Applied Food Research 3 (2023) 100252

Contents lists available at ScienceDirect

Applied Food Research

journal homepage: www.elsevier.com/locate/afres

Study of stingless bee (*Heterotrigona itama)* propolis using LC-MS/MS and TGA-FTIR

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ARTICLE INFO

Keywords:
Propolis
Stingless bee
Meliponini
Maceration
Ultrasound
Antioxidant

ABSTRACT

Propolis, especially meliponini propolis is getting more popular in the functional food market, mainly due to its pharmacological importance. The quality of propolis is largely dependent upon the extraction technology and its solvent system. The present study investigated the performance of propolis extraction from maceration with and without ultrasonic pretreatment (30 min) in both water and 20% ethanol systems. Maceration was chosen to avoid degradation of heat sensitive compounds. Ultrasonic pretreatment was introduced to enhance propolis extraction from recalcitrant raw material. The yield of propolis was 4.0-5.5%. The results found that maceration with ultrasonic pretreatment, especially in aqueous ethanol increased the phenolics (17.043 mg GA/g), tannins (5.411 mg GA/g) and flavonoids (0.83 mg Q/g) content, as well as antioxidant capacity of propolis (80% at 1 mg/mL). Mass spectral based principal component analysis revealed that solvent system had higher effect (> 30%) on the variance of propolis quality rather than extraction technology. The variance of chemical composition had also led to the difference of antioxidant capacity among propolis samples. Alcohol precipitation would remove polymeric substance from propolis which was then characterised by thermogravimetric analysis coupled with Fourier transform infrared spectrometer (TGA-FTIR) after acid hydrolysis. The substance was putatively identified as hygroscopic lignocelluloses (14.4% moisture, 36.6% hemicelluloses and celluloses, 40% lignins), and started to decompose at 203°C, involving 4 steps of degradation mechanism at the highest derivative weight of 12.09%/min.

1. Introduction

Meliponiculture is a fast-growing industry nowadays, partly due to the large population of stingless bees which are mostly found in the warm and humid environment of tropical and subtropical regions. Ranz (2019) reported about 60 species of stingless bees, mainly from the two main genera of *Melipona* and *Trigona* in the Indo-Malayan region of Asia (Michener, 2000). *Heterotrigona itama* from the Apidae family is the most abundant species found in Southern part of Malaysia (Rasmussen & Cameron, 2009). *H. itama* also exhibited higher radical scavenging activity than *Geniotrigona thoracica* (Ibrahim et al., 2016).

Many apiaries have shifted their focus from honey to propolis in recent years. Propolis is another product developed by meliponiculturists, beside harvesting ready to eat honey and beebread. It has been commercialized as a complementary and functional food substance. Unlike honey bees, meliponines produce larger volume of propolis in a beehive, and the phytochemical profile of meliponini propolis is also more diverse due to the flora rich environment in tropical regions. A small variance of chemical profile was reported by (Salatino, Pereira, & Salatino, 2019) who revealed that meliponini propolis might have more glycosylated flavonoids. This was because stingless bees do not have glycosidases in saliva for glycolysis compared to honey bees. Publication regarding meliponini propolis is relatively limited compared to honey bee propolis (Al-Hatamleh et al., 2020). The demand for meliponini propolis is in increasing trend, mainly because of its potential pharmacological importance in antioxidant (Da Silva et al., 2020), anti-inflammatory (Zhang et al., 2020), anti-microbial (Ibrahim et al., 2016), anti-cancer (Mohamed et al., 2020) and wound healing (Martinotti & Ranzato, 2015) properties. Comprehensive review on the chemistry and therapeutic effects of propolis from stingless bees have been published recently (Sanches et al., 2017). Most importantly, the recent randomized and placebo-controlled trials demonstrated the therapeutic benefit of propolis in treating Covid-19 patients (Silveira et al., 2021; Kosari et al., 2021).

Bees use propolis to build their hives and to cover any holes from the attack of invaders, climate change and waterproofing from rainfall.

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https://doi.org/10.1016/j.afres.2022.100252
Received 26 May 2022; Received in revised form 18 December 2022; Accepted 22 December 2022
Available online 23 December 2022

Propolis from stingless bees consists mostly of plant resin (50%), wax (30%), essential oil (10%), pollen (5%), bee salivary secretion and other minor constituents such as sugars, amino acids, vitamins, phenolics, terpenoids, tannins and alkaloids (Salatino & Salatino, 2021; Syed Salleh *et al.*, 2021). According to Anjum et al. (2019), more than 300 compounds have been identified in propolis, mostly consists of vegetative resin and plant exudates like polyphenols, coumarins, lignins and tannins and aromatic acids. The quality and composition of propolis is strongly dependent upon the geographical origin, collecting season, bee species and botanical sources available in the surrounding area of bee hives, in addition to the solvent and extraction technology. Propolis has been extracted from the raw material using different extraction techniques ranged from conventional to modern methods. Literature survey found that maceration (Syed Salleh et al., 2021; Mohd Suib et al., 2021; Ibrahim et al., 2016; Abduh et al., 2020; Ishizu et al., 2018) was widely used as non-thermal technique for propolis extraction. It was applied to avoid the degradation of heat sensitive compounds, but longer extraction time, usually 3 to 10 days is required for the process (Wyan, Charland, & Mojica, 2021). There were few studies reporting the application of modern techniques like supercritical fluid (Paviani et al., 2011), microwave (Hamzah & Leo, 2015), ultrasound assisted extraction (Chong & Chua, 2020; Sulaeman et al., 2021; Trusheva and Trunkova, 2007) for propolis extraction. However, the yield of propolis extracted from supercritical fluid was reported to be lower because many phenolic compounds are less soluble in carbon dioxide. Microwave assisted extraction was prone to extract beewax, and thus lower selectivity in extracting bioactive compounds (Trusheva & Trunkova, 2007). Probably, the high content of beewax could be minimized or eliminated by storing the propolis extract in a freezer overnight at -16˚C (Bankova et al., 2019). This would help to solidify the beewax, and then filtering it out the next day as recommended by the method for the extraction of *Apis mellifera* propolis. Oroian et al., (2020) demonstrated higher extraction yield and selectivity of ultrasound assisted extraction than maceration and microwave assisted extraction. Different extraction variables such as extraction time, temperature, solvent and particle size have also been optimized by previous researchers. Trusheva & Trunkova (2007) reported that 30 min was the optimum time of ultrasound assisted extraction to obtain high flavonoids in propolis. Ultrasound is likely to be suitable for propolis extraction from the recalcitrant raw material. Somehow, it is important to monitor the frequency and temperature control condition in ultrasound assisted extraction as these are two factors contributing to the result variation in beewax and balsamic content of propolis (Vilas-Boas, et al., 2022). Such explanation has been proven in a recent international inter-laboratory collaborative trial on the method performance of propolis extraction participated by 12 laboratories from 9 countries.

In the present study, the technique of maceration was applied to extract propolis with and without pretreatment of ultrasound in two different solvent systems, namely water and 20% ethanol. The obtained propolis samples were then characterised for the chemical composition of phenolics, flavonoids and tannins. Methanol was then added to precipitate polymeric substances such as polysaccharides and/or tannins from propolis solution. The addition of alcohol would precipitate polysaccharides via a change in molecular structure conformation and an increase of intra-molecular hydrogen bonding with a concomitant decrease in the dielectric constant of solvent system (Ai et al., 2020)). Tannin is plant polyphenol that usually found in the tree bark, leaves, buds, stems, fruits, seeds and roots with antioxidant property. However, it also exhibits antinutritional property by impairing the digestion of nutrients for body absorption (Popova & Mihaylova, 2019). Therefore, alcohol precipitation has been widely applied in the production of botanical extract like traditional Chinese medicine as the refining process (Tai et al., 2020). The high performance and sensitivity analytical tools of thermal gravimetric analysis coupled with Fourier transform infrared spectroscopy (TGA-FTIR) and liquid chromatography integrated with tandem mass spectrometry (LS-MS/MS) were used to analyse propolis samples. An unsupervised multivariate data analysis approach like principal component analysis (PCA) was statistically used to cluster propolis according to the data similarity.

2. Materials and methods

2.1. Sample and chemicals

The raw material of propolis (*Heterotrigona itama*) was collected from a bee farm in Senai (Johor, Malaysia) in the month of October 2022. The bee form was surrounded by a jaboticaba (*Plinia cauliflora*) plantation. The raw material was stored in a refrigerator at 4 ˚C overnight to ease cutting process for the next day (Kumar et al., 2008). It was cut into small piece around 0.5 to 1.0 cm.

Analytical grade of ethanol, methanol, sodium carbonate, ascorbic acid (99%), formic acid and Folin-Ciocalteau reagent were purchased from Merck (Darmstadt, Germany). The standard chemicals like gallic acid, quercetin (95%) and Trolox (97%) were obtained from Sigma-Aldrich (Missouri, USA). Aluminium chloride hexahydrate (97%) was sourced from Qrec (Selangor, Malaysia).

2.2. Maceration with and without pretreatment

The cut raw propolis (100 g) was put into 2 Schott bottles (1 L) each. One of the bottles was filled up with distilled water and the other bottle was filled up with 20% aqueous ethanol. The total volume of the mixture was 1 L and the bottles were kept in a room at 28 ˚C for 7 days (Bankova et al., 2019).

Similarly, another 2 Schott bottles consisted of 100 g raw propolis were topped up with distilled water and 20% aqueous ethanol to 1 L, respectively. The mixture was then subjected to ultrasonic pretreatment in an ultrasonicator bath (Daihan Scientific Co. Ltd, Wonju-si, Gangwon-do, South Korea) for 30 min and left them at room temperature (28 ˚C) for 7 days (Bonkova et al., 2019).

The mixture was vigorously mixed by shaking the capped bottles for about 2 min daily. The supernatant was harvested from the mixture using filter paper (Whatman, Grade 4, 125 mm diameter) and concentrated by a rotary evaporator (Heidolph, Laborota, Germany) at 40˚C. After concentrated, the supernatant was further dried in an oven at 40˚C for about 3 days to obtain dried propolis. The dried propolis was stored at 4˚C for the subsequent analysis. All experiments were conducted in triplicate unless otherwise stated.

2.3. Determination of proximate phytochemical content

The total phenolic content (TPC) of propolis was determined according to the procedures described by Kurek-Górecka et al. (2022). A 0.5 mL sample (0-3 mg/mL) was mixed with 0.5 mL Folin-Ciocalteau reagent and 0.5 mL 10%w/v sodium carbonate solution. The mixture was then made up to 5 mL with distilled water. The solution was then incubated at room temperature (28˚C) for 60 min and the absorbance was measured at 760 nm using a UV-VIS spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan). Gallic acid (1 – 5 ppm) was used as positive control and its calibration curve was constructed to determine the TPC of sample expressed in milligram of gallic acid equivalent per gram propolis extract (mg GA/g).

The total flavonoid content (TFC) of propolis was determined according to the method reported by Abduh et al. (2020). A 2 mL sample (0-5 mg/mL) was mixed with 5% aluminium chloride and incubated at 28˚C for 30 min. The absorbance of sample was recorded using a UV/VIS spectrophotometer at 415 nm. Quercetin (1–5 ppm) was used as positive control and its calibration curve was prepared to determine the TFC of sample expressed in milligram of quercetin equivalent per gram propolis extract (mg Q/g).

The total tannin content (TTC) of propolis was quantified using the method of casein precipitation as reported by Monteiro et al. (2014). About 1 g casein was mixed with 6 mL sample (0-3 mg/mL) and topped

up with 12 mL distilled water. The solution was stirred with a magnetic stirrer for 3 h at 25°C. Subsequently, the precipitate in the solution was separated out using filter paper. The filtrate was tested for non-tannin phenolics using the TPC method as stated above. Therefore, TTC can be calculated using Eq. (1).

$$\text{Total tannin content (TTC)} = \text{TPC} - \text{TPC}_{\text{non-tannin}} \quad (1)$$

2.4. Alcohol precipitation of propolis

Another set of propolis samples prepared as above were concentrated in a rotary evaporator to about 20 mL. Methanol (20 mL) was then added into the concentrated propolis solution and centrifuged to harvest precipitate formed at the bottom of flasks. The TPC of precipitate was analyzed using Folin-Ciocalteau assay. Acid hydrolysis (pH 2.5) was also carried out on the precipitate. Two drops of formic acid were added into the precipitate which was re-constituted in water (2000 mg/L). The solution was vigorously mixed for 5 min. The pH of the solution was recorded using a calibrated digital pH meter (Laquatwin, Horiba, Kyoto, Japan), whereas total sugar was measured as Brix value using a refractometer (MA885 Milwaukee, Wisconsin, USA). The pH and total sugar content were measured before and after acid hydrolysis (Ferreira et al., 2020).

2.5. Ultra performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS)

An UPLC-MS/MS was used for high throughout mass screening of propolis samples. This hybrid system integrated UPLC (Waters Acquity, Milford, MA) with a triple quadrupole-linear ion trap tandem mass spectrometer (Applied Biosystems 4000 Q TRAP; Life Technologies Corporation, Carlsbad, CA). Compounds were separated by a C18 reserved phase column (Acquity, 150 × 4.6 mm, 1.7 μm) and then ionized by an electrospray ionisation (ESI) source for mass detection.

The mobile phase of UPLC system was consisted of solvent A (water with 0.1% formic acid) and solvent B (CH_3CN). The gradient of the mobile phase was: 0–5 min, 10% B; 5–15 min, 10–90% B; 15–20 min, 90% B; 20–21 min, 90–10% B; 22–30 min, 10% B for column equilibration. The flow rate was 0.15 mL/min and the injection volume was 5 μl. All samples were filtered with 0.2-μm nylon membrane filter prior to injection.

The scan mode of enhanced mass spectra (EMS) integrated with two parallel Enhanced product ion (EPI) runs was setup using information dependent acquisition (IDA) to acquire the mass fragments of compounds. The acquisition was performed in both positive and negative modes ranged from m/z 50–1500. The capillary and voltage of the ion source were maintained at 400°C and 5.5 kV (−4.5 kV), respectively. Nitrogen was used for nebulization (40 psi), solvent drying (40 psi) and as curtain gas (10 psi). The scan rate was 1000 amu/s. Data acquisition and data processing were performed using Analyst 1.4.2.

2.6. Thermogravimetric analysis coupled to Fourier Transform Infrared Spectroscopy (TGA-FTIR)

The alcohol precipitated sample (25.798 mg) was put on a ceramic pan and analyzed by a thermogravimetric analyser (Q500, TA Instruments, New Castle, USA) coupled to Fourier transform infrared spectrometer (Nicolet^{TM}, ThermoFisher Scientific, Massachusetts, USA) in a dynamic programmed temperature ranged from 20 and 1000°C at a constant heating rate of 20°C per min under an inert atmosphere supplied with high purity nitrogen gas (99.5% nitrogen and 0.5% oxygen). The high value of the first derivative weight of sample was scanned using the FTIR spectrometer from 500 to 4000 cm^{-1} at 8 cm^{-1} resolution.

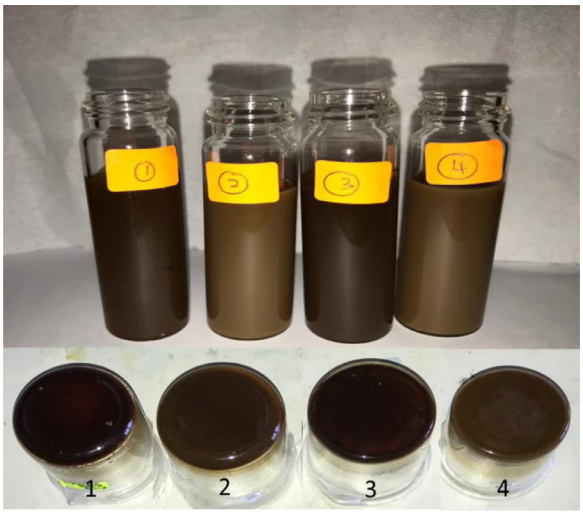

Fig. 1. The appearance of concentrated propolis solution and its dried extract, (1) maceration in 100% water, (2) maceration in 20% ethanol, (3) maceration with ultrasonic pretreatment in 100% water and (4) maceration with ultrasonic pretreatment in 20% ethanol.

2.7. Statistical analysis

Statistical analysis was conducted for the data of extraction yield, TPC, TFC and TTC. A pattern recognition tool of principal component analysis (PCA) was also used to cluster samples according to their similarities after statistical dimensional reduction. The software of MarkerView 1.1 (AB SCIEX, Foster City, Canada) was used to perform the PCA with Pareto scaling.

3. Results and discussion

3.1. Physical property and propolis extraction

The colour of propolis which will affect the appearance and acceptance of customers is very important for product development. Fig. 1 shows the images of concentrated propolis solution after extraction. It was found that propolis extracted by water (sample 1 and 3) appeared to be darker than samples extracted by aqueous ethanol (sample 2 and 4). The propolis solution extracted by maceration either with or without ultrasonic pretreatment by 20% ethanol was yellowish and having a sticky sensation.

The yield of propolis was between 4 and 5.5% as presented in Fig. 2. The results found that maceration with ultrasonic pretreatment showed to have higher performance in extracting propolis from the raw material, particularly in the binary solvent system of 20% ethanol. Ultrasound is a kind of energy created through sound wave which induces the production of cavitation bubbles within the liquid. The bubbles would increase in size and then collapse continuously over the extraction time. The phenomenon would generate pressure and increase temperature, and thus enhancing the penetration of solvent into sample matrix to release compounds into solvent (Xu et al., 2017). The use of binary solvent system would facilitate the extraction of water and ethanol soluble compounds, mostly phenolics and flavonoids. This explains that higher yield of propolis could be obtained from aqueous ethanol medium. The variance was about 1% for different solvent systems, and 0.5% for different extraction technologies.

A group of researchers reported that the yield of propolis was ranging from 4.91 to 12.85% from the raw material collected from different regions of Indonesia (Fikri et al., 2019). It was noticed that the yield of propolis from the region of Banten was comparable with the result of this study. The researchers also used similar ratio of solid-to-solvent,

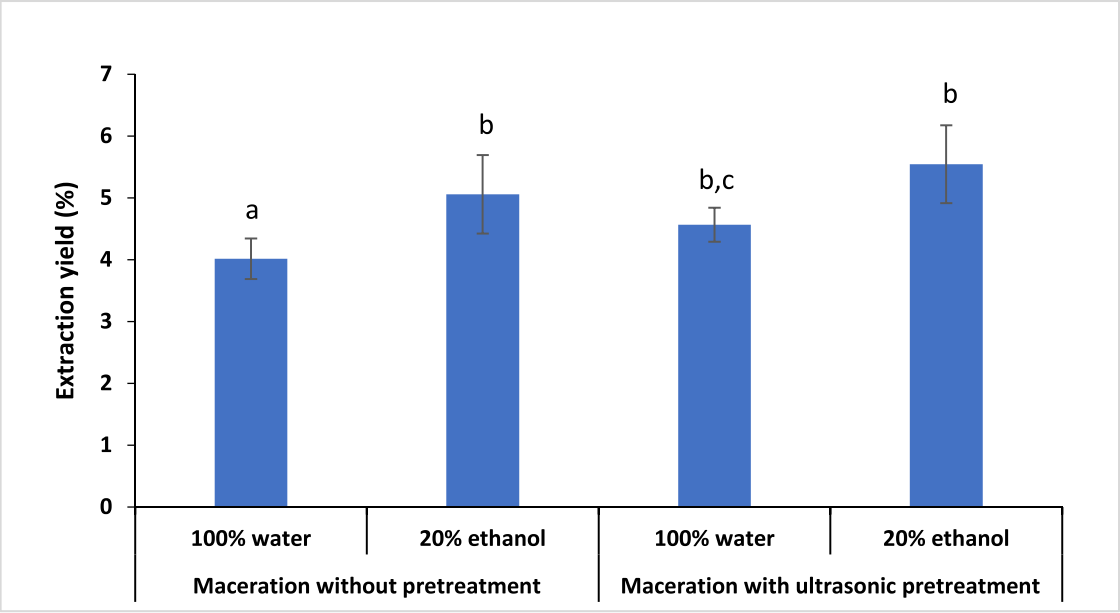

Fig. 2. Yield of propolis extraction using different solvent systems in different extraction technologies such as maceration with and without ultrasonic pretreatment. Different small letters indicate the significant difference of paired two samples for means by T-test

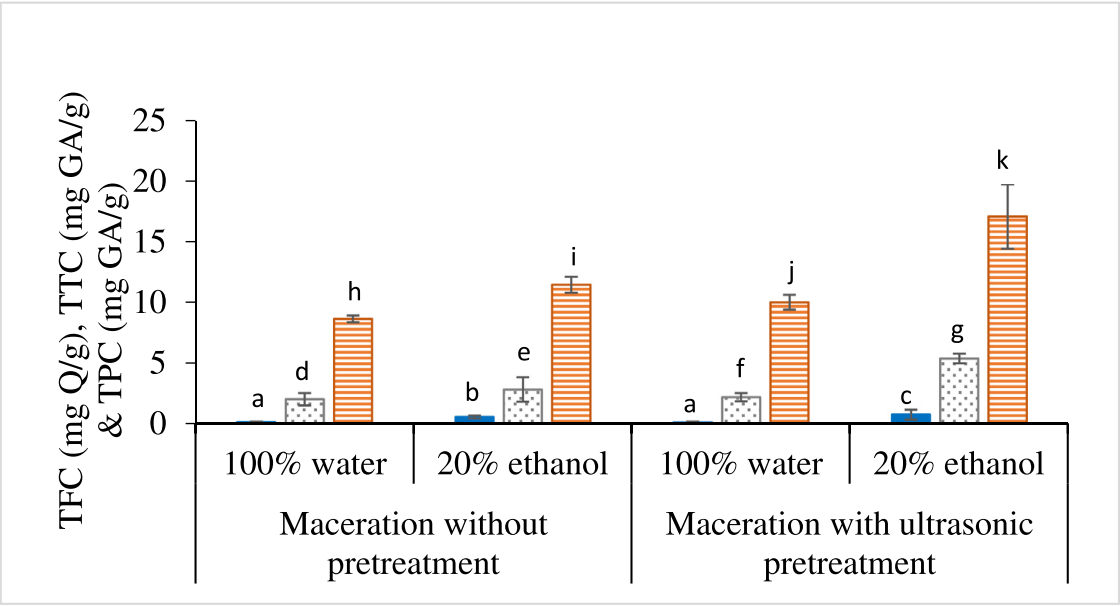

Fig. 3. Total flavonoid content (TFC, blue solid bar), total tannin content (TTC, dot bar) and total phenolic content (TPC, line bar) of propolis. Different small letters indicate the significant difference of paired two samples for means by T-test

1:10 in both water and aqueous ethanol system assisted by ultrasound in the extraction. They also reported higher yield of propolis was obtained from aqueous ethanol, even though the variance of yield was not statistically different.

3.2. Chemical composition of propolis

Fig. 3 shows the proximate chemical composition of propolis extracted from different solvent systems using different extraction technologies. The results revealed that propolis samples had the lowest content of flavonoids, followed by tannins and phenolics. The TFC and TPC reported in the present study were higher than those results reported by previous researchers who sourced the raw material of *H. itama* from different geographical origins (Asem et al., 2020). The results were also comparable with the TFC and TPC of propolis harvested from the beehive of *Tetrigona melanoleuca* and *Tetrigona binghami* (Awang et al., 2018). However, the observation was not in line with previous studies which reported higher flavonoids than phenolics in propolis (Syed Salleh et al., 2021). Anyhow, the chemical variation in propolis mostly contributed to the difference of plant preference and vegetation pollinated by bees (Awang et al., 2018). There was also limitation in the colorimetric assays as the non-specific Folin-Ciocalteau reagent in TPC as-

Table 1
Mass fragments detected in propolis samples extracted from different solvent systems using different extraction technologies.

Extraction	m/z (-)	m/z (+)	Putative compound	Refs.
Maceration in water	407/255(-152)/151/107		gallyol pinocembrin	Falcão et al. (2010)
	425/273(-152)/215/177/151		gallyol afzelechin	Ben Said et al. (2017)
Maceration in 20% ethanol	265/97/80		isomagnolol	Xu et al. (2010)
	407/255/151		gallyol pinocembrin	Falcão et al. (2010)
	441/289/151	441/337/314/210/170	gallyol catechin	Zhao et al. (2013)
		338/321/303/177(-161)/109/69	feruloyl hexose	Da Cruz et al. (2022)
	339/183(-156)		esculetin-6-O-glucoside	Vuković et al. (2018)
Ultrasonic pretreated maceration in water	265/97		isomagnolol	Xu et al. (2010)
	407/255(-152)/151		gallyol pinocembrin	Falcão et al. (2010)
	423/271(-152)/213/151/107		gallyol naringenin	Ye et al. (2012)
	425/273(-152)/215/177/151	425/257/173/153	gallyol afzelechin	Ben Said et al. (2017)
	439/421/337/298/287(-152)/270/151/122		(Epi)catechin 3-O-vanillate	Šuković et al. (2020)
	441/395/289(-152)/271/213/187/151		gallyol catechin	Zhao et al. (2013)
	447/431/267/148/149		luteolin hexoside	Kang et al. (2016)
	653/447(-206)/417/355/285/149		kaempferol sinapoyl hexoside	Lin et al. (2014)
	793/537(-256)		amentoflavone	Pereira et al. (2015)
	315/300/255/151/145/133/		isorhamnetin	Fathoni et al. (2017)
Ultrasonic pretreated maceration in 20% ethanol	407/255(-152)/151	407/287/267/197/179/153	gallyol pinocembrin	Falcão et al. (2010)
	409/351(-58)/306/295/255(-154)	409/353/267/153	protocatechyl pinocembrin	Falcão et al. (2010)
	423/271(-152)/213/151/107		gallyol naringenin	Ye et al. (2012)
	425/273(-152)/215/177/151	425/407/299/283/257/187/183/153/155	gallyol afzelechin	Ben Said et al. (2017)
	439/353(-86)/287(-152)/151		gallyol aromadendrin	Chen et al. (2016)
	441/289(-152)/231/151	441/317/275/233/219/177	gallyol catechin	Zhao et al. (2013)
	847(2M-1)/721(-126)/441(-406)/423(-424)/287(-136)/151(-136)		gallyol naringenin dimer	Ye et al. (al. (2012)
	447/431/267/148/149		luteolin hexoside	Kang et al. (2016)
	653/447(-206)		kaempferol sinapoyl hexoside	Lin et al. (2014)
	315/300/255/151/145bp/133/		isorhamnetin	Fathoni et al. (2017)

say could simultaneously oxidize non-phenolic organic compounds and some inorganic substances (Pękal & Pyrzynska, 2014). Indeed, the Folin-Ciocalteau assay can be used to estimate the antioxidant capacity of samples because it follows the basic mechanism of oxidation-reduction reaction as reported by Prior et al. (2005). Hence, Fig. 3 also illustrates that the antioxidant capacity of propolis extracted from 20% ethanol is higher, especially sample pretreated with ultrasonication.

The present study has almost similar extraction procedure with the study of Escriche & Juan-Borrás (2018) who performed three types of double extractions, namely maceration-maceration, maceration-ultrasonication and ultrasonication-ultrasonication for comparison. The double extraction of 30 min of ultrasonication after 24 h of maceration was close to the extraction method of the present study. They reported that the phenolic content of propolis from the double extraction of maceration-ultrasonication did not have significant difference with the double extraction of maceration-maceration and ultrasonication-ultrasonication. They also concluded that the extraction method had lesser influence on the extraction yield and phenolic content, but the variation was more likely to be propolis origin. Besides propolis origin, the lack of consensus in using the standard chemical and solvent of Folin-Ciocalteau assay has also complicated the comparison of TPC in propolis (Osés et al., 2020). This had also been proven for TFC in which different standard chemicals used in the assay would produce different results. Somehow, the TPC and TFC of stingless bee propolis in the present study were lower than the values reported by Osés et al. (2020) who collected 13 different propolis samples from different geographical areas, mostly from European countries.

The use of Al(III) preferred to form complex with flavonols and flavones which could exhibit high absorbance around 400 nm in the TFC assay. The maximum absorption strongly depends on the presence of a double bond in the positions of C2–C3 on the skeleton of flavonols and flavones. However, flavanones and flavononols were unable to detect based on the colorimetric assay. They do not have a double bond at C2-C3, and thus not showing absorbance at 415 nm. The mass screening results proved the statement that many flavanones and flavononols were detected in propolis samples as presented in Table 1. Few glycosylated flavonoids were also detected from propolis samples, except propolis extracted from maceration in water.

The tannin content of propolis is relatively limited reporting in literature. The tannin content of Brazilian propolis was ranging from 0.6 to 4.1% (Mayworm et al., 2014) and Chilean propolis was between 15.5 and 20.2% (Alvear et al., 2021). The results of previous researchers were higher than the TTC reported in this study ranged from 0.2-0.5%. TTC was found to be positively correlated with TPC. The ratio of TTC to TPC (0.22–0.32) was comparable with Brazilian propolis (0.13-0.28). Both hydrolyzable (gallic and/or ellagic) or condensed tannins (proanthocyanidins) fragments were detected based on the mass screening using LC-MS/MS. Kiziltas & Erkan (2021) revealed that tannin was the compound contributing to the dark colour of propolis. Possibly, tannins were attributed to the woody plant of Jaboticaba (Myrtaceae) because the raw material of propolis was harvested from the Jaboticaba plantation.

3.3. Principal component analysis for sample clustering

The propolis samples were then subjected to an unsupervised statistical PCA using mass screening spectra for sample clustering (Fig. 4). The huge and complex masses detected in samples was able to reduce into 7 principal components without compromising data information. The mass spectral analysis demonstrated that solvent system was the major contributor to the variance among samples. This can be seen from the score and loading plots either at the positive (Fig. 4a and b) or negative (Fig. 4c and d) ion modes. The first principal component (PC1) had explained 32.8% and 32.5% of the total variance. The combination of PC1 and PC2 covered for more than 50% of the total variance.

PCA was also applied on the data of chemical analyses, namely extraction yield, TPC, TTC, and TFC of 4 different propolis samples. The proximate chemical content had covered all major components in propolis. Again, the score plot shows solvent system is a very important factor to differentiate propolis samples (Fig. 4e). The PC1 had covered for 89.3% of total variance in which extraction with water was located at the negative region, whereas the aqueous ethanol was located at the positive region of the score plot. The variance of chemical content was

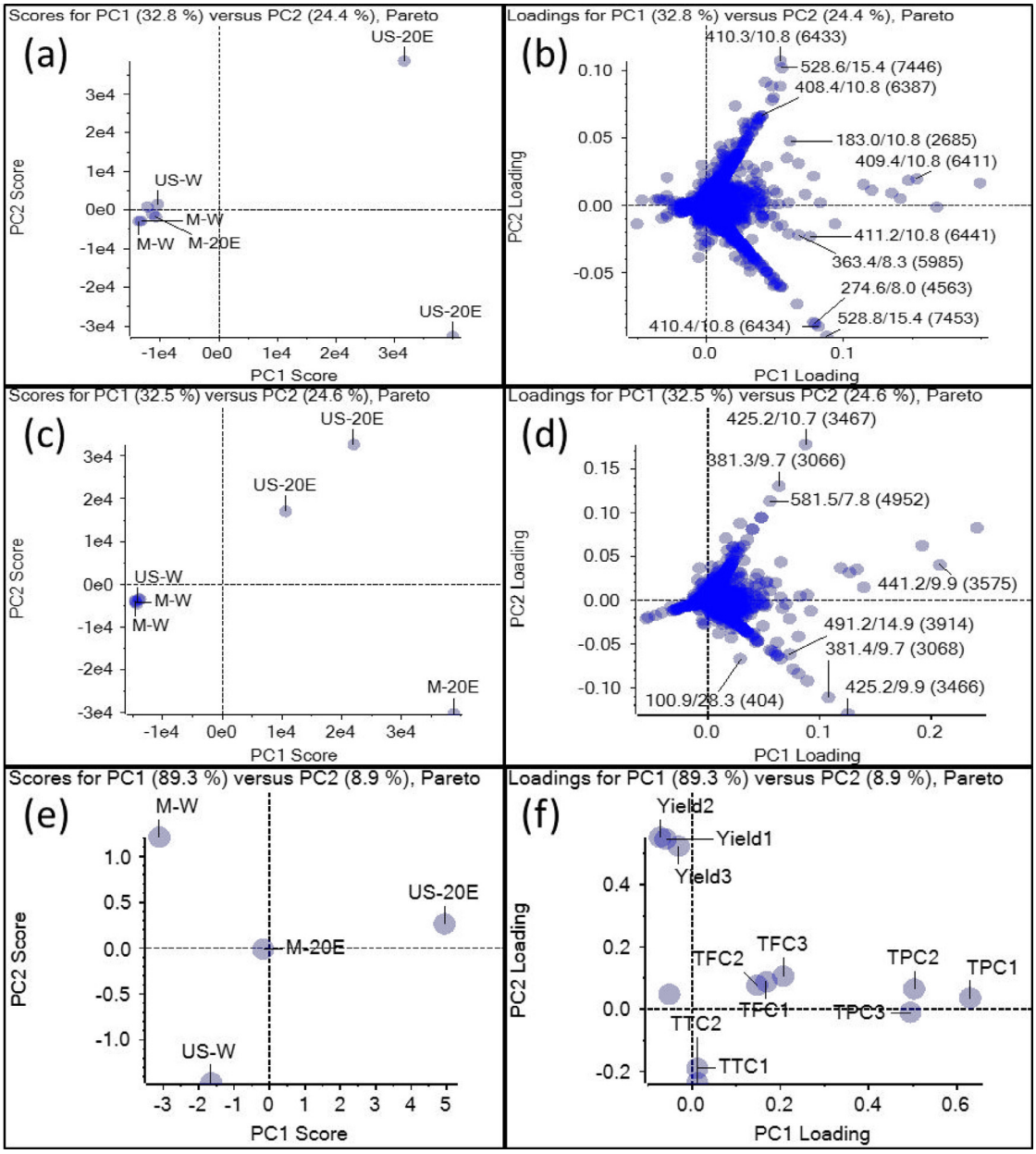

Fig. 4. Principal component analysis of four propolis samples, namely maceration in 100% water (M-W), maceration in 20% ethanol (M-20E), maceration with ultrasound pretreatment in water (US-W) and maceration with ultrasound pretreatment in 20% ethanol (US-20E): (a) Score plot of propolis samples prepared from different solvent systems using different extraction technologies at the positive ion mode of LC-MS/MS; (b) Loading plot of propolis samples prepared from different solvent systems using different extraction technologies at the positive ion mode of LC-MS/MS; (c) Score plot of propolis samples prepared from different solvent systems using different extraction technologies at the negative ion mode of LC-MS/MS; (d) Loading plot of propolis samples prepared from different solvent systems using different extraction technologies at the negative ion mode of LC-MS/MS; (e) Score plot of propolis samples prepared from different solvent systems using different extraction technologies for chemical analysis; (f) Loading plot of propolis samples prepared from different solvent systems using different extraction technologies for chemical analysis.

mainly contributed by TPC in the propolis samples as shown in the loading plot (Fig. 4f).

3.4. Alcohol precipitation for polymeric substance

An equal portion of alcohol volume was added into the concentrated propolis solution to precipitate large molecules. Most probably, the large and highly water soluble molecules would be precipitated due to the restriction of compound solubility resulted from the reduction of solvent polarity. Water has the highest relative polarity and the addition of methanol will reduce solvent polarity. In the present study, the relative polarity of water system was reduced from one to 0.881, whereas the relative polarity of 20% ethanol system was reduced from 0.931 to 0.881.

Acid hydrolysis was then carried out on the propolis precipitate for further investigation. It was found that the initial pH of propolis precipitate was 5.5. This mild acidic solution might be contributed by the existence of coniferyl, coumaryl or sinapyl residues in lignins. The Brix values of propolis precipitate was also increased to twice after hydrolysis. The value was increased from 0.9% to 3.2% which indicated that propolis precipitate might largely contain cellulosic components. Acid hydrolysis might break down celluloses into mononsaccharides, disaccharides or oligosaccharides, and thus contributing to the increment of total sugar (Sakamoto et al., 2020). PCA also found that the propolis

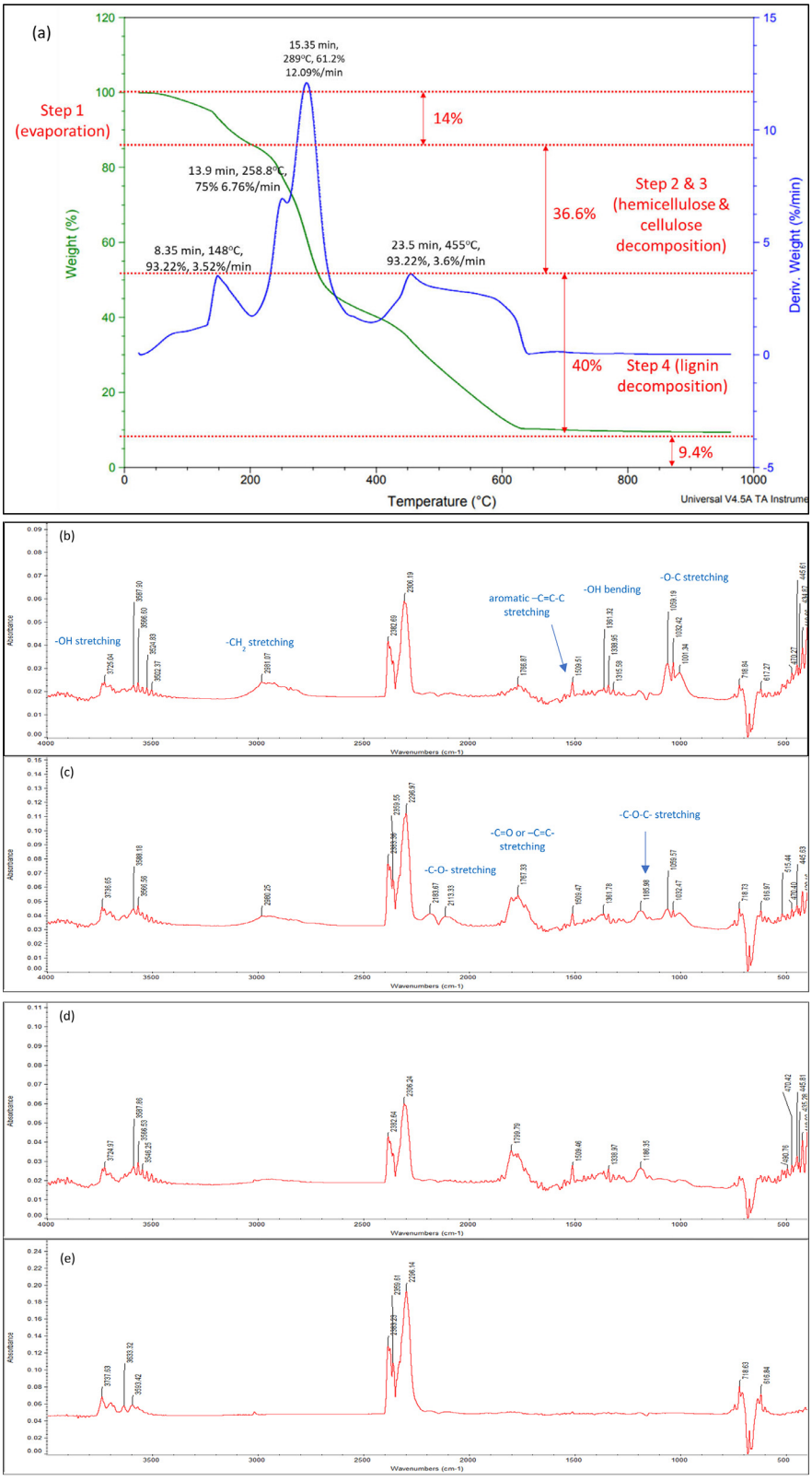

Fig. 5. (a) Thermogravimetric curves of propolis precipitate, (b) FTIR Spectrum at 13.9 min, (c) FTIR spectrum at 16.5 min, (d) FTIR spectrum at 20.9 min and FTIR spectrum at 29.5 min.

precipitates had almost similar mass profile, and therefore, they were combined for TGA-FTIR.

The precipitate of propolis was then characterised by TGA-FTIR which is a powerful analytical technique for unknown polymer and its thermal behavior. The degradation of the substance could be divided into 4 steps based on the thermogravimetric curve (Fig. 5a). The first step was dehydrating process in which about 5.6% of residual free water on the surface of substance was started to evaporate at the onset temperature of 22.4°C. Further increasing the temperature, the physically bound moisture was liberated from the inner structure of substance. The bound moisture achieved the highest evaporation rate of 3.52%/min at 148.1°C as indicated in the derivative thermogram. The

substance could be a hygroscopic compound incorporated with 14% of moisture. (Baaka et al., 2017) mentioned that some easily degraded small molecules like organic acids might be degraded at this stage.

The second step was likely the degradation of tannins or hemicellulose residues through inter- and intra-molecular hydrogen bonds which began at 208˚C (Zhao & Umemura, 2015; Baaka et al, 2017; Nardeli et al., 2019). The process of decarboxylation to release carbon dioxide was observed at the maximum heating temperature of 254.3˚C. The degradation also contributed to the vibrational stretching of symmetric and asymmetric CH2 (2981.07 cm^{-1}), and vibrational stretching of CH, CH2 and CH3 (2306.19 and 2382.69 cm^{-1}) which were detected in FTIR spectrum (Fig. 5b). The stretching -C-O-C (1001.34-1059.19 cm^{-1}) related to the presence of glycosidic bond in carbohydrates (Ismail et al., 2021; Deus et al., 2021). Da Costa Lopes *et al.* (2013) reported that the linkage of C-O-C explained the skeletal vibration of pentose and hexose units which are commonly present in hemicellulose. The absorption at 1185.98 cm^{-1} was assigned to -C-O asymmetric stretching vibration arising from the pyran-derived ring structure of condensed tannins (Chupin et al., 2013) (Fig. 5c). The small bands around 1400–1300 cm^{-1} associated with the -OH bending of -C-OH group which is also one of the characteristics of carbohydrates. Hence, the bands were confirmed by the presence of unconjugated C=O stretching in the acetyl group of hemicelluloses.

The intensity of the absorption bands was further increased in the third step of degradation mechanism, mostly attributed to the decomposition of celluloses. The highest weight loss rate of 12%/min was observed just after 1.45 min of derivative weight for hemicelluloses. This was the major decomposition step of most thermochemical conversions or called as devolatilization of biomass. Therefore, the derivative thermal curve demonstrated dual peaks for the maximum degradation temperatures at 254.3˚C and 294˚C. Previous studies reported that hemicellulose was decomposed at the lower range of temperature than cellulose (Yang et al., 2007; Lv et al., 2010). The observation of this study was in line with the finding of (Subhedar & Gogate, 2014) who reported the degradation of cellulose started around 250˚C and nearly decomposed totally into volatile products at 400˚C. Based on the thermogram, the components of hemicelluloses and celluloses might cover for about 36.6% of the total mass of polymer (Fig. 5a).

The decomposition of lignin could be involved in the fourth step of degradation mechanism. This can be seen from the increment of stretching C=C and C=O bands at 1799.79 cm^{-1}, and stretching C-O-C at 1186.35 cm^{-1} (Fig. 5d). The first derivative thermogram showed another small thermal peak at the maximum degradation temperature of 460˚C and the weight loss indicated that lignin composed approximately of 40% of the total mass (Fig. 5a). The lignin component also explained its TPC (0.652 ± 0.188 mg GA/g). The low TPC would also have low antioxidant capacity. The polymer decomposition was slowly continued until completely reached the degradation at 625˚C at the final step of mechanism. Only the strong CO2 absorption bands (2383.61–2296.14 cm^{-1}) and small O-H stretching (3593.42-3737.63 cm^{-1}) were detected at this stage in the FTIR spectrum (Fig. 5e). The polymer was also consisted of 9.4% of inorganic component as ash in the crucible after pyrolysis. The degradation of this polymer occurred in a broad temperature interval (141.8–625˚C) because of various functional groups interrelated complexly with each other. Nevertheless, the polymer showed the highest degradation temperature at 289˚C and lignin almost covered for half of the total mass of polymer. Lignin appeared to be the major contributor of recalcitrant component in biomass and this had explained the recalcitrant structure of raw propolis in this study (Yoo et al., 2020).

4. Conclusion

Maceration was a useful technique for propolis extraction and the introduction of ultrasonic pretreatment for a short period of time would increase the quality of propolis in term of phenolics, flavonoids and tannins, as well as antioxidant capacity. The effect of solvent was found to be higher than the extraction techniques as proven in the pattern recognition tool of PCA. The polymeric substance was removed by alcohol precipitation and then characterised by a high performance hyphenated technique of TGA-FTIR. Possibly, the polymeric substance was tannin bonded lignocelluloses which could be removed by alcohol precipitation for propolis quality improvement.

Ethical statement - studies in humans and animals

The authors declare that neither human or animal is involving in this study.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

Acknowledgement

The authors would like to thank Jabo Plantation Sdn Bhd for giving research grant (4C541 and 4C542) to support the cost of experimental works.

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