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Phytochemical Analysis and Antioxidant Activities of Ethanol Extract of Stingless Bee Propolis from Indonesia

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Abstract. The objective of this study was to evaluate the phytochemical constituents and antioxidant activities of the ethanol extracts of Indonesian stingless bee propolis. The stingless bee propolis was obtained from South Sulawesi, Bintan, and Lampung, and was prepared through maceration with ethanol. The free radical scavenging and antioxidant activity of the extract were assessed against 1,1-diphenyl-2-picrylhydrazyl (DPPH), while the total phenolics and flavonoids were also determined to assess their corresponding effect on the antioxidant activity of the propolis extract. Total phenols and flavonoids contents were analyzed by Folin-Ciocalteu colorimetric and aluminum chloride ($AlCl_3$) methods, respectively. The results showed the ethanol extracts of Indonesian stingless bee propolis containing phytochemical compounds such as phenolic and flavonoid compounds. The antioxidant activity (IC_{50}) ranged from 150.20 to 207.63 mg/L, while total phenols and flavonoids ranged from 152.46 ± 55.61 to 327.86 ± 38.15 mg GAE/g extract and 6.16 ± 1.24 to 17.08 ± 0.53 mg QE/g extract, respectively. Indonesian stingless bee propolis might contain greatly diverse phytochemical compounds and antioxidant activities.

Keywords: Propolis, stingless bee, ethanol extract, antioxidant, natural medicines

INTRODUCTION

Propolis is a bee product known for its biological and pharmacological properties for centuries. This dark resinous substance is collected by bees from various plant sources, typically mixed with wax, to close cracks and caulking cracks, to minimize and close holes, and to make the surface of the nest cavity smooth, moist, and has a stable temperature [1]–[4]. Some reviews stated that *Populus* plants are the main resin source in the propolis component, especially for propolis originating from temperate regions. For the tropical and subtropical areas, the main resin source is relatively diverse and usually comes from the flowers of the *Baccharis dracunculifolia* plant, which is a native resin plant in Brazil, while *Macaranga* is known as resin plant source in Taiwan. Meanwhile, *Cistus spp.* and *Ambrosia deltoidea* are also known as a resin source plant in such temperate regions [5]–[7]. The composition of propolis depends on time, vegetation, and collection area [8]. Propolis contains vitamins B1, B2, B6, C, and E as well as minerals such as magnesium (Mg), calcium (Ca), potassium (K), sodium (Na), copper (Cu), zinc (Zn), manganese (Mn), and iron (Fe), with Fe and Zn are the most abundant. Propolis contains essential amino acids

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that are needed for cell regeneration, with arginine and proline are classified as the most contained amino acid that could reach 45.8% content. The mineral content is also strongly influenced by the environment in which the plants grow [9].

Since ancient times, the Egyptians have used propolis as a preservative balm. In addition to the Egyptians, the Greeks and Romans used propolis as an antiseptic drug and medicine to accelerate the healing of various diseases. Propolis has been reported to have various health benefits related to digestive disorders, allergies, and gynecological, oral, and dermatological problems [10], radioprotector, tissue regeneration stimulator, and immunomodulators, as well as treatment of burns, venous ulceration, suppurative osteitis, arthritis, postoperative wound complications [11], cardioprotective, vasoprotective, antioxidant, anti atherosclerosis, anti-inflammatory and antiangiogenic [12].

Stingless bees are a very diverse and abundant group of eusocial bees that occupy the tropical and subtropical parts of the world, originating from Africa. There are more than 500 species of stingless bees worldwide from 18 genera. *Trigona* is the most common genus, which includes 130 species from 10 sub-genera, followed by *Melipona* that consists of 50 species [13]. Stingless bee hives are found mostly in tree trunks, old walls, in termite mounds, and underground cavities. The nest is built using wax removed from the metastoma terga, mixed with plant resins and gums. Various plants are pollinated by stingless bees, namely guava, mango, orange, cucumber, sunflower, etc. The stingless bee propolis has broad-spectrum pharmacological properties such as antibacterial, antimicrobial, antioxidant, anti-herpes, anti-ulcer, antihypertensive, and anticancer activity [7,14,15]. Supplementation of 20 drops of 30% propolis solution as a standard antituberculosis drug for the treatment of pulmonary tuberculosis patients has been shown useful to accelerate the effects of treatment and weight recovery [2, 16]. *Trigona* bee propolis has a hepatoprotective effect on rat liver injury due to carbon tetrachloride (CCl4) exposure [17] – [19]. Research on Brazilian propolis has long been carried out and its products have been marketed internationally. Indonesia, despite being a tropical region which produces a lot of propolis, still has a very limited amount of research regarding Indonesian propolis. The comparative research on nutrient content and bioactive compounds in Brazilian and Indonesian propolis has been carried out [20], but the biological activity test still needs to be expanded.

As comparison to the stinging bee, the phytochemical compounds of stingless bee propolis are less explored. Several studies reported the composition of stingless bee. Dos Santos *et al*. 2017 [21] found di- and trigalloyl and phenylpropanyl heteroside derivatives, flavanones, diterpenes, and triterpenes contained in propolis produced by Melipona quadrifasciata anthidioides. Unfortunately, the compound greatly depends on the environment. The recent findings showed stingless bee propolis from tropical area mainly contained triterpenes and phenolic lipids [22]. Sawaya *et al*. 2005 [23] found the chemical composition of propolis from three species of *Scaptotrigona* were different between the seasons.

Indonesia has the potential to cultivate and produce stingless bee propolis. With a forest area of 200 million hectares, Indonesia has the potential to produce stingless bee propolis of *Trigona spp.* as much as 2,243 tons per 4 months or 6,729 tons per year. The uniqueness of the *Trigona spp.* includes easier cultivation and higher resistance to diseases compared to *Apis mellifera*, more diverse phytochemical component due to the diversity of flavors and colors of raw propolis, and higher yields, especially the products of *Trigona spp.* propolis from South Sulawesi [24].All *Trigona spp*. honeycomb extracts from five regions in Indonesia contain flavonoids and are shown to have anticancer activity against breast cancer cells [25]. The presence of bioactive compounds in propolis differs from the respective regions of origin in Indonesia, namely octadecane (found in propolis from southern Sulawesi), tricosane (found in propolis from southern Kalimantan) and nonacosane (found in propolis from Banten) [26].

Indonesia has abundant biodiversity for the produced bee species and propolis types, with a very diverse resinous vegetation source. The difference in chemical composition in quantity and quality in propolis greatly affects biological activity. Research conducted by Mahani *et al.* 2011[24] stated that the flavonoid content produced by Indonesia-origin *Trigona spp*. is higher than *Apis mellifera*. Thus, this study was aimed to analyze the phytochemical compounds of Indonesian stingless bee propolis from South Sulawesi, Bintan, and Lampung which had not been conducted.

MATERIAL AND METHODS

Material

Raw propolis was taken from South Sulawesi, Bintan, and Lampung. Ethanol, methanol, *n*-hexane, ethyl acetate, silica gel 60 F_{254}, 1,1-diphenyl-2-picrylhydrazyl, ascorbic acid, Folin-Ciocalteu reagent, Na_2CO_3, gallic acid, quercetin, $AlCl_3$, potassium acetate, HCl, $FeCl_3$, ether, Liebermann Burchard reagent (CH_3COOH anhydrous and concentrated H_2SO_4), Mg powder, concentrated HCl, amyl alcohol, ammonia, Mayer and Dragendorff reagent were used for analysis without further purification and purchased from Merck-Sigma Chemical Company. The UV-Vis spectra were recorded by Genesis 10-S with 1 cm quartz cells.

Methods

Extraction and Identification of Bioactive compounds of Stingless Bee Propolis from Indonesia

Characterization of chemical composition of propolis was carried out by extraction method according to Susana *et al*. 2018 [27]. Propolis (1 g) was mixed with 25 mL of 70% ethanol and continuously stirred for 48 h at 20 °C in a place protected from light. The mixture was stored at -20 °C for 24 h, then filtered. The ethanolic extract of propolis was concentrated in a rotary evaporator at 40 °C and propolis extract residue was obtained. Phytochemical contents of dried propolis extract, including alkaloid, flavonoid, saponin and terpenoid, was evaluated based on Harborne 1998 [28]. The phytochemical analysis is a qualitative test to examine the presence of active compounds contained in propolis extract. Furthermore, bioactive compounds of propolis extract were characterized by total phenolic compounds analysis (folin-ciocalteu method), total flavonoids analysis (aluminum nitrate method), and antioxidant analysis (DPPH method) with three replications.

Total phenolic compounds analysis (Folin-Ciocalteu method)

Determination of total phenolics compounds by the colorimetric method[29]. Gallic acid (GA) was used as a standard on total phenolic compounds analysis. A solution of gallic acid with different concentrations and 0.4 mL of Folin-Ciocalteu reagent was added into a test tube and left for 4–8 minutes, then 4.0 mL of 7% Na2CO3 solution was added into the mixture followed by homogenization before adding 10 mL of H2O. The mixture was incubated for 2 h at room temperature. Ethanol extract of propolis (10 mg) was dissolved with 10 mL methanol. Solution ethanol extract of propolis (10 mL) was pipetted and mixed with 0.4 mL of Folin-Ciocalteu reagent. The mixture was incubated for 4–8 minutes before adding with 4.0 mL of 7% Na2CO3 solution and shaken until homogeneous. H2O was added into a volume of 10 mL before incubation for 2 h at room temperature. Absorption is measured at 744.8 nm using a ThermoUV-Vis light spectrophotometer Genesys 10-S.

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Total Flavonoids Analysis (Aluminum Nitrate Method)

Ethanol extract of propolis (50 µl) was placed into a tube containing 950 µL of 80% ethanol. Solution with a volume of 500 µL was mixed with 100 µL of 10% AlCl3, 100 µL of 1 M CH3CO2K, and 4 mL of 80% ethanol. The mixture was left idle for 40 minutes at 20 °C. The absorption of test solutions and quercetin as standard were evaluated at 415 nm using a Thermo UV-Vis light spectrophotometer Genesys 10-S [30].

Antioxidant analysis (DPPH method)

Ethanol extract of propolis was added into each test tube with 0.6 mL of 0.4 mM DPPH solution. Methanol was added into the solutions until a volume of 3 mL, followed by homogenization. The mixture was incubated for 30 minutes at 37 ºC. The absorption was measured at 517 nm light using a UV-Vis light spectrophotometer.

RESULTS AND DISCUSSION

Physical characterization of propolis including color, texture and aroma were evaluated (data not shown). Color, texture and aroma of propolis were influenced by the area of origin, plant flora, type of bee and the propolis processing system employed by farmers. Origin of propolis affected the propolis quality significantly due to differences in their bioactive compounds content. These secondary metabolite compounds from plants based on the

environment where the beehive is taken by bees to compile and fill the gaps in the hive as protection from weather or other threats.

TABLE 1. Phytochemical test of several types of stingless bee propolis from Indonesia

No.	Phytochemical test	Types of propolis		
		South Sulawesi	Bintan	Lampung
1.	Alkaloids (Dragendorff)	+	+	+
	Alkaloids (Mayer)	-	+	-
2.	Flavonoids	+	+	+
3.	Polyphenols	+	+	+
4.	Saponin	+	-	-
5.	Terpenoid	-	-	-

Ethanol extract of stingless bee propolis from Indonesia contains bioactive compounds such as alkaloids, polyphenols and flavonoids indicating potential antioxidant activities to improve human health (Table 1). Moreover, 241 compounds were identified in propolis for in 2000–2012 such as flavonoids, phenylpropanoids, terpenes, stilbenes, lignans, coumarin, and prenylated derivatives related to the diversity of geographical locations, plant sources, and species of bees [7]. Flavonoids in propolis are derived from plants [31]. Twelve different flavonoids, namely pinocembrin, acacetin, chrysin, routine, luteolin, kaempferol, apigenin, myricetin, catechin, naringenin, galangin, and quercetin; two phenolic acids (kainic acid and cinnamic acid) and one resveratrol derivatives have also been investigated in propolis extract [32]. Tannins phlobaphene, catechins, chalcones, aurones, flavanones, flavonols, xanthones, pentacyclic triterpenoids, and gutiferones were found in Brazilian red propolis [33]. Although bioactive compounds of Indonesian propolis have not been studied in detail, they are very promising due to the very high forest biodiversity of this country.

TABLE 2. Yields and antioxidants activity of stingless bee propolis from Indonesia

Propolis	Yield (%)	Total phenolic (mg GAE/g)	Total flavonoids (mg QE/g)	IC_{50} (ppm)
South Sulawesi	9.20 ± 0.13^{a}	152.46 ± 55.61^{a}	10.64 ± 1.49^{b}	150.20 ± 16.02^{a}
Bintan	14.84 ± 4.22^{a}	158.81 ± 78.06^{a}	6.16 ± 1.24^{a}	204.09 ± 20.89^{b}
Lampung	13.70 ± 3.60^{a}	327.86 ± 38.15^{b}	17.08 ± 0.53^{c}	207.63 ± 7.66^{b}

*Data were expressed as mean ± SEM, n=3
**Different letters in the same column indicate a significant difference ($p<0.05$)

Propolis has been known as the strong antioxidant agents. Phenols and flavonoids may play an important role on this activity [34]. Flavonoids, phenols and triterpenes are the main phytochemical compound found in propolis. Indonesian stingless bee propolis from South Sulawesi, Bintan, and Lampung exhibit high level of total phenols and flavonoids ranged from 152.46 to 327.86 mg GAE/g propolis extract and 6.16 to17.08 mg QE/g propolis extract, respectively. The IC_{50} of antioxidant activity (DPPH methods) ranged from 150.20 to 207.63 ppm (Table 2). The result was much higher than the findings from Fikri *et al*. 2019 [35,36] that the total phenols and flavonoids of Indonesian stingless bee propolis from the other three regions ranged from 10.00 to 28.65 mg/g GAE and 0.76 to 3.39 mg/g QE, respectively. The differences were probably due to sugar content. Some raw propolis was obtained by squeezing and the others by aspiration that might affect the sugar content. The present finding was comparable to the findings from Sawaya *et al.* 2009 [22] who found the IC_{50} of stingless bee from Brazil ranged from 183 to 593 ppm.

There was an interesting result. Our study showed propolis from Lampung exhibited the highest antioxidant activity, while propolis from South Sulawesi possessed the highest total phenols and flavonoids. In contrast, Sant'ana *et al*.2011 [37] found that total phenols and flavonoids were correlated with IC_{50}. However, Ghasemi *et al*. 2009 [38] found no correlation between total phenols and/ or flavonoids with antioxidant activity. It is known that only flavonoid with certain structure, particularly hydroxyl position in the molecule act as radical scavenger. Furthermore, propolis is a mixture of different compounds with distinct activities.

CONCLUSION

Indonesian stingless bee propolis from South Sulawesi, Bintan, and Lampung contained alkaloids, polyphenols and flavonoids. The content was greatly diverse in total phenols, flavonoids and antioxidant activities. Stingless bee propolis from Lampung possessed the highest total phenols and flavonoids, while propolis from Sulawesi South exhibited the highest antioxidant activity.

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