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FULL PAPER

Integrated green extraction method in enhancing total phenolic content and antioxidant activities of *Heterotrigona itama* propolis

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Abstract

Propolis, a natural resinous by-product from the beehive, has captured researchers' interest due to the presence of its rich phytonutrient properties, including antioxidant, antiviral, antifungal antibacterial, and anti-inflammatory effects. With the increased awareness of environmental protection, there is a high demand for a greener alternative to extraction methods from natural sources. In the present study, several extraction methods have been tested to enhance the total phenolic content and antioxidant activities of the *Heterotrigona itama* propolis extract: a) thermal extraction, b) enzyme-assisted extraction, and c) combination of thermal and enzyme-assisted (TE) extraction. Total phenolic content (TPC) was determined using the Folin-Ciocalteu method, while the antioxidant activities were analysed using ferric reducing antioxidant power (FRAP) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay. Generally, the TE extraction has increased the total phenolic content and antioxidant activities of propolis extract compared to the single extraction technique. The TE propolis extract showed the highest total phenolic content and DPPH free radical scavenging activities at 194.03±4.40 mg GAE/g and 90.95±1.69 mg AAE/g, respectively. Meanwhile, the thermal treatment of propolis extract obtained the highest FRAP value of 136.34±3.04 mg AAE/g. Conversely, enzyme-assisted propolis extract showed the lowest total phenolic content (35.95±2.32 mg GAE/g) and antioxidant activities (FRAP value, 43.45±0.67 mg AAE/g; DPPH value, 26.73±0.94 mg AAE/g). These findings indicated that the combination of thermal and enzyme-assisted (TE) technique is a highly efficient extraction method and could enhance the propolis extract's total phenolic content and antioxidant activities.

1. Introduction

Propolis, also known as bee glue, is a natural resinous mixture produced by bees from substances collected from parts of plants, buds, and exudates (Wagh, 2013). Propolis is a natural product containing numerous chemical compositions that are potentially responsible for its biological activities. The main constituents of stingless bee propolis are phenolic and aromatic acids, terpenes and carbohydrates (Campos *et al*., 2015). Previous studies found that the propolis' compounds significantly differ in composition, which may consequently cause inconsistencies in the biological activities (Freitas *et al*., 2008; Choudhari *et al.,* 2012). This occurrence was due to the influence of species, botanical origins, surrounding environment, and extraction of the propolis (Salatino *et al*., 2005; Huang *et al*., 2014). According to Bogdanov and Bancova (2017), propolis includes 40 to 70% of balsam composed of flavonoids and phenolic acids. These bioactive compounds have pharmacological effects like antioxidants (Rosli *et al*., 2016). The high amount of total phenolic content was due to the presence of free phenolic compounds in propolis extracts. Naturally, the majority of the antioxidative phenolic compounds in natural sources exist in covalent bound form. The extraction process is, therefore, required to release the

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phenols and subsequently enhance the antioxidant capacity (Xu *et al*., 2007).

Heating or thermal treatment is a conventional extraction method that is simple and easy to perform. However, the heating or thermal process could be associated with the loss of secondary metabolites due to hydrolysis, air oxidation and extended extraction time (Epifano *et al.,* 2018). Previously, Olczyk *et al*. (2016), discovered that 7% propolis treated at 60°C produced more free radicals. According to their findings, the quantities of free radicals rose as the heating temperature of the sample increased. Despite the thermal treatment of propolis, ethanol extraction is the most widely utilised approach (Kubiliene *et al*., 2015). This approach was ideal for generating low-wax propolis extracts with a high concentration of biologically active compounds. However, the major drawback of using this technique is that it may contain strong residual flavour, which will then limit its use in the cosmetics and pharmaceutical industries as ethanol extracts are not suitable for the treatment of certain diseases in ophthalmology, otorhinolaryngology, paediatrics, or in cases of alcohol intolerance (Pietta *et al*., 2002; Kubiliene *et al*., 2015).

Greener alternative extraction methods are being introduced to process safer products. According to Chemat *et al.* (2012), green extraction can be explained based on the discovery and design of the extraction process as an alternate solution to reduce energy consumption, allows the usage of alternative solvents and renewable natural products and at the same time, able to secure the safety and high quality of the extract. One of the three alternative method is the enzyme-assisted extraction. These methods are notable for their quick extraction time, low organic, non-hazardous solvent content and ease of handling. Moreover, it produces a higher extraction yield while using less energy (Chemat *et al*., 2012; Khoddami *et al*., 2013). Enzyme-assisted extraction, has been widely used in the pharmaceutical and food industries due to its several advantages. It is environmentally friendly and can reduce solvent consumption and increase the efficiency of extracting bioactive compounds (Cheng *et al*., 2019; Wen *et al*., 2019). Furthermore, the enzyme has been explicitly used to extract phenolic compounds from natural resources (Yazdi *et al*., 2019). Nowadays, there is a demand to integrate or combine several extraction methods to enhance extraction yields of phytonutrients from natural resources (Yin *et al*., 2018; Luo *et al*., 2019). Combination methods also help achieve a synergistic effect, minimal degradation and enhanced extraction yield (Wen *et al*., 2019). The purpose of this study is to determine and compare the employed thermal, enzyme-assisted and integrated TE extraction approaches in enhancing total phenolic content and antioxidant activities using water as an extractant. The investigation was carried out for the *Heterotrigona itama* propolis, which is obtained from one of the commonly found stingless bee species in the region of study.

2. Materials and methods

2.1 Raw material preparation

Propolis from *H. itama* was obtained from a beekeeper's farm in Mantin, Negeri Sembilan, Malaysia. The propolis was kept at -20°C before being cut into a propolis chunk. The propolis chunk was then ground before being kept at -20°C for further investigation.

2.2 Extraction and experimental design

A single-factor experiment was used to determine the conditions of *H. itama* propolis extraction. Three different techniques were implemented in this study: thermal, enzyme-assisted and integration of thermal and enzyme-assisted (TE) extraction. An analytical comparison was performed for total phenolic content (TPC), antioxidant activity based on ferric reducing antioxidant power (FRAP) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity. . The following subsections describe the applied extraction techniques.

2.2.1 Thermal extraction

The treatment was performed according to the method of Daou and Zhang (2012), with slight modifications. About 2 g of propolis sample was extracted using 50 mL distilled water. The mixture was heated at 121°C, 0.1 MPa for 15 mins (ES-315-TOMY, Japan). It was then centrifuged at 2°C, 10,000 rpm for 10 mins (Eppendorf AG, Germany) before being filtered using Whatman no. 1 filter paper. The extract was kept at 4°C for 24 hrs to remove the wax. All extractions were carried out in triplicate.

2.2.2 Enzyme-assisted extraction

With modification, the hydrolytic cellulase enzyme extraction was performed as described by Pontillo *et al*. (2021). The enzyme solution at the concentration of 1% was added into 2 g of propolis samples and immersed in 50 mL distilled water with adjusted pH at 7.00 using a certain amount of 0.5 M NaOH. The mixture was then incubated at 50°C for 60 mins at 160 rpm (Innova 4000, New Brunswick Scientific, Canada). The enzyme was deactivated by heating at 90°C for 10 mins before centrifuging at 20°C, 10,000 rpm for 10 mins and filtered using Whatman no. 1 filter paper. The extract was kept at 4°C for 24 hrs to remove the wax. All extractions were carried out in triplicate.

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2.2.3 Integration of thermal and enzyme-assisted extraction

Integrated of thermal and enzyme-assisted (TE) propolis extract was a combination of thermal and enzyme-assisted extraction techniques. The TE propolis sample was initially carried out with the thermal extraction method and kept cool at 30°C before undergoing the enzyme-assisted extraction using cellulase enzyme.

2.3 Determination of total phenolic content

The propolis extract was mixed with a 5 mL Folin-Ciocalteu reagent (Merck, USA) and incubated at 30°C for 5 mins. Subsequently, 4 mL of 7.5% of sodium carbonate solution was added and vortexed before being incubated in a dark place for 2 hrs. The absorbance at 765 nm was measured against the reagent blank using UV-Vis spectrophotometer, VARIAN, Cary 50. The amount of total phenolic content was expressed as gallic acid (Sigma-Aldrich) equivalents (mg GAE/g extract) from a calibration curve (Koh *et al*., 2012).

2.4 Determination of antioxidant activities

2.4.1 Ferric reducing antioxidant power (FRAP) assay

FRAP assay was performed according to Haida and Hakiman (2019) method, with some modifications. Approximately 150 µL of the aliquot extract were mixed with 2850 µL of freshly prepared FRAP working solution (0.3M acetic acid, 0.01 M 2,4,6-tripyridyl-s-triazine (TPTZ) in hydrochloric acid solution, and 0.02 M ferric chlorides at the ratio 10:1:1) for 30 mins in the dark. A UV-Vis spectrophotometer (VARIAN, Cary 50) was used to compare the absorbance changes of the blue-coloured ferrous-tripyridyltriazine complex at 593 nm. The ascorbic acid (Sigma-Aldrich) calibration curve was used to calculate the FRAP value.

2.4.2 DPPH free radical scavenging assay

The 2,2-Diphenyl-1-picryl-hydrazyl hydrate (DPPH) free radical scavenging method is commonly used to measure antioxidant capacity. The process is based on electron transfer, which results in a violet solution. Therefore, the stable free radical will be reduced in the presence of the antioxidant molecule from the extract and result in discolouration from violet to colourless. A freshly made 2850 µL of DPPH methanolic solution was combined with 150 µL extract and vortexed thoroughly. The mixture was incubated in dark conditions for 30 mins at room temperature. The absorbance was read at 515 nm using a UV-Vis spectrophotometer. The ascorbic acid (Sigma-Aldrich) was employed as the standard to set up the DPPH calibration curve (Koh *et al*., 2012).

2.5 Statistical analysis

The Minitab software Version 18 (Minitab, LLC) was used to analyse the collected data from this study. The experiment was complemented with three replicates. The differences between different propolis extracts are represented as mean value±standard deviation (n=3) and compared using one-way analysis of variance (ANOVA) followed by Turkey's test at the significance level of $p<0.05$ (Nur Diyana *et al*., 2020).

3. Results and discussion

3.1 Total phenolic content of propolis

Figure 1 displays the total phenolic content of propolis extracts using different extraction methods: thermal, enzyme-assisted and integrated TE extraction. It shows that the the integrated TE extraction exhibits the highest total phenolic content (TPC) in the propolis extract sample, followed by thermal and enzyme-assisted extraction (194.03±4.40, 77.38±9.43 and 35.95±2.32 mg GAE/g, respectively). The initial thermal process in the TE technique has caused the substrate's cell walls and membranes to degrade, while subsequently releasing the phenols. The afterwards introduction of the cellulase has further released the phenols, since the enzymatic activity was able to increase the permeability of the cell wall, resulting in higher bioactive compounds extraction including phenols (Cheng *et al*., 2015). A single thermal treatment was found to yield a low amount of TPC. This finding coincided with the study by Hamzah and Leo (2015), who reported that the phenolic content of the propolis decreased when the extraction temperature was elevated to more than 120°C, due to the deteriorating structure of the phenolic compounds at high temperature. In contrast, the lower amount of total phenolic content obtained from single cellulase extraction in Figure 1 was due to the polarity of the water used as the extractant. Polar water characteristics have limited its ability to dissolve less polar compounds such as phenolic (Mokhtar *et al*., 2019).

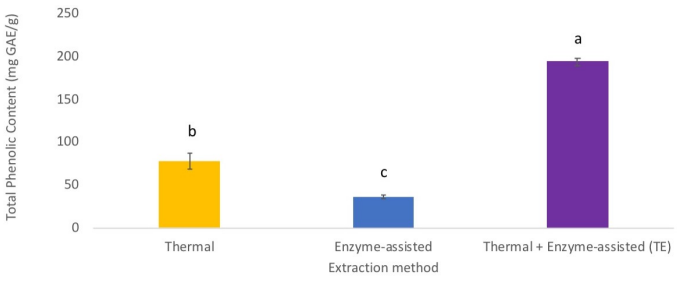

Figure 1. Total phenolic content (TPC) of single and integrated TE propolis extraction methods. Data are reported as mean±standard deviation of triplicate analyses. Bars with different notations are significantly different between different extract ($p<0.05$).

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3.2 Determination of antioxidant activities

3.2.1 Ferric reducing antioxidant power of propolis

FRAP was measured in propolis extracts as shown in Figure 2(a). It was observed that the FRAP activity of propolis from *H. itama* in the thermal extract was the highest with the value of 136.34±3.04 mg AAE/g, followed by integrated TE and single enzyme-assisted propolis extracts (114.71±1.48 and 43.45±0.67 mg AAE/g, respectively). The FRAP value represents the concentration of electron-donating antioxidants associated with the reduction of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) by antioxidants present in the samples (Halvorsen *et al.,* 2002). This is in accordance with Xu *et al*. (2007) findings, where FRAP value increases during thermal extraction at 120°C. Similarly to the findings for TPC, the FRAP value of cellulose extraction of propolis extract was the lowest among the three methods. Due to propolis's resinous properties, most bioactive compounds were not dissolved in water, resulting in a lack of substrate active sites to bind and further react. This could be explained in combination with TE extract, whereby the heating treatment initiated the substrate's degradation, thus, stimulating the enzyme catalysis. Greater exposition between enzyme and substrate during the interaction resulted in FRAP value increment. Enzymatic extraction of antioxidants, as mentioned by Pinelo *et al.* (2008), may prevail via hydrolytic degradation of the polysaccharides cell wall, which is linked by hydrophobic bonding. Nonetheless, direct enzymatic breakdown of the ether and ester linkages between the bioactive compounds and the plant cell wall polymers also can occur. In the enzyme-assisted method, several operational conditions such as pH and reaction temperature, extraction time, enzyme concentration, and substrate particle size are some of the factors that must be considered (Cheng *et al*., 2015). Improvement of these parameters may enhance the antioxidant activities of propolis extract. This is consistent with a study published by Laroze *et al.* (2010), which found that increasing enzyme concentration by 5 to 10% resulted in a significant increase in FRAP value at 42% of raspberry wastes.

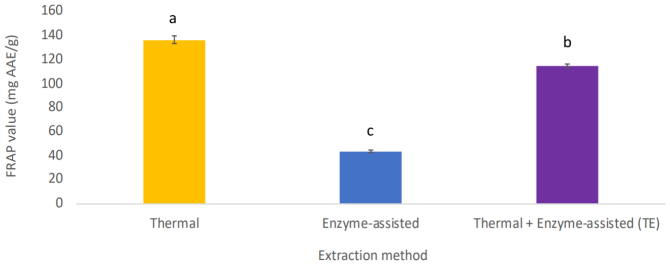

Figure 2(a). Ferric reducing antioxidant power (FRAP) of single and integrated TE propolis extraction methods. Data are reported as mean±standard deviation of triplicate analyses. Bars with different notations are significantly different between different extract (p<0.05).

3.2.2 DPPH free radical scavenging activity

In Figure 2(b), *H. itama* propolis extracted using integrated TE extraction has exhibited higher activity in scavenging free radicals of DPPH (90.95±1.69 mg AAE/g) compared to both single thermal and enzyme-assisted propolis extracts (69.57±4.60 and 26.73±0.94 mg AAE/g, respectively). Similar trends can be seen in DPPH free radical scavenging activity and total phenolic content (TPC) of single and integrated TE propolis extraction methods, whereby the most potent antioxidant activity was obtained in the combination of TE propolis extracts. Several studies have been published on propolis's ability to scavenge free radicals and protect against oxidative damage (Campos *et al*., 2015; Hamzah and Leo, 2015; Rosli *et al*., 2016; Galeotti *et al*., 2018). Phenolic compounds are well-known as radical scavengers. Their presence in plants may help protect cells against oxidation damage caused by free radicals based on their ability to donate hydrogen atoms to free radicals. The hydroxyl group in propolis extract is responsible for facilitating free radical scavenging (Soobrattee *et al*., 2005). According to Oberoi and Sandhu (2015), numerous studies have suggested that phenolic compounds inhibit free radicals. A linear correlation of total phenolic content with antioxidant capacity has also been reported by Aryal *et al.* (2019) with significant correlation-coefficient values of selected wild vegetables extracts.

4. Conclusion

Based on the findings, it can be observed that TE propolis extracted using thermal and enzyme-assisted extraction has exhibited the more prominent potential to combat free radicals since the extracts showed higher total phenolic content and antioxidant activities. Furthermore, this finding also discovered that the integration of thermal and enzyme-assisted extraction technique was more efficient than the single technique and resulted in higher total phenolic content as well as antioxidant activities of propolis extract.

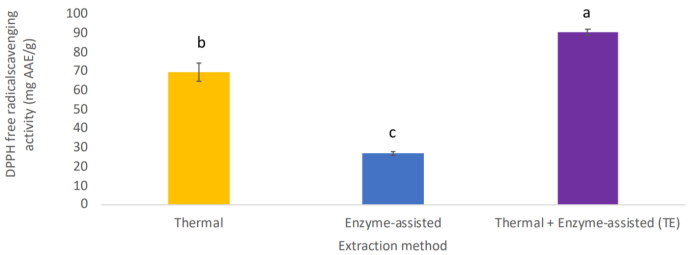

Figure 2(b). DPPH Free radical scavenging activity of single and integrated TE propolis extraction methods. Data are reported as mean±standard deviation of triplicate analyses. Bars with different notations are significantly different between different extract (p<0.05).

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Conflict of interest

The authors declare no conflict of interest.

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