Effects of Solvent and pH on Stingless Bee Propolis in Ultrasound-Assisted Extraction

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Abstract: Ultrasound-assisted extraction was used to extract propolis from a dark and resinous substance harvested from a beehive of Heterotrigona itama, which is commonly known as stingless bees. The propolis extracts were prepared using ethanol and water at different pH values of 3, 6, and 9. The yield of the ethanolic extract was significantly higher than the water extract, but there were no significant differences at different pH values. The ethanolic extract was found to have a lower 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity than the water extract at pH values of 6 and 9. However, the acidic propolis extracts, particularly the ethanolic extract, were found to have the highest antioxidant capacity. The addition of 20% polyethylene glycol 400 in the solvent systems was unlikely to improve propolis extraction. This can be seen from the antioxidant capacity and metabolite profile of the propolis extracts. Gas chromatography–mass spectrometry (GC–MS)-based high throughput screening of the propolis extracts showed them to have small metabolites of hydrocarbons, esters, terpenes, and alkaloids, as well as high antioxidative 2,4-di-tert-butylphenol. The detection of mangostin, mangiferin, and a few flavanones in the acidic ethanolic extract by liquid chromatography tandem mass spectrometry LC–MS/MS proved its high antioxidant capacity compared to the water extract.

Keywords: Heterotrigona itama; stingless bees; propolis; ultrasound-assisted extraction; 2,4-di-tert-butylphenol; mangostin; mangiferin

1. Introduction

Propolis, which is also known as bee glue, is a natural resinous material collected by bees from different sources of plant exudates, buds, leaves, and barks. It is mainly composed of resin (50%), wax (30%), essential oils (10%), pollen (5%), and other organic compounds (5%) [1]. It is used to seal holes in beehive and to protect larvae, young bees, and the queen bee from microbial infection, as well as to maintain constant humidity and internal hive temperature of around 35 °C [2]. Previous researchers have reported that propolis exhibits remarkable pharmacological properties such as antioxidant, anti-microbial, anti-septic, anti-inflammatory, anesthetic, and anti-tumor diabetic activities [2]. These properties are mainly attributed to the presence of active compounds in propolis extracts. Therefore, the quality of propolis is strongly dependent on its geographical origin, extraction method, and operating conditions.

Ultrasound-assisted extraction was found to be effective for recovering phenolic compounds from propolis [3]. This effectiveness includes a shorter extraction time, lesser solvent consumption, higher yield, and total phenolics [4]. Ultrasonic energy produces cavitation bubbles that continuously enact the processes of bubble formation, growth, and implosion to produce high shear forces for
complete extraction. This is done by accelerating mass transfer between propolis and a solvent [5]. This technique had been also proven to produce a higher extraction yield than microwave-assisted and maceration extraction in recent publications [3,6].

A solvent and its ionic strength are very important to effectively extract bioactive compounds from propolis. Methanol was reported to be the effective solvent of propolis extraction [7]. However, ethanol and water are still the solvents of choice because they are considered green solvents [8]. The use of water as a solvent is highly restricted because of the poor solubility of organic compounds during extraction. Mello et al. [9] and De Moura et al. [10] revealed about 10-times lower total phenolics in a water extract than in an ethanol extract using raw propolis harvested from the beehives of Apis mellifera from Brazil. Hence, ethanol is likely to be the most popular solvent for propolis extraction [11]. Nevertheless, the performance of extraction could be increased by changing the pH of the solvent. This is because the change of ionic strength may affect the solubility of compounds. According to Yeo et al. [12], the antioxidant activity of their propolis extract was increased if extraction was conducted in an alkaline condition. However, the work of Jug et al. [13] showed that acidic water and ethanol (pH 3) produced a slightly higher total flavonoid count of a propolis extract than their neutral solvent system counterparts (pH 7). On the other hand, Kubiliene et al. [14] reported that the addition of polyethylene glycol in their extraction solvent could enhance propolis extraction.

In the present study, a propolis extract was prepared using ultrasound-assisted extraction in both ethanol and water at different pH values of 3, 6, and 9. The present study tried to use non-toxic solvents. The raw and dark colored propolis of stingless bees, scientifically named Heterotrigona itama, was used for extraction. The effects of solvent and pH on the propolis extract were chromatographically and spectroscopically characterized and then compared for the antioxidant capacity in terms of free radical scavenging activity.

2. Materials and Methods

2.1. Chemicals and Raw Material

The raw material of propolis was harvested from a stingless bee farm located in Melaka, Malaysia. The propolis of Heterotrigona itama was stored in a fridge at 4 °C overnight and then manually cut into small pieces (~1 cm) using a knife. The small pieces of propolis were stored again in the fridge before analysis. Analytical grade ethanol, methanol, formic acid, ammonium hydroxide, and polyethylene glycol 400 (PEG-400) were sourced from Merck (Darmstadt, Germany), and 1,1-diphenyl-2-picrylhydrazyl (DPPH) and L-ascorbic acid (99%) were purchased from Sigma-Aldrich (St. Louis, USA).

2.2. Ultrasound-Assisted Extraction

A ratio of 1 to 10 (solid to solvent) was used to extract propolis (10 g) in 100 mL of a solvent. Four solvent systems consisted of 96% ethanol, water, 20% PEG-400-added ethanol and 20% PEG-400-added water. Formic acid (0.1 M) and ammonium hydroxide (0.1 M) were used to adjust the pH of the solvent systems to 3, 6, and 9, as well as to avoid matrix interferences during metabolite analysis. Ultrasound-assisted extraction was carried out in an ultrasonic cleaner bath (Daian Scientific Co. Ltd, Wonju-si, Gangwon-do, South Korea) for 15 min at 70 °C [15]. The frequency of the bath was 40 kHz. The mixture was centrifuged at 6000 ×g for 15 min using a centrifuge (Hettich universal 320R, Germany). The supernatant was harvested and filtered with filter paper. The extraction process was repeated three times using the remaining propolis and fresh solvent. Three parts of the supernatants were combined and concentrated using a rotary evaporator (Heidolph, Laborota, Germany). The concentrated propolis extract was dried in an oven at 50 °C until dry. The weight of the propolis extract was recorded, and the yield of the extraction was determined using Equation (1). The process of extraction was performed in triplicate. A one-way analysis of variance was carried out on the data using Microsoft Excel 2016 (Microsoft, Redmond, Washington D.C., USA).
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\text{Extraction yield (\%)} = \frac{\text{Weight of dry extract (g)}}{\text{Weight of propolis (g)}} \times 100\%
\]

2.3. Antioxidant Assay

The antioxidant capacity of the propolis extract was determined using a DPPH assay. A serial dilution of the propolis extract ranging from 0.0625 to 2.000 mg/mL (100 µL) was prepared and added into 100 µL of methanolic DPPH (0.11 mM in 70% methanol) in a 96-well microplate. The absorbance of the solution was read at 517 nm using a microplate spectrophotometer (BioTek Epoch, Winooski, VT, USA) after incubation for 30 min in a dark room. Ascorbic acid was used as the standard chemical for the construction of a calibration curve. Methanolic DPPH was used as a control. The scavenging activity was determined using Equation (2). The results were expressed in IC50 as the scavenging activity at 50% inhibition.

\[
\text{Scavenging activity (\%)} = \left(1 - \frac{\text{Absorbance of sample}}{\text{Absorbance of control}}\right) \times 100\%
\]

2.4. Attenuated Total Reflectance–Fourier Transform Infrared Spectroscopy (ATR–FTIR)

The functional groups of the compounds in the propolis extracts were evaluated using attenuated total reflectance (ATR)–FTIR spectrometry (Spectrum One, Perkin Elmer, MS, USA). Two milligrams of propolis extracts were used for scanning from 4000 to 400 cm⁻¹ at a resolution of 4 cm⁻¹ with 32 scans. A PEG-400 spectrum was used as the background for samples prepared by the PEG-400-added solvent system.

2.5. Gas Chromatography–Mass Spectrometry (GC–MS)

Gas chromatography–mass spectrometry (GC–MS) (Agilent 7890A, Wilmington, DE, USA) was used to analyze the volatile compounds in the propolis extracts. The GC–MS was connected to a headspace sampler. A fused silica capillary BP-5 (5% phenyl/95% dimethyl polysiloxane) column was used for compound separation. Helium was used as the carrier gas and flowed at 1.0 mL/min. The oven temperature was programmed as 50 °C for 2 min, increased to 280 °C at 10 °C/min, and maintained at 280 °C for 10 min. The temperature of the injector and detector was set at 250 °C. One microliter of sample was injected in the splitless mode and analyzed in the MS full-scan mode (40–650 m/z). The electron ionization was set at 70 eV. Data acquisition was performed using the Chemstation software (Rev. E.02.00) from Agilent Technologies, Santa Clara, USA. The detected compounds were identified using the National Institute of Standards and Technology (NIST, G1036A) library search.

2.6. High Performance Liquid Chromatography Tandem Mass Spectrometry (LC–MS/MS)

High performance liquid chromatography (HPLC) was used to analyze the propolis extracts prepared by different solvent systems at different pH values. The HPLC system (Ultimate 3000, Dionex Corporation, Sunnyvale, USA) was integrated with a diode array detector. The mobile phase consisted of acidified water with 0.1% formic acid (A) and acetonitrile (B). A C18 reversed phase column (XSelect HSS T3 XP, 2.1 mm × 100 mm × 2.5 µm) was used to separate compounds with a flow rate of 0.15 mL/min. The gradient of the mobile phase was 0–10 min, 10% B; 10–12 min, 10%–90% B; 12–20 min, 90% B; 20–22 min, 90%–10% B; and 22–30 min, 10% B. All samples (2 mg/mL) were dissolved in 50% aqueous methanol and filtered through a 0.2 µm nylon filter prior to injection. The sample injection volume was 5 µL, and the measurement was performed at 290 nm.

The mass range of 100–2000 m/z was set for compound screening. The TOF MS (time-of-flight mass spectrometry) scan was acquired with two dependent product ion scans using rolling collision energy. Nitrogen gas was used as a nebulizing (40 psi) and curtain (20 psi) gas. The voltage of the ion spray was set at 4500 V for the negative ion mode. The declustering and the focusing potentials were set at 40 and 300 V, respectively.
3. Results and Discussion

3.1. Extraction Yield

In the present study, ultrasound-assisted extraction produced a high yield of the propolis extract using ethanol. The yield varied from 35.7% to 42.6% at different pH values, as shown in Figure 1. Those yields were comparable (31%–46%) with the findings of Korean researchers who reported the ultrasonic extraction of propolis harvested from Suwon, Daegu, and Jeju using ethanol with a similar solid-to-solvent ratio of 1:10 [16]. However, the yield of the water extracts was approximately six times lower than that of the ethanolic extracts. The lower yield of the water extract had also been proven by Indonesian researchers who compared the extraction yield of stingless bee propolis using both solvents, namely water and ethanol, in ultrasound-assisted extraction [17]. The researchers reported that the yield of water extracts ranged from 4.9% to 11.8%. The results of the present study were in the range of data reported by previous researchers (Figure 1). This observation suggests that the yield of propolis extracts strongly relies on the geographical origin of samples, since bees collect the buds and exudates of different plants from their surrounding region. A low yield of water extracts indicated that propolis contains less water-soluble compounds [14]. The yield of the propolis extracted by PEG-added solvent systems is not presented due to the high boiling point of PEG (290 °C) required for solvent evaporation to get the dried propolis extract.

![Figure 1. Extraction yield of propolis extracts using ethanol (green bar) and water (blue bar) as solvents at different pH values.](image)

3.2. Antioxidant Activity by DPPH Assay

Figure 2 compares the IC₅₀ of the propolis extracts, namely ethanol, water, PEG-400 in ethanol, and PEG-400 in water, at different pH values. The comparison of IC₅₀ for ethanol and water is presented in Figure 2a, whereas Figure 2b shows the IC₅₀ of the PEG-400-added solvent systems in ethanol and water. The individual solvent systems with and without PEG are further compared in Figure 2c,d. A high IC₅₀ value suggests a low antioxidant capacity because a higher concentration of the sample was required to inhibit 50% of free radicals. The results showed that the antioxidant capacity of the ethanolic extract was lower than that of the water extract, even though the ethanolic extract appeared to have a higher extraction yield. This was because water extract was found to have a lower IC₅₀ at pH values of 6 and 9 (Figure 2a). However, the water extract prepared at pH 3 was found to have a lower antioxidant capacity due to its higher IC₅₀ than the ethanolic extract at the same pH. The addition of 20% PEG-400 to the solvent system did not improve the antioxidant capacity of the propolis extracts, although Kubiliene et al. [14] reported that the addition of PEG-400 could increase the phenolic content of propolis extracts. The IC₅₀ of the extracts using the PEG-400-added solvent system was found to have higher values than that of their counterparts (Figure 2c,d). Phenolic compounds usually have a direct relationship with the antioxidant property of samples. Overall, acidic solvent has been found to have a better solubility of compounds with radical scavenging activity from propolis. Hence, acidic ethanol (IC₅₀ = 0.1731 ± 0.0223 mg/mL) appeared to be the most
effective solvent for preparing the propolis extract in the present study. The antioxidant capacity of this acidic ethanolic extract was in the range of IC_50 values (0.0700–0.9320 mg/mL) reported for ethanolic Romanian propolis by Duca and coworkers [18].

![Figure 2. Comparison of 50% radical inhibition by the propolis extracts: (a) ethanol (green bar) and water (blue bar), (b) polyethylene glycol (PEG)-400 in ethanol (green line bar) and PEG-400 in water (blue dot bar), (c) ethanol (green bar) and PEG-400 in ethanol (green line bar), and (d) water (blue bar) and PEG-400 in water (blue dot bar) at different pH values.](image)

3.3. Characterization of Propolis Extracts by ATR–FTIR

FTIR was used to rapidly screen for the functional groups of compounds in the propolis extracts. The FTIR spectrum of ethanol extract was found to have significant bands at 2920 and 2848 cm\(^{-1}\), which were assigned to symmetric and asymmetric C–H stretching, respectively (Figure 3). A small broad band at 3549–3246 cm\(^{-1}\) was also observed for O–H stretching. The other significant bands in the ethanol extract were in the region of 1700–900 cm\(^{-1}\): 1685 cm\(^{-1}\) (C=O stretching), 1591 cm\(^{-1}\) (C=C stretching), 1448 cm\(^{-1}\) (C–H bending), 1375 cm\(^{-1}\) (O–H bending), and 1269 cm\(^{-1}\) (C–O stretching). The PEG-400-added solvent system was found to have a broad band from 1155 to 1000 cm\(^{-1}\) corresponding to C–O stretching from PEG-400. Three bands (935, 883, and 835 cm\(^{-1}\)) explained the out-of-plane C–H bending of PEG-400. The spectra of the PEG-400-added solvent system also did not show any additional functional groups of metabolites in the propolis extracts. Therefore, PEG-400 did not have significant improvement for propolis extraction in this study. On the other hand, the water extract was found to only have C=C stretching and bending at 1585 and 1001 cm\(^{-1}\), respectively.
Figure 3. Spectra of FTIR for the propolis extracts prepared using ethanol, water, and PEG-400 in ethanol and PEG-400 in water at pH 3.

3.4. Characterization of Propolis Extracts by GC–MS and LC–MS/MS

The propolis extracts were analyzed by both GC–MS and LC–MS/MS. GC–MS was used to detect small and non-thermolabile metabolites (<350 da), whereas larger metabolites (250–1000 da) were scanned in LC–MS/MS. The results found that the addition of PEG-400 in the extraction solvents did not improve or increase the detected metabolites in the propolis extracts. This observation was in line with the findings of an antioxidant assay, in which the extracts prepared by PEG-400-added ethanol and water at pH values of 3, 6, and 9 did not show an increment in antioxidant capacity. The water extract of propolis was found to have small metabolites, mostly hydrocarbons (4-dodecene, 2-tetradecene, methyl cyclohexane, and 1-heptadecene), esters (methyl benzoate and methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl)propionate), carboxylic acid (phenylpropanoic acid), and alkaloids (2-ethylacridine and 2-methyl-3-phenyl-3H-indole), as detected by GC–MS. On the other hand, esters like 11-butyl-2-oxo-1-azaspiro[5.5]undecan-10-yl acetate, methyl 14-methylpentadecanoate, and methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl)propionate, in addition to the other metabolites, such as amyrin (triterpene), 1-hexylnaphthal-1-ylmethanone, and 2,4-di-tert-butylphenol, were detected as volatile metabolites in the ethanol extracts. It is also interesting to note that 2,4-di-tert-butylphenol was detected in both the ethanol (~3.4%) and water (~0.9%) extracts, but it was not detected in the PEG-400-added solvents. The compound has been identified as an artefact that is used as antioxidant in plastics and as a chemical intermediate in the production of synthetic resins, paints, and some plasticizers. The European Commission has also notified the World Trade Organization to identify 4-tert-butylphenol as a very high concern substance due to its potential in endocrine-disrupting properties [19]. On the other hand, Zhao et al. [20] reviewed various natural sources and bioactivities of 2,4-di-tert-butylphenol including its antioxidant capacity in term of free radical scavenging. Therefore, 2,4-di-tert-butylphenol was likely to be the key compound exhibiting antioxidative properties in the ethanol and water extracts. The absence of that compound could most likely explain the lower antioxidant capacity of the propolis extract prepared by the PEG-400-added solvent system.

The high sensitivity of LC–MS/MS was able to detect polyphenols in the propolis extracts, especially in the ethanol extract. The detection of mangostin and mangiferin was found to be possible because there was an orchard nearby the bee hive [21]. The loss of 90 amu from the sugar moiety was very common in C-glycosylated polyphenol. The ethanolic extract of pH 3 was also rich in flavanones such as galloyl naringenin, galloyl eriodictyol, and galloyl pinocembrin (Figure 4). The compounds were tentatively identified based on the detection of fragment ions. Possibly, the polyphenols were attached to the group of galloyl because of the detection of a neutral loss of 152 amu in the mass.
fragmentation pattern of the compounds. However, the configuration and bonding position of the galloyl group could not be distinguished in LC–MS/MS. There were also a few lithocholic acid derivatives detected in the ethanol extracts. The existence of polyphenols was attributed to a high antioxidant capacity, especially in the acidic ethanol extract.

![Image](image.png)

**Figure 4.** Mass spectra of detected flavanones; (a) mangostin, (b) mangiferin, (c) galloyl pinocembrin, (d) galloyl naringenin and (e) galloyl eriodictyol in the acidic ethanolic propolis extract. The characteristic ions of the compounds are marked in blue circles.

4. Conclusions

This study found that ethanolic extracts had higher yields than water extracts with no significant difference at pH values of 3, 6, and 9. However, the water extracts were found to have a better antioxidant activity at pH values of 6 and 9 but a lower such activity in an acidic medium than the ethanol extracts. The antioxidant capacity was the highest for the acidic ethanolic propolis extract. The addition of PEG-400 did not show any improvement in the performance of the ultrasound-assisted extraction in both solvent systems. The results of analysis showed that the high radical scavenging activity of the acidic ethanolic propolis extract could have been due to the presence of 2,4-di-tert-butylphenol, mangostin, mangiferin, and a few known flavanones with remarkable antioxidative behavior.

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