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Original Article

ANALYSIS OF PROPOLIS STINGLESS BEE BIOACTIVE COMPOUNDS FROM SEVERAL REGIONS IN INDONESIA

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ABSTRACT

Objective: Propolis is a bee product collected by honeybees from a resinous substance of various plant sources. Its antioxidant activities are different from various geographic origins. This study aimed to analyze bioactive compounds by lC-MS/MS, compare antioxidant activity total phenolic and flavonoid contents in stingless bee propolis samples from several regions in Indonesia.

Methods: The propolis samples were taken from stingless bee hives of *Tetragonula clypearis* from Sumbawa, *Tetragonula laeviceps* from Magelang, *Tetragonula biroi* from Bogor, and *Geniotrigona thoracica* from South Kalimantan). Analysis of bioactive compounds was identified by lC-MS/MS. The quantification of the chemical compound determined its total phenolics and flavonoid (TPC and TFC) contents. The antioxidant activity was determined by 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging assay.

Results: The results of this study obtained 10 compounds from *G. thoracica*, 8 compounds each from *T. laeviceps* and *T. biroi*, and 7 compounds from *T. clypearis*. The results of TPC was ranging from 57.24±5.35 to 139.39±15.79 mg GAE/g. The TFC was ranging from 22.13±0.79 to 37.20±3.63 mg QE/g. With the highest TPC and TFC was propolis of *G. thoracica*. The IC_{50} of antioxidant activity was ranging from 11.12 to 162.72 ppm.

Conclusion: The compounds contained in propolis *T. clypearis, T. laeviceps, G. thoracica* and *T. biroi* have a potential as a new herbal candidate as antioxidant agents.

Keywords: Stingless bee, Propolis, lC-MS/MS, Phenolic, Flavonoid, Antioxidant

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INTRODUCTION

Propolis is one of the traditional medicines produced by honey bees sourced by plant resinous near the beehive colony [1]. Stingless bees produce propolis in large quantities compared to sting bees [2]. This is because stingless bees do not have stings to protect themselves, their nests and colonies, so by producing propolis stingless bees are able to protect themselves from predators [3]. Propolis has many biological activities such as antioxidant, anti-inflammatory, immunostimulant, hepatoprotective, anti-diabetic, anti-hypertensive and antimicrobial [4-7].

Propolis contains natural antioxidant properties from secondary metabolite compounds in the form of phenolics and flavonoids, which are able to reduce hydroxyl and superoxide radicals, which then neutralize free radicals so that they can protect cells, maintain the integrity of cell and tissue structures and are able to protect lipid membranes from damaging reactions [8]. Previous research has evaluated the antioxidant activities of propolis from different botanical and geographic origins [9]. Propolis from ten countries were compared its antioxidant activities by the β-carotene bleaching and 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay systems. Propolis extract from some countries had relatively strong antioxidant activities and also has high total phenolics and flavonoid contents [9]. Previous research also compared different solvent of extract propolis, it showed that water and methanol extracts exhibited stronger DPPH free radical scavenging activity [10].

More than 180 compounds contained in propolis have been identified, but they are relatively different in each region depending on the majority of plants as a source of resin from that place [11]. The compound content contained in propolis depends on the plant, geographical location, environmental conditions and also the bee species [5]. The bioactive compounds contained in propolis are rich in flavonoids and phenolics [12]. These compounds are also believed related to activity of some pharmacological activities.

Research on Indonesian propolis has been evaluated from different types and origins of bees. However, there are several types of stingless bees propolis that still lack references regarding the compound profile and benefits, such as *Tetragonula clypearis* (Sumbawa, West Nusa Tenggara), *Tetragonula laeviceps* (Magelang, Central Java), *Tetragonula biroi* (Bogor, West Java), and *Geniotrigona thoracica* (Tanah laut, South Kalimantan). Therefore, propolis from these bees was used in this research to determine the compound profile and biological properties of the compounds contained therein, including antioxidant properties, total flavonoid content, and total phenolic content. The chemical compounds were determined by using lC-MS/MS. It is hoped that the profile of chemical compounds contained in propolis can be the basis for standardizing and identifying marker compounds that are characteristic of propolis in Indonesia as a traditional medicine.

MATERIALS AND METHODS

Sample preparation

This study used stingless bee propolis samples from *Tetragonula clypearis* (Sumbawa, West Nusa Tenggara), *Tetragonula laeviceps* (Magelang, Central Java), *Tetragonula biroi* (Bogor, West Java), and *Geniotrigona thoracica* (Tanah laut, South Kalimantan).

Chemical and reagent

Ethanol 96%, ethanol 70%, methanol pro analysis, water, aluminum chloride ($AlCl_3$), sodium carbonate (Na_2CO_3), folin-ciocalteu, gallic acid, quercetin, 1,1-diphenyl-2-picrylhydrazyl (DPPH), formic acid

0,1%, acetonitrile (ACN), isopropanol (IPA), and H_2O (HPLC grade) were purchased from Merck KGaA, Darmstad, Germany.

Extraction

The extraction method used maceration with a ratio of 1: 5. First 100 grams of sample is weighed and mashed using a blender with the aim of facilitating the stirrer process and added ethanol 96% as a solvent as much as 500 ml. Next, the samples were stirred using a macerator for 8 h at 350 rpm then incubated for 24 h at room temperature. The filtered using a funnel buchner with filter paper pores of 10 µm so as to get Ethanol Extract Propolis 96% (EEP 96%) and extract precipitate in the form of resin. Then, EEP 96% filtrate was diluted with addition of aquadest until it reached a ethanol 70% concentration and incubated at 50 °Cfor 30 min. The sample extract solution was stored overnight at-4 °C Furthermore, the propolis extract was separated from the wax using a funnel buchner. After being filtered, a rotary vacuum evaporator was used for the evaporation process at 60 °Cuntil the propolis 70% viscous extract was obtained [13].

Determination of total phenolic level

Quantitative analysis of total phenolic content was based on previous study with some modification. 20 µl of diluted 70% ethanol extract propolis and gallic acid solution were mixed with 100 µl of 1:4 diluted folin-ciocalteu. Next, 75 µl of sodium carbonate (Na_2CO_3) 1M were added and incubated for 120 min at room temperature. Absorbance was measured at a wavelength of 765 nm using a microplate reader [13].

Determination of flavonoids levels

Quantitative analysis of total flavonoid content was based on previous study with some modification. 50 µl of diluted 70% ethanol extract and quercetin solution were mixed with 50 µl of aluminum chloride ($AlCl_3$) 10%. Then, it incubated for 15 min at room temperature. $AlCl_3$ were able to react with flavonoid compounds and form complexes. Absorbance was measured at a wavelength of 415 nm using a microplate reader [13].

Antioxidant activity test DPPH method

This antioxidant test was based on a previous study with some modification. The ability of the sample to reduce the presence of free radicals can be tested by using the DPPH analysis method. Antioxidant activity in neutralizing free radicals was by donating 1 hydrogen atom (H) to DPPH radical. A total of 20 µl of diluted samples was added to 180 µl DPPH 150 µM and 20 µl quercetin as a positive control solution was added to 180 µl DPPH 150 µM and incubated for 40 min in the dark with room temperature; the absorbance measured at 515 nm using microplate reader [13].

LC-MS/MS analysis

LC-MS/MS analysis was conducted by using UHPLC Vanquish Tandem Q Exactive Plus Orbitrap HRMS Thermo Scientific (Thermo Fisher Scientific, Massachusetts, US) with Accucore C18, 100 x 2.1 mm, 1.5 µm ThermoScientific column. A 5 mg propolis extract was diluted with 1 ml methanol and filtered with 0.2 µm PTFE membrane. A 2.0 µl of samples was injected to the column with 0.2 ml/min flow rate. The temperature was 30 °C, mass range 100-500 m/z, positive ionization mode, and mass tolerance 5 ppm. The chemical compound databases were using mzcloud and chemspider. The mobile phase using 0.1% formic acid in H_2O (Phase A) and 0.1% formic acid in acetonitrile (Phase B) with gradient: 0-1 min (5% B), 1-25 min (5-95% B), 25-28 min (95%B), 28-30 min (5%B) [14].

RESULTS

Propolis extract

Extraction results obtained from 4 propolis samples *T. clypearis, T. laeviceps, G. thoracica,* and *T. biroi* in the form of thick brown extract and yield data was shown in table 1.

Table 1: Yield of propolis extract

Indicator	*Tetragonula clypearis*	*Tetragonula laeviceps*	*Tetragnoula biroi*	*Geniotrigona thorasica*
Initial Weight (g)	100	100	100	100
Total Extract (g)	13.435	14.772	1.95	5.5
Yield (%)	13.43	14.77	1.95	5.50

The crude extract has a sticky texture. The difference yields its extract, which have various regions and bee species. Propolis sticky texture, which is different from each region caused differences in the remaining solvent in the samples so as to obtain filtration results with different volumes. This affects the final weight of each propolis sample.

Total phenolic and flavonoid contents

The value of total phenolics and flavonoid content was carried out colorimetrically using gallic acid as a standard for TPC and quercetin for TFC. The data was presented in table 2. The results of TPC were ranging from 57.24±5.35 to 139.39±15.79 mg GAE/g. The TFC was ranging from 22.13±0.79 to 37.20±3.63 mg QE/g. The *G. thoracica* has the highest TPC and TFC.

Antioxidant activity

The IC_{50} of the antioxidant activity in the sample was calculated using the absorption of the linear regression equation, in which the sample concentration (in logarithm) as x values and %inhibition as y values with the equation y = ax+b as seen in table 3. The IC_{50} of antioxidant activity was ranging from 11.12 to 162.72 ppm. The highest antioxidant activity was samples from *T. clypearis,* and the lowest was *T. laeviceps.*

Table 2: Total phenol and flavonoid content

Samples	Total phenolic content (mg GAE/g extract)	Total flavonoid content (mg QE/g extract)
Tetragonula clypearis	67±7.43	28.66±1.61
Tetragonula laeviceps	57.46±15.26	22.13±0.79
Geniotrigona thoracica	139.39±15.79	37.20±3.63
Tetragonula biroi	57.24±5.35	24.25±0.57

Data were presented in mean±SD, n=3

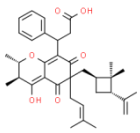
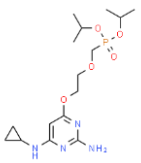
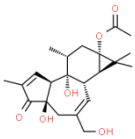
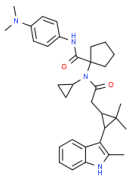
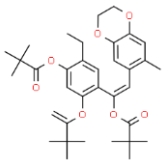
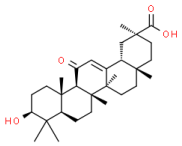
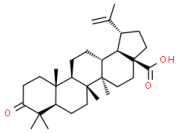
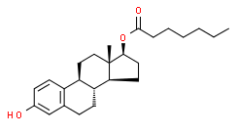
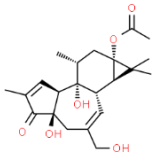
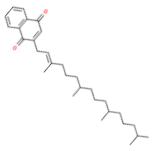
Table 3: IC_{50} of antioxidant activity

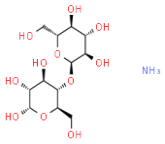
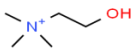
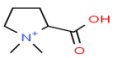
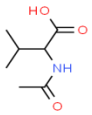
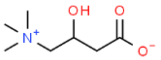
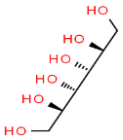
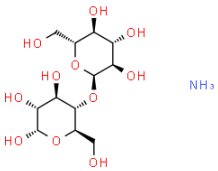
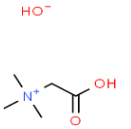
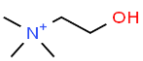
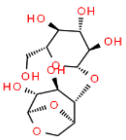
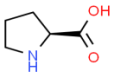
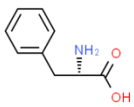
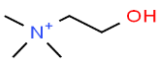
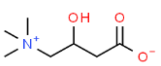
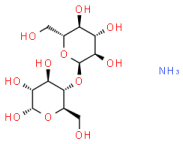
Samples	Equation	IC_{50}	Category
Tetragonula clypearis	y = 21.368x+27.53	11.12±0.99	Very Strong
Tetragonula laeviceps	y = 0.031x+44.96	162.72±9.12	Weak
Geniotrigona thoracica	y = 0.135x+46.10	28.87±6.05	Very Strong
Tetragonula biroi	y = 0.078x+44.78	67.69±12.76	Strong

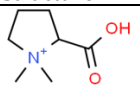
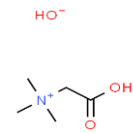
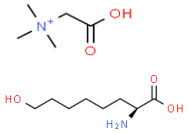
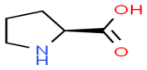
LC-MS/MS analysis

LC-MS/MS analysis results showed the secondary metabolites of various propolis extracts as seen in table 4. The data obtained in the form of molecular weight, retention time (minutes), the molecular structure of each compound, and the percentage area. Retention time the property of each compound differs depending on the size of time it takes for the solute to pass through the chromatography column. Retention time calculated as the time from injection to sample detection.

Table 4: Chemical compounds of propolis extract

No	RT (min)	Chemical formula	Compound name	Structure	Molecular weight
Propolis *Geniotrigona thoracica* (Pematang Siantar, *South Sumatra)*					
1.	25.744	$C_{35}H_{44}O_6$	Brasiliensophyllic acid A		560.31
2.	21.27	$C_{16}H_{29}N_4O_5P$	Diisopropyl [(2-{[2-amino-6-(cyclopropylamino)-4-pyrimidinyl]oxy}ethoxy)methyl]phosphonate		388.189
3.	21.332	$C_{22}H_{30}O_6$	Prostratin		390.2
4.	26.17	$C_{33}H_{42}N_4O_2$	1-(Cyclopropyl{[2,2-dimethyl-3-(2-methyl-1H-indol-3-yl)cyclopropyl]acetyl}amino)-N-[4-(dimethylamino)phenyl]cyclopentanecarboxamide		526.33
5.	21.552	$C_{35}H_{46}O_7$	(E)-1-{2-[(3,3-Dimethyl-1-buten-2-yl)oxy]-4-[(2,2-dimethylpropanoyl)oxy]-5-ethylphenyl}-2-(7-methyl-2,3-dihydro-1,4-benzodioxin-6-yl)vinyl pivalate		578.32
6.	23.894	$C_{30}H_{46}O_4$	18-β-Glycyrrhetinic acid		470.34
7.	23.643	$C_{30}H_{46}O_3$	NP-005821		454.34
8.	20.65	$C_{25}H_{36}O_3$	Estradiol enanthate		384.27
9.	22.638	C26H30O6	Fuscaxanthone C		438.02
10.	22.108	C30H44O2	Demethylphylloquinone		436.333
Propolis *Tetragonula laeviceps* (Magelang, Central Java)					

No	RT (min)	Chemical formula	Compound name	Structure	Molecular weight
1.	1.128	$C_{12}H_{25}NO_{11}$	4-O-α-D-Glucopyranosyl-α-D-glucopyranose ammoniate (1:1)		359.14
2.	1.08	$C_5H_9NO_2$	D-(+)-Proline		115.06
3.	1.056	$C_5H_{13}NO$	Choline		103.1
4.	1.127	$C_7H_{13}NO_2$	DL-Stachydrine		143.1
5.	1.054	$C_{15}H_{19}N_4O_2P_3$			380.1
6.	1.098	$C_7H_{13}NO_3$	N-Acetylvaline		159.1
7.	1.107	$C_7H_{15}NO_3$	DL-Carnitine		161.1
8.	1.057	$C_6H_{14}O_6$	L-Iditol		182.1
Propolis *Tetragonula clypearis* (Sumbawa, West Nusa Tenggara)					
1.	1.128	$C_{12}H_{25}NO_{11}$	4-O-α-D-Glucopyranosyl-α-D-glucopyranose ammoniate (1:1)		359.14
2.	1.054	$C_{15}H_{19}N_4O_2P_3$			380.1
3.	1.084	$C_5H_{11}NO_2$	Betaine		117.1
4.	1.056	$C_5H_{13}NO$	Choline		103.1
5.	1.102	$C_{12}H_{20}O_{10}$	Bis-beta-D-fructofuranose 1,2':2,3'-dianhydride		324.1
6.	1.08	$C_5H_9NO_2$	D-(+)-Proline		115.1
7.	2.415	$C_9H_{11}NO_2$	L-Phenylalanine		165.1
Propolis *Tetragonula biroi* (Bogor, West Java)					
1.	1.056	$C_5H_{13}NO$	Choline		103.1
2.	1.107	$C_7H_{15}NO_3$	DL-Carnitine		161.10
3.	1.054	$C_{15}H_{19}N_4O_2P_3$			380.1
4.	1.128	$C_{12}H_{25}NO_{11}$	4-O-α-D-Glucopyranosyl-α-D-glucopyranose ammoniate (1:1)		359.14

No	RT (min)	Chemical formula	Compound name	Structure	Molecular weight
5.	1.127	$C_7H_{13}NO_2$	DL-Stachydrine		143.1
6.	1.084	$C_5H_{11}NO_2$	Betaine		117.1
7.	1.129	$C_8H_{17}NO_3$	(2S)-2-Amino-8-hydroxyoctanoic acid		175.1
8.	1.08	$C_5H_9NO_2$	D-(+)-Proline		115.1

Based on the result of the lC-MS/MS analysis, 33 compounds were identified, that is 10 compounds from *Geniotrigona thoracica*, 8 compounds each from *Tetragonula laeviceps* and *Tetragonula biroi*, and 7 compounds from *Tetragonula clypearis*. Several compounds has antioxidant activity, namely prostratin, fuscaxanthone C, demethylphylloquinone, D-(+)-Proline, Choline, DL-Stachydrine, N-Acetylvaline, DL-Carnitine, Betaine l-Phenylalanine, and l-iditol.

DISCUSSION

The propolis crude extract has a sticky texture and it is also known as bee glue. The difference in the results of the yield of all samples caused by the propolis sticky texture, which varies regions, affecting the results of sample filtration before evaporation. Propolis sticky texture, which is different from each region caused differences in the remaining solvent in the samples so as to obtain filtration results with different volumes. This affects the final weight of each propolis sample [10].

Total phenolic and flavonoid content possessed by each propolis is influenced by regional differences and most of it is also influenced by the type of plants used as a source of bee feed [5]. These types of plants vary depending on the geographical location. Plant origin will affect the physicochemical parameters that determine the quality of propolis. Flavonoids are one of the compounds derived from phenolic compounds which are widely distributed in various types of plants [15]. Flavonoid and phenolic total levels will interpret the antioxidant activity of a sample. The total flavonoid content has a good correlation with antioxidant properties, in which the higher total flavonoid content has a stronger antioxidant activity [5].

Antioxidants are divided into five categories, namely very strong (<50 ppm), strong (51-100 ppm), moderate (101-150 ppm), weak (151-200 ppm) and very weak (>200 ppm) [16]. The antioxidant activity contained in propolis is influenced by the phenolic content. The flavonoid components contained in it in the form of (chrysin or quercetin) and organic acids in the form of (ferulic acid or caffeic acid) are active compounds that have antioxidant activity as indicated by their ability to reduce DPPH free radicals. Even though it belongs to the same category, the propolis sample with a smaller IC_{50} will have the strongest antioxidant activity. The smaller of IC_{50} value of a sample, was the stronger of antioxidant activity [5]. Some of the compounds in propolis analyzed by lC/MS-MS mentioned above have an important role in inhibiting free radicals in each sample. So, it can be said that the secondary metabolites contained in extract of propolis have potential as antioxidant compounds [17-24].

CONCLUSION

The results of this study obtained 10 compounds from *G. thoracica*, 8 compounds each from *T. laeviceps* and *T. biroi*, and 7 compounds from *T. clypearis*. The results of TPC were ranging from 57.24±5.35 to 139.39±15.79 mg GAE/g. The TFC was ranging from 22.13±0.79 to 37.20±3.63 mg QE/g. With the highest TPC and TFC was propolis of *G. thoracica*. The IC_{50} of antioxidant activity was ranging from 11.12 to 162.72 ppm. The compounds contained in propolis *T. clypearis, T. laeviceps, G. thoracica* and *T. biroi* have a potential as a new herbal candidate as antioxidant agents.

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AUTHORS CONTRIBUTIONS

Diah Kartika Pratami and Sania Citra Alfifah: contributed to sample preparation, carried out the experiments, analyzed the data and administration of the research; Izzul Islam: contributed to the interpretation of the results; Muhamad Sahlan: conceived and planned the experiments, support the financial research; Sri Angky Soekanto: supervised and verified the result; All authors provided critical feedback and helped shape the research, analysis and manuscript.

CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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