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Short communication

Chemical Composition and Antioxidant Activity of Stingless Bee Propolis from Different Extraction Methods

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Abstract

Propolis has been used as therapeutic agent since ancient time and considered as high value materials. In order to obtain maximum yield of extract production with excellent biological activity, extraction techniques need to be established. This study aimed to optimize extraction protocol and screen total phenolic, total flavonoid and antioxidant activity. Propolis was collected from Apiary of UniSZA, Besut Campus. Propolis was extracted with 70% and 95% ethanol using different extraction methods, which were: maceration, sonication and soxhlet. Total phenolic content and flavonoid content were determined using spectrophotometric method. The antioxidant activity was evaluated using 2, 2-diphenyl-1-picrylhydrazyl (DPPH assay). Extraction by maceration at 5 days of ethanolic extract showed the highest total phenolic content of 46.68±2.08 mg gallic acid equivalents per g extract propolis (GAE/g). While, 70% ethanolic from 30 minutes of sonication method displayed the highest total flavonoid content compare to that 95% ethanol with value 107.27±4.10 mg quercetin equivalents per g extract (QE/g). The lowest total flavonoid content was 95% ethanolic extract by soxhlet extraction at 6 hours (26.71±3.58QE/g). The propolis extract of 70% ethanol from sonication (30 minutes) also showed a good antioxidant activity with IC_{50} value 11±0.55µg/mL Trolox equivalents per g extract. Finding from this study showed the most specification of extraction methods is important to extract specific compounds and quality extract for propolis.

Keywords: *stingless bee, propolis, phenolic, flavonoid, antioxidant*

1. Introduction

Heterotrigona itama is a stingless bee species belong to Apidae family and acts as pollinator [1][2]. This species is well - known among beekeepers in Malaysia. Propolis is the ones of natural products produced by stingless bees besides honey and beebread. Propolis is resinous substances that produce by bees or stingless bees. It is a gummy and waxy resinous substance collected from various plant sources [3]. Propolis is a sticky at room temperature and hard and brittle at low temperature [4]. It is used in hive as building materials as well as defensives substances from insects or microorganism [5]. The colors of propolis ranges from yellow to dark brown depend on the origin of resin while has aromatic smell and bitter to almost sweet [1]. Propolis has been established as health-related products and therapeutic agent since ancient time and considered as high value materials. Propolis contains various bioactive constituents that related to antioxidant such as total phenolic contents, total flavonoid contents and others [6]. In particular, flavonoids have been described as the main group of phenolic compounds responsible for biological properties. The composition of propolis is depends on its botanical sources and geographical origin and vegetation [7]. Factors that may affect the amount of chemical compound and biological activity in propolis are extraction methods and solvents used for extraction of propolis [3]. Extraction is a technique to breakdown the plant cell wall to releases phytochemical in the samples [8]. There are many extraction method has been established such as maceration, sonication, soxhlet, microwave and many more. Usually, solvent used to extract are ethanol, methanol, dichloromethane, hexane, ethyl acetate and others. Extraction techniques need to be optimized in order to obtain maximum yield of extract production with excellent biological activity under optimum extract condition. So, this study is aimed to optimize extraction protocol by maceration, sonication and soxhlet and screen total phenolic, total flavonoid and antioxidant activity with different percentage of solvent extraction and different parameter of extraction based of time period.

2. Methodology

2.1. Chemical and reagents

All the chemicals and reagents used in this experiment is analytical grade reagents. Ethanol, methanol and dimethyl sulfoxide (DMSO) were purchased from Merck Sdn. Bhd, Selangor, Malaysia; Folin-Ciocalteu's reagent, 2,2- diphenyl-picryl-hidrazyl (DPPH), quercetin, gallic acid, aluminium chloride, sodium carbonate,

potassium acetate were acquired from Sigma-Aldrich (M) Sdn. Bhd. (Kuala Lumpur, Malaysia); Trolox® was purchased from Calbiochem® (Selangor, Malaysia).

2.2. Sample Collection

The propolis samples were obtained from Apiary Farm, Universiti Sultan Zainal Abidin (Besut Campus). The propolis samples were frozen at -80 ºC and ground in the grinder to obtain powder form. These processes must be handled quickly as the propolis samples can easily be sticky at room temperature and difficult to handle. Then, the propolis samples were kept back in -80ºC for further analysis.

2.3. Sample Extraction

2.3.1. Maceration Extraction

Approximately 18g of propolis were extracted in 60 mL of 70 % and 95% ethanol. Propolis samples were macerated at room temperature for 1, 3, 5 and 7 days. The solutions were filtered and concentrated under vacuum pressure at 45°C. The extracted propolis were kept in 4°C prior analysis. The crude propolis samples were labelled as 70M-1d (ethanol 70%- maceration- 1 day), 70M-3d (ethanol 70%- maceration- 3 days), 70M-5d (ethanol 70% - maceration- 5 days), 70M-7d (ethanol 70% - maceration- 7 days), 95M-1d (ethanol 95% - maceration- 1 day), 95M-3d (ethanol 95% - maceration- 3 days), 95M-5d (ethanol 95% - maceration- 5 days) and 95M-7d (ethanol 95% - maceration- 7 days).

2.3.2. Sonication Extraction

Approximately 18g of propolis were extracted in 60 mL of 70 % and 95% ethanol. The samples were sonicated at 10, 30, 60 and 120 minutes using ultrasonic bath at 37°C. Furthermore, the solutions were filtered, concentrated under vacuum pressure at 45°C and kept in 4°C until further analysis. The crude propolis were labelled as 70S-10m (ethanol 70% - sonication – 10 minutes), 70S-30m (ethanol 70% - sonication – 30 minutes), 70S-60m (ethanol 70% - sonication – 60 minutes), 70S_120m (ethanol 70% - sonication – 120 minutes), 95S_10m (ethanol 95% - sonication – 10 minutes), 95S-30m (ethanol 95% - sonication – 30 minutes), 95S-60m (ethanol 95% - sonication – 60 minutes) and 95S-120m (ethanol 95% - sonication – 120 minutes).

2.3.3. Soxhlet Extraction

Approximately 5g of propolis were extracted in 150 mL of 70 % and 95% ethanol. Propolis samples were subjected to 2, 4, 6 and 8 hours soxhlet extraction. Then, the solutions were filtered, concentrated under vacuum pressure at 45°C and kept in 4°C prior analysis. The crude propolis were labelled as 70SH-2h (70% ethanol – soxhlet – 2 hours), 70SH-4h (70% ethanol – soxhlet – 4 hours), 70SH-6h (70% ethanol – soxhlet – 6 hours), 70SH-8h (70% ethanol – soxhlet – 8 hours), 95SH-2h (95% ethanol – soxhlet – 2 hours), 95SH-4h (95% ethanol – soxhlet – 4 hours), 95SH-6h (95% ethanol – soxhlet – 6 hours), and 95SH-8h (95% ethanol – soxhlet – 8 hours).

2.4. Percentages of Extraction Yield

The percentages of extraction yield was calculated as following formula:

$$\% \text{ yield} = \frac{\text{Weight of propolis extracts (g)}}{\text{Weight of propolis raw powder (g)}} \times 100\%$$

2.5. Total phenolic content

Total phenolic content was determined using Folin-Ciocalteau colorimetric modified method with gallic acid as a standard [9]. The principle of this method is based on the oxidation and reduction in alkaline condition, which the phenolate ion is oxidized. The MO^{6+} and W^{+} complex ion in follin –ciocalteu is reduced and change the reaction turn to blue color [4]. The total phenolic content was expressed as mg gallic acid equivalents per g of sample extract (mg GAE/ g extract). Briefly, 1mg/mL of gallic acid were prepared as stock solution in a microtubes and were made serial dilution to produce final concentration at 60, 80, 100, 120, 140, 160, 180 and 200 mg/mL. For propolis extract, 5 mg/mL were prepared as stock solution and 60 µL were pipetted from stock solution and were added into microtubes to produce final concentration (1mg/mL) of samples extract. Then, gallic acid and propolis extract were make up to 100 µL with methanol. After that, 200 µL of Folin-Ciocalteau reagent were added into microtubes and vortex thoroughly to mix up the solution. Then, 800 µL of 7.5% sodium carbonate (Na_2CO_3) were added and the contents were mixed thoroughly and the blue color solution was developed. (Sodium carbonate act as stop reaction and excluded in final volume in microtubes). The microtubes were allowed for 2 hours at room temperature in the dark. The absorbance of the mixture was measured at 765nm. Total phenolic content of samples extracts were calculated using linear regression equation obtained from Gallic Acid Equivalent (GAE) calibration curve and following formula:

$$C = cV/m$$

Where,

C: total phenolic content (mg of GAE / g of samples extract)

c: the concentration of gallic acid (mg/mL) established from the calibration curve

V: final volume of propolis extract

m: the weight of propolis extract

2.6. Total flavonoid content

A calorimetric assay was used to quantify total flavonoid content in the propolis [11] with some modification. The flavonoid content was expressed as mg of quercetin equivalent (QE) per g of sample extract. Therefore, quercetin of various concentrations was used as a standard for the calibration curve. Accurately weighted of 0.5 µg/ mL of standard was dissolved in 1 mL of methanol. The standard were made serial dilution in micro tubes from the stock solution to produce final concentration of 0.78125, 1.5625, 3.125, 6.25, 12.5, 25, 50, 100 µg/ mL. For propolis extract, 140 µL of stock solution (5 mg/mL) of each propolis extracts were added into micro tubes to get 1 mg/mL of final concentration of samples extracts. Then, 150 µL of 10% aluminium chloride (AlCl3) were added into micro tubes for both standard and propolis samples. Subsequently, 150 µL of 1M of potassium acetate were added into all micro tubes and then make up to 700 µL with distilled water to complete the final volume of each micro tubes. Absorbance was taken at 415 nm after 30 minutes incubation at room temperature in the dark to complete the reaction. The total flavonoid content of samples extracts were calculated using linear regression equation obtained from quercetin Equivalent (QE) calibration curve and following formula:

$$C = cV/m$$

Where,

C: total flavanoid content (mg of QE / g of samples extract)

c: the concentration of quercetin (mg/mL) established from the calibration curve

V: final volume of propolis extract

m: the weight of propolis

2.7. Assessment of antioxidant activity

The antioxidant capacity of the extracts (IC_{50}) were estimated and compared with trolox (positive control) using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical according to [14] Jo et al. (2012) with slight modification. An amount of 5 mg/mL of the stock solution of standard (trolox) and propolis extracts was dissolved in 1 mL of dimethyl sulfoxide (DMSO). All standard and samples were made serial dilution in 96-well micro titre plate from the stock solution to produce final concentration of 7.8125, 15.625, 31.25, 62.5, 125, 250, 500 µg/ mL in each respectively wells. The samples and standards then were mixed with 0.125 mM DPPH in methanol, to produce a final DPPH concentration of 0.1 mM in final volume of 250 µL each well. Absorbance was taken at 517 nm after 30 minutes incubation at room temperature in the dark to complete the reaction. The percentage of inhibition was calculated using the following formula:
Percentage of inhibition:
[1-(A517nm, sample / A517nm, control) x 100]

2.8. Statistical analysis

Assays were performed in triplicate (n=3) and the results were expressed as mean values with standard deviation. The significant differences were analyzed by one-way analysis of variance (ANOVA) and followed by post hoc turkey's test.

3. Results

3.1. Percentages of Extraction Yield

The yield of extraction is depends on solvents, methods and times of extraction used. Based on the solvent extraction between 70% and 95% ethanol, 95% of ethanol revealed higher extraction yield compare to extract in 70% ethanol in all extraction methods. These results showed that increasing in percentages of ethanol gave the higher extraction yield. Based on results (Table 1), extraction method using soxhlet at 4 hours (95SH-4h) gave the highest extraction yield with 49.29%. After 4 hours extraction, the extraction yield decreased gradually as they were at final equilibrium.
On the other hand, only 3.90% of extraction yield by 1-day maceration (70M-1d) indicated the lowest yield compared to all methods. Similar results reported in [12] as extraction of propolis using soxhlet extractor produced higher extraction yield compared to maceration technique as well as reduced extraction time. Sonication technique produce intermediate percentages of extraction yield compared to soxhlet and maceration.

Table 1: Value of percentages yield extraction of different extraction methods which were maceration, sonication and soxhlet method with different time period in 70% and 95% ethanol of propolis.

Maceration extraction (%)							
70M-1d	70M-3d	70M-5d	70M-7d	95M-1d	95M-3d	95M-5d	95M-7d
3.90	7.05	8.74	8.88	38.53	36.48	39.72	38.10
Sonication extraction (%)							
70S-10m	70S-30m	70S-60m	70S-120m	95S-10m	95S-30m	95S-60m	95S-120m
11.06	10.36	9.17	10.91	33.04	31.94	27.00	24.09
Soxhlet extraction (%)							
70SH-2h	70SH-4h	70SH-6h	70SH-8h	95SH-2h	95SH-4h	95SH-6h	95SH-8h
12.59	36.87	12.18	13.45	44.32	49.29	40.96	38.48

3.2. Total Phenolic Content

Phenolic content by Folin–Ciocalteu reagent work on the oxidation-reduction reaction. Blue color is formed by the reduction of mixture of tungstates and molybdates under basic condition adjusted by sodium carbonates [13]. All three methods showed that, extraction used 70% ethanol produced higher phenolic content compared to 95% of ethanol extraction.
In this study, the optimum time to extract phenolic content in propolis is by maceration for 5 days using 70% ethanol (70M-5d) with 46.68 ± 2.08 mg/mL (GAE/g). After 5 days extraction, the phenolic content was decrease gradually. There were highly significant different ($p<0.05$) in 70% of ethanol while there is no significant different $p>0.05$) in 95% of ethanol for maceration. Maceration technique contributed to higher phenolic even though this method produced low extraction yield.
However, total phenolic content in 70% ethanol extract with sonication technique showed the least value compared to all samples by 6.21 ± 0.07 mg/mL (GAE/g) (70S-120m). From this study, phenolic content in sonication technique was decreasing when exposed to longer of extraction time.

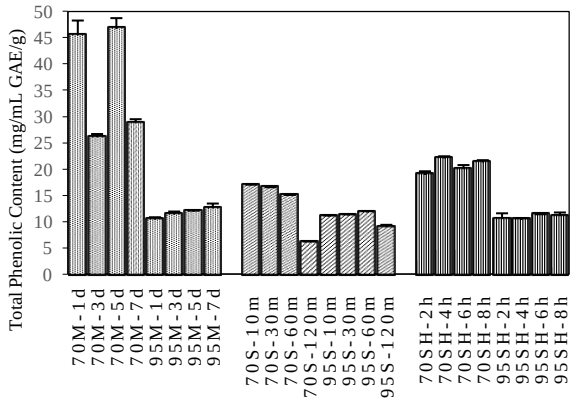

Fig. 1: The graph of total phenolic content for maceration, sonication and soxhlet technique.

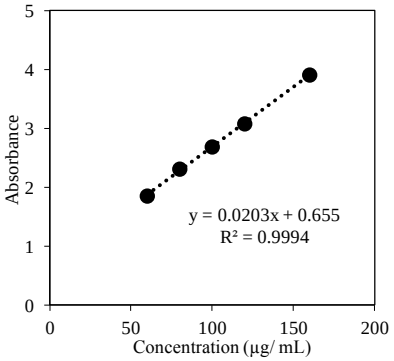

Fig. 2: Calibration curve of gallic acid as standard for total phenolic content.

3.3. Total Flavonoid Content

Flavonoids content in propolis was screening using aluminium chloride ($AlCl_3$) colorimetric method that relies on spectophotometric detection of color complexes formed between Al (III) with C-4 ketone groups and either C-3 or C-5 hydroxyl group of flavonoid in alkaline medium [14]. In this study, 70% of ethanol also produced higher flavonoid content compared to that of 95% ethanol. Khacha-ananda *et al* [15] reported that ethanol used in extract propolis was to generate flavonoid and fatty acid.

Sonication technique produced intermediate percentages of extraction yield however this technique can gave out highest flavonoid content. The optimum time to extract flavonoid was at 30 minutes in 70% ethanol (70S_30m) with value 107.27 mg/mL ± 4.10, QE/g compared to all methods and there were significant different ($p<0.05$). After 30 minutes extraction, the amount of total flavonoids decreases with extraction time.

The least total flavonoid was found in soxhlet extraction with 95% of ethanol at 6 hours extraction (26.71 ± 3.58 mg/mL QE/g) and there were no significant different between all samples when extracted using 95% ethanol. Even though soxhlet extraction gave higher yield but produced the smallest amount of flavonoid contents. Both total flavonoid and phenolic content were higher in 70% ethanol compared to 95% ethanol. In this study, total flavonoid was extracted more compared to total phenolic content in all method extraction.

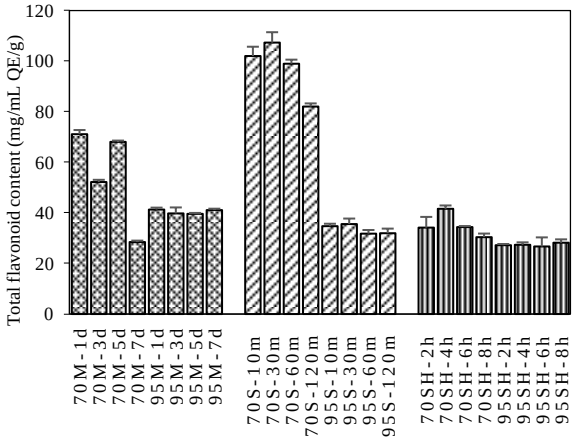

Fig. 3: Total flavonoids content for maceration, sonication and soxhlet technique.

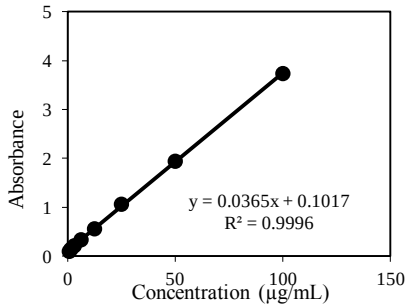

Fig. 4: Calibration curve of quercetin as standard for total flavonoids content.

3.4. Antioxidant activity

Antioxidant is a group of molecules that inhibit or remove free radical and delay or prevent cell damages in the body [14]. In this study DDPH assay was used to analyze antioxidant activity of propolis. DPPH assay is a method that accepted electron or hydrogen to become a stable free radical which noticeable by the changes of purple to yellow color [16]. The results are expressed as IC_{50}. The lower IC_{50} value indicates the stronger antioxidant activity in propolis. The radical scavenging activities of propolis extracts with different solvent percentages and extraction methods were compared to trolox, which used as standard.

Extraction using 95% and 70% ethanol were found to have antioxidant activity in all methods extraction. However, 70S-30m showed the stronger antioxidant activity of 87.55% inhibition with IC_{50} value by 11 ± 0.55 µg/mL Trolox equivalents/g extract. On the other hand, maceration in 95% ethanol for 1 days showed the weaker antioxidant activity with IC_{50} value 31.3 ± 3.56 µg/mL Trolox equivalents/g extract. All extraction methods showed inhibition more than 85% with different IC_{50} value. Thus, propolis has a natural antioxidant that can be used to neutralize oxidative stress.

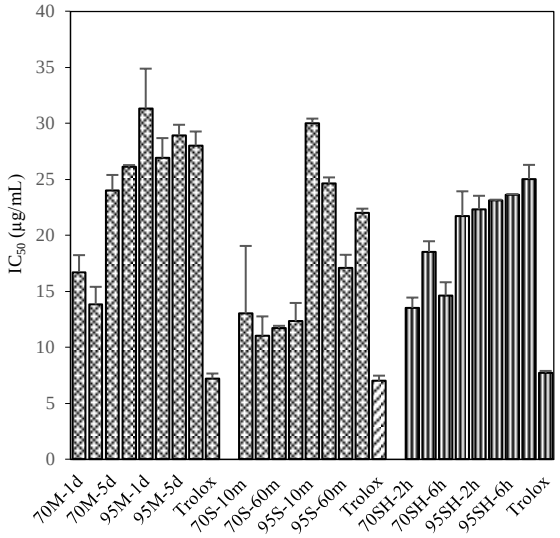

Fig. 5: The IC_{50} value for total antioxidant activity of propolis extracted from different extraction methods. Both 70% and 95% have an antioxidant activity. Trolox was used as a standard.

Table 2: Overall results of extraction yield, total flavonoid, phenolic and antioxidant capacity of ethanol extracts of propolis from three different extraction methods.

Samples	Extraction yield (%)	Total phenolic content mg/mL (GAE/g)*	Total flavonoid content mg/mL (QE/g)*	DPPH assay µg/mL IC_{50} value*
Maceration extraction				
E70M_1 day	3.9	45.41 ± 2.79^{a}	71.07 ± 1.65^{a}	16.70 ± 1.51^{a}
E70M_3 days	7.05	26.13 ± 0.58^{b}	52.17 ± 0.88^{b}	13.80 ± 1.58^{a}
E70M_5 days	8.74	46.68 ± 2.08^{c}	68.04 ± 0.51^{c}	24.00 ± 1.37^{b}
E70M_7 days	8.88	28.88 ± 0.65^{d}	28.46 ± 0.56^{d}	26.10 ± 0.15^{bc}
E95M_1 day	38.53	10.43 ± 0.44^{e}	41.29 ± 0.76^{e}	31.30 ± 3.56^{dfg}
E95M_3 days	36.48	11.40 ± 0.56^{e}	39.77 ± 2.36^{e}	26.90 ± 1.76^{ebd}
E95M_5 days	39.72	12.01 ± 0.12^{e}	39.6 ± 0.36^{e}	28.90 ± 0.95^{fce}
E95M_7 days	38.1	12.61 ± 0.87^{e}	$41.08\ 0.55^{e}$	28.00 ± 1.25^{gbed}
Trolox	-	-	-	7.20 ± 0.44^{h}
Sonication extraction				
E70S_10 min	11.06	16.98 ± 0.10^{a}	101.96 ± 3.68^{a}	13.00 ± 0.40^{a}
E70S_30 min	10.36	16.53 ± 0.05^{a}	107.27 ± 4.10^{b}	11.00 ± 0.55^{ac}
E70S_60 min	9.17	15 ± 0.11^{b}	98.92 ± 1.61^{c}	11.70 ± 1.14^{bd}

E70S_120 min	10.91	6.21 ± 0.07^{c}	82 ± 1.24^{d}	12.30 ± 0.36^{bc}
E95S_10 min	33.04	$11.04 \pm 0.17d^{e}$	34.73 ± 0.96^{e}	30.00 ± 6.03^{d}
E95S_30 min	31.94	11.22 ± 0.22^{ef}	35.56 ± 2.17^{e}	24.60 ± 1.74^{d}
E95S_60 min	27	11.78 ± 0.28^{f}	31.77 ± 1.44^{e}	17.10 ± 0.32^{d}
E95S_120 min	24.09	9 ± 0.43^{g}	31.94 ± 1.82^{e}	22.00 ± 1.64^{d}
Trolox	-	-	-	7.00 ± 0.45^{de}
Soxhlet extraction				
E70SH_2h	12.59	18.92 ± 0.70^{a}	34.16 ± 4.23^{a}	13.50 ± 0.92^{a}
E70SH_4h	36.87	22.07 ± 0.40^{b}	41.59 ± 1.32^{b}	18.50 ± 0.95^{b}
E70SH_6h	12.18	20.13 ± 0.67^{ac}	34.37 ± 0.40^{a}	14.60 ± 1.19^{a}
E70SH_8h	13.45	21.30 ± 0.43^{bc}	30.38 ± 1.42^{ac}	21.70 ± 2.21^{c}
E95SH_2h	44.32	10.58 ± 1.04^{de}	27.21 ± 0.47^{c}	22.30 ± 1.21^{c}
E95SH_4h	49.29	10.36 ± 0.36^{d}	27.37 ± 0.96^{c}	23.10 ± 0.06^{cd}
E95SH_6h	40.96	11.41 ± 0.03^{e}	26.71 ± 3.58^{c}	23.60 ± 0.06^{ce}
E95SH_8h	38.48	11.1 ± 0.68^{f}	28.19 ± 1.32^{cd}	25.00 ± 1.27^{de}
Trolox	-	-	-	7.70 ± 0.17^{f}

All values are mean ± standard deviation n=3
Same letter indicated that the samples have n significant different
Different letter indicated that the samples are significantly different

4. Discussion

Three extraction methods (maceration, sonication and soxhlet) with different percentages of ethanol (70% and 95%) were employed in order to get the highest extraction yield, total phenolic and flavonoid content and antioxidant activity. Based on this study, the percentages of yield depended on method of extraction, solvent used to extract, time extraction and temperature.

Extraction by soxhlet technique in 95% ethanol revealed the highest extraction yield compared to other techniques. Soxhlet extraction is performed by heating and condensation to evaporate the organic solvent to concentrate the product [15]. Longer time of extraction led to produce higher extraction yield due to longer time of samples and solvent in contact each other and have more mass transfer [16]. After 4 hours extraction, the extraction yield decrease gradually as they were at final equilibrium.

On the other hand, maceration technique in 70% ethanol showed the least value of extraction yield. Maceration only involved the soaking of the samples without any vibration cause the sample and solvent less in contact as compared to soxhlet extraction [9]. Ethanol is a solvent with intermediate polarity. Due to exposure to high temperature and continuous solvent recycle during the extraction process, it contributes to increase solubilization of components from raw materials [18].

However, maceration technique contributed to higher phenolic even though this methods produced low extraction yield. Khacha-ananda *et al* [15] stated that ethanol at concentration more than 70% did not assist to extract the phenolic compound in propolis extraction process. Extended time of extraction by sonication until 120 minutes lead to degradation of phenolic content in propolis and supported by Gullian & Terrats [19] which stated that total phenolic in samples will generally degraded due to longer time extraction and exposed to high temperature.

The optimum time to extract flavonoid is at 30 minutes sonication in 70% ethanol extraction. After that, the total flavonoid decreasing with the increasing of extraction time. Sonication works by acoustic cavitation. This cavitation provided a good penetration of solvent into the samples facilitating better extraction of bioactive compound in the propolis [21]. Khacha-ananda *et al.* [15] reported that ethanol used in extract propolis was to generate flavonoid and fatty acid as 70 % of ethanol gave out high flavonoid content compared to 95% of ethanol extraction.

Antioxidant activity mostly correlated with phenolic and flavonoid in the samples. In this study, total flavonoid contents were most contributed to antioxidant activity of propolis compared to phenolic content as flavonoid was recorded highest value in all samples. Surprisingly, sonication in 70% ethanol at 30 minutes showed the highest total flavonoid content and stronger antioxidant activity even though the extraction yield of sonication was intermediate. There is relationship between flavonoid and antioxidant activity. Flavonoid has ability to donate hydrogen and scavenging free radicals with its basic backbones of diphenylpropanes (C_6-C_3-C_6) with a central pyran ring [21]. The ability scavenge free radicals allow them to interact with reactive oxygen species (ROS) which can lead to oxidative stress and damage the tissue.

5. Conclusion

In the present study, propolis extracted by maceration for 5 days showed the most efficient technique to obtain the extract with better phenolic content. Extraction by sonication at 30 minutes in 70% of ethanol showed higher flavonoid content and antioxidant activity than those two other methods. At the same time, sonication can reduce the time of extraction, effective mixing and reduce thermal gradient. Findings from this study showed that extraction technique is important to obtain better yield, specific compound to extract and quality extract for propolis.

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References

[1] Megan, H., Robert, S., Ann,e D. (2013) Pot - honey a legacy of stingless bees. Australian stingless bee. Edited by Patricia, V., Silvia, R.M., Pedro, David, W.R. Springer, New York 35-72.

[2] Siok, P.K., Nyuk, L.C., Yusof, Y., Sheau, W.T., Lee, S.C. (2014) Total phenolic contents and colour intensity of Malaysian honeys from the *Apis* spp. and *Trigona* spp. bees. *Agriculture and Agricultural Science Procedia* 2, 150 – 155.

[3] Niken, P., Ritonga, H., Uslinawaty, Z. (2014) Properties and flavonoids content in propolis of some extraction method of raw propolis. *International Journal of Pharmacy and Pharmaceutical Sciences* 6(6), 975-1491.

[4] Medic-Saric, M., Bojic, M., Rastija, V., Cvek, J. (2013) Polyphenolic profiling of Croatian propolis and wine. *Food Technology and Biotechnology* 51(2), 159-170.

[5] Bankova, V., Popova, M., Trusheva, B. (2014) Propolis volatile compounds: chemical diversity and biological activity: a review. *Chemistry Central Journal* 8(28), 1-8.
[6] Sun, C., Wu, Z., Wang, Z., Zhang, H. (2015) Effect of ethanol/water solvents on phenolic profiles and antioxidant properties of Beijing propolis extracts. *Evidence-Based Complementary and Alternative Medicine* 2015, 1-9.
[7] Araujo, K.S.D.S., Joaquim, D.S.J., Marcello, O.S., Fernanda, D.B., Ilsamar, M.S., Robson, B., Tarso, D.S.A., Sergio, D.A., Sandra, M.B.M. (2016) Physicochemical properties and antioxidant capacity of propolis of stingless bees (Meliponinae) and apis from two regions of Tocantins, Brazil. *Acta Amaznica* 46(1), 61 – 68.
[8] Azwanida, N.N. (2015) A review on the extraction methods use in medicinal plants, principle, strength and limitation. *Medical Aromatic Plants* 4(196), 2167-0412.
[9] Ainsworth, A.E. & Gillespie, K.M. (2007) Estimation of total phenolic content and other oxidation substrates in plant tissues using folin-ciocalteu reagent. *Nature Protocols* 2(4), 875-877.
[10] Bhaigyabati, T., Devi, P.G., Bag, G.C. (2014) Total flavonoid content and antioxidant activity of aqueous rhizome extract of three *Hedychium* species of Manipur Valley. *Research Journal of Pharmaceutical, Biological and Chemical Sciences* 5(5), 970-976.
[11] Cunha, I., Sawaya, A.C., Caetano, F.M., Shimizu, M.T., Marcucci, M.C., Drezza, F.T., Carvalho, P.D.O. (2004) Factors that influence the yield and composition of Brazilian propolis extracts. *Journal of the Brazilian Chemical Society* 15(6), 964-970.
[12] Jadhav, A.P., Kareparamban, J.A., Nikam, P.H., Kadam, V.J. (2012) Spectrophotometric estimation of ferulic acid from *Ferula asafoetida* by Folin—Ciocalteu's reagent. *Der Pharmacia Sinica* 3, 680-684.
[13] Bag, G.C. & Devi, P.G. (2015) Research article assessment of total flavonoid content and antioxidant activity of methanolic rhizome extract of three *Hedychium* species of Manipur Valley 1. *International Journal of Pharmaceutical Sciences Review and Research* 30(28), 154–159.
[14] Jo, W.S., Yang, K.M., Park, H.S., Kim, G.Y., Nam, B.H., Jeong, M.H. & Choi, Y.J. (2012) Effect of microalgal extracts of *Tetraselmis suecica* against uvb-induced photoaging in human skin fibroblasts. *Toxicological Research* 28(4), 241-248.
[15] Khacha-Ananda, S., Tragoolpua, K., Chantawannakul, P., Tragoolpua, Y. (2013) Antioxidant and anti-cancer cell proliferation activity of propolis extracts from two extraction methods. *Asian Pacific Journal of Cancer Prevention* 14(11), 6991-6995.
[16] Ansari, A.Q., Ahmed, S.A., Waheed, M.A., Juned, S. (2013) Extraction and determination of antioxidant activity of *Withania somnifera Dunal*. *European Journal of Experimental Biology* 3, 502-507.
[17] Baldosano, H.Y., Castillo, M.B.M.G., Elloran, C.D.H., Bacani, F.T. (2015) Effect of particle size, solvent and extraction time on tannin extract from *Spondias purpurea* bark through soxhlet extraction. *In Proceedings of the DLSU Research Congress 3*.
[18] Paviani, L.C., Fiorito, G., Sacoda, P., Cabral, F.A. (2013) Different solvents for extraction of Brazilian green propolis: composition and extraction yield of phenolic compounds. *In III Iberoamerican Conference on Supercritical Fluid* 1-5.
[19] Gullian, K.M. & Terrats, P.M. (2017) Optimization of the ultrasound-assisted extraction of phenolic compounds from *Brosimum alicastrum* leaves and the evaluation of their radical-scavenging activity. *Molecules* 22(8), 1286.
[20] Kothai, S., Jayanthi, B. (2015) Environmental impact on stingless bee propolis (*Tetragonula iridipennis*) reared from two different regions of Tamilnadu– a comparative study. *International Journal of ChemTech Research* 7(7), 3081-3088.
[21] Choe, J.H., Kim, H.Y., Kim, Y.J., Yeo, E.J. & Kim, C.J. (2014) Antioxidant activity and phenolic content of persimmon peel extracted with different levels of ethanol. *International Journal of Food Properties* 17(8), 1779-1790.