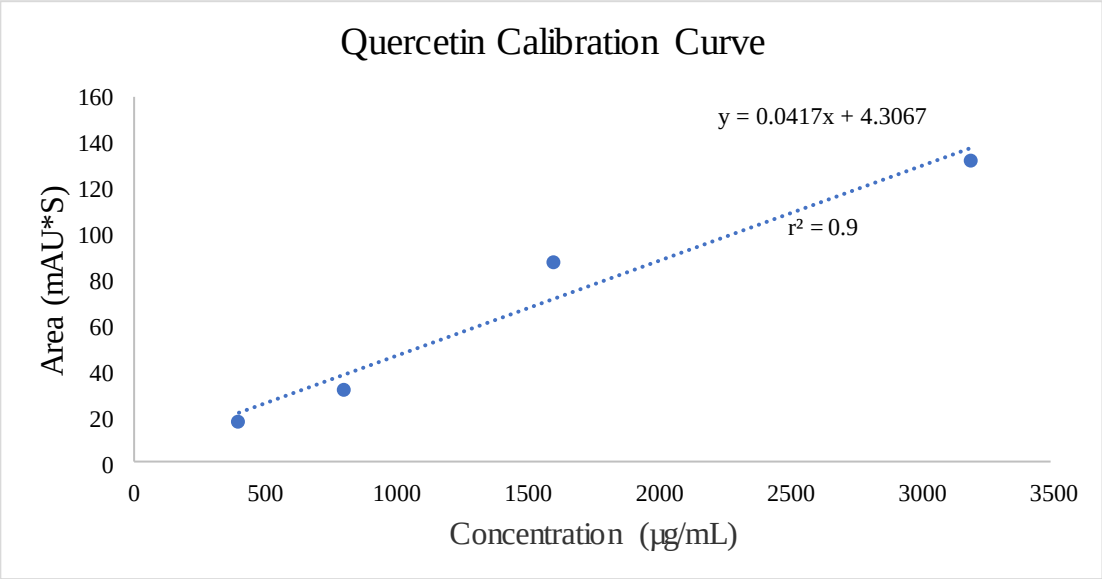
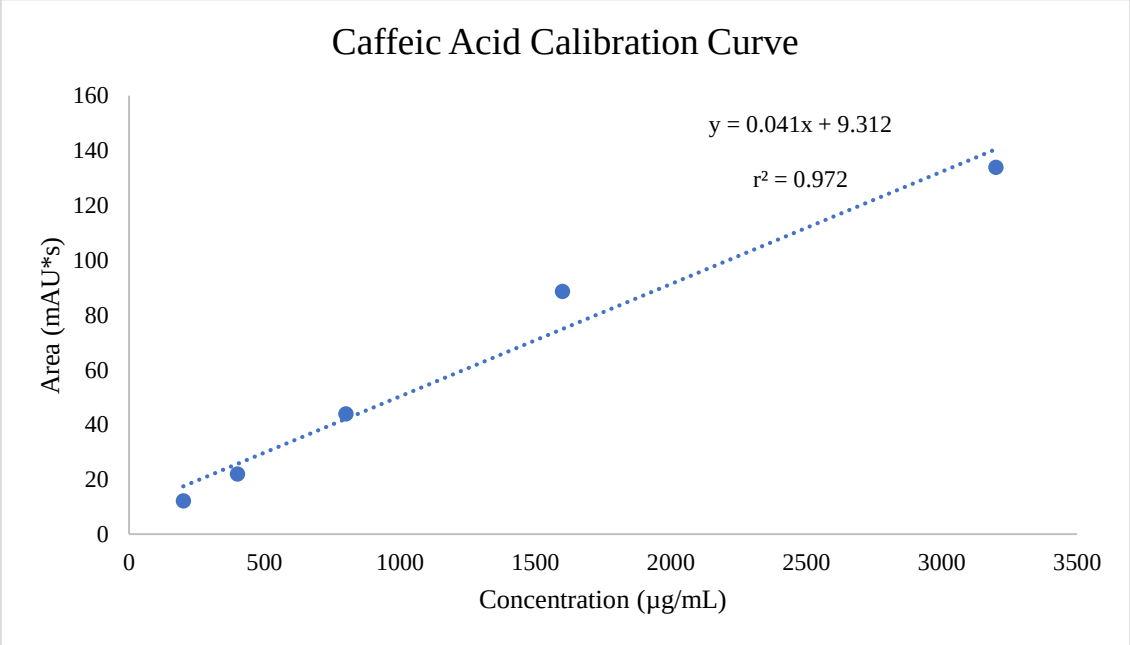
SUPPLEMENTARY INFORMATION



Supplementary Figure 1: Calibration curve of quercetin.



Supplementary Figure 2: Calibration curve of caffeic acid.

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