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Original Article

Phytochemical Profile and Antioxidant Activity of Propolis Ethanolic Extract from Tetragonula Bee

Diah Kartika Pratami[1], Abdul Mun'im[2], Andini Sundowo[3], Muhamad Sahlan[4,5]*

ABSTRACT

Objective: This research aimed to determine the phytochemical composition and the antioxidant properties *in vitro* of three types propolis collected from the region of South Sulawesi Province of Indonesia. **Methods:** Samples from three types of propolis: smooth, rough and mix were extracted with 96% ethanol. The propolis ethanolic extracts (PEE) was dried and studied their antioxidant properties by using FRAP (Ferric Reducing Antioxidant Power) and DPPH radical scavenging assays. Total phenolic compounds were quantified by Folin-Ciocalteu, and total flavonoid contents were also quantitatively determined by the $AlCl_3$ colorimetric method with a microplate reader. The chemical compounds were identified by an ultraperformance liquid chromatography TOF mass spectrometer (UPLC-TOF-MS) using the MS^E mode. **Results:** The sequence of potential antioxidant activity of PEE is smooth propolis > mix propolis > rough propolis. Which showed by EC_{50} value with DPPH assays are 25.54; 31.66; and 69.96 µg/mL, respectively and also showed by EC_{50} value with FRAP assays consecutively were 26.41; 32.10; and 34.62 µg/mL.Smooth propolis has the lowest EC_{50} value of all the types of propolis examined, contains total flavonoid content 791.06+13.06 mg QE/g extract and total phenolics content 426.91+61.08 mg GAE.g^{-1} extract. Chemical component identified by UPLC-TOF-MS using the MS^E mode were (-)-Sesamin $C_{20}H_{18}O_6$; Curcumin $C_{21}H_{20}O_6$; 8-epi-Helenalin $C_{15}H_{18}O_4$; and Kushenol F $C_{25}H_{28}O_6$. **Conclusion:** Smooth propolis which taken from inside the nest was the most potent antioxidant among of all the types of examined propolis. The antioxidant activity was influenced by the phenolic content of Propolis.

Key words: DPPH, FRAP, TPC, TFC, Microplate method, UPLC-TOF-MS.

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INTRODUCTION

Bees produce propolis, a mixture of bee saliva and gum produced by leaf buds and stems, which come out through plant's skin collected by bees.[1] The physical properties of raw propolis are hard and wax-like when fresh, but soft and very sticky when warm. It has a characteristic aromatic smell and odor; its range color varies from light to dark brown, red, yellow, or green, depending on its age and source.[2] Chemical composition, which bees collect from the resinous plant parts, may influence its biological effect.[3] The composition of propolis depends on diverse vegetation, phytogeographic region, and time of the collection.[4]

Results from previous researchers found more than 300 components contained in propolis i.e. aromatic acids; aromatics esters; flavanones; flavones and flavonol; chalcones and dihydrochalcones; terpenoids; acyclic hydrocarbons and esters; alcohols; aliphatic acids (short-chain); aliphatic esters; aliphatic fatty acids (long-chain) and esters; amino acids; aromatic hydrocarbons; acetophenones and other ketones; glycerol derivatives; steroids; sugars and sugar alcohols; and miscellaneous ingredients.[2]The quantification of the phytochemical content of propolis can be determined throught total flavonoid and total phenolics content. These parameters are related to the biological activity.[5]

All species stingless bees (Hymenoptera: Apidae) produce propolis in this study belong to the genus Tetragonula, *Tetragonula fusco balteata*; *T. laeviceps*; *T. biroi*; *T. sapiens*.[6] The genus is small body size. *Tetragonula spp* is one species of bees producing propolis honey more than other bee species.[7]

Propolis contained one of the biological activity was well known as an antioxidant. Propolis showed the most potent antioxidant of all the bee products including honey, royal jelly, and bee pollen.[8] Antioxidant activity of propolis was originated from their polyphenolic substances. The use of propolis with antioxidant capacity for prevention and treatment of diseases related to the increase of oxidative stress such as cancer, aging, and cardiovascular diseases.[4]

This research aimed to determine the phytochemical composition and the antioxidant properties *in vitro* of three types propolis collected from the region of South Sulawesi Province of Indonesia.

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MATERIALS AND METHODS

Samples

Tetragonula Beehives were taken from Masamba, North of Luwu district, South Sulawesi Province of Indonesia. The types of beehive were smooth (taken from inside the nest), rough (taken from outside the hive) and mix (a combination of both).

Chemicals

Quercetin, 2,2-Diphenyl- 1-picrylhydrazyl (DPPH), Foline-Ciocalteu, ferric chloride,2,4,6-Tri(2-pyridyl)-s-triazine(TPTZ), acetic acid (glacial) anhydrous, $AlCl_3$ anhydrous and sodium carbonate were purchased from Sigma Chemical Company (Sigma Aldrich, Singapore). Gallic acid and ethanol were obtained from Merck (Darmstadt, Germany).

Preparation of Propolis Extracts

PEE was extracted by Muhamad Sahlan method (2013).[9] The beehive sample (1 kg), from three types of propolis: smooth, rough and mix, were macerated with 5 L of 96% ethanol, allowed to stand for 16 h. After that, filtrate and residue were separated by filtration. The water was added to extract until 70 % ethanol-water v/v and incubated on water bath 50°C for 30 mins, to separate propolis with wax. Then, the solution was frozen in the refrigerator overnight. Propolis separated with wax apparently at room temperature incubation; separate the wax and propolis by filtration. The filtrate was evaporated using rotary vacuum evaporator (Rotavapor R-205, Büchi, Switzerland) to give a viscous residue and then was dried using oven vacuum. We obtained the dried propolis ethanolic extracts and wax free.

Determination of Percentage Yield (%)

The percentage of yield was determined using the dry weight of extract (a) and soaked samples material (b) using Equation 1,

$$\text{Percentage Yield (\%)} = \frac{a}{b} \times 100$$

The extraction yield was calculated for each type of PEE in triplicate.

Phytochemical Screening

The qualitative phytochemistry test was conducted as shown in Table 1 according to Indonesian Materia Medika(1995)[10] and Harborne (1998).[11]

Determination of Total Phenolic Content

TPC method was based on the microplate method given by Ahmad *et al* (2017)[12] with some modifications. A total of 25µL of the sample solution or the standard solution was mixed with 100 µL of 1:4 diluted Folin–Ciocalteu reagent and shaken for 60 s in a 96-well microplate and incubated for 4 min. Then add the mixture with 75 µL of sodium carbonate solution (100 g L^{-1}), and shaken for 60s. Incubate it two h at room temperature. The absorbance was measured at λ 765 nm using a microplate reader 96 well™ (Versa Max ELISA Microplate Reader, USA).

Gallic acid solution (5–300 mgL^{-1}) was used as standards. The calibration curve of standards (gallic acid) was measured by the absorbance from microplate reader instrument and was calculated using SoftMax 6.5.1 software. The equation formula was Y = 0.0633x + 0.0099 and R^2 = 0.9947, where Y is the yield of GAE (total phenolic content) and X is the absorbance of gallic acid or samples. All determinations were carried out in triplicate.

Determination of Total Flavonoid Content

TFC was determined by the $AlCl_3$ microplate method given by Massoumeh Farasat (2014)[13] with some modification. A total of 20 µL of each sample or standard solution were mixed with 20 µL of 10 % $AlCl_3$, 20 µL of CH_3COOK(1M) and 140 µL of distilled water, and shaken for 60s. Incubate it 30 min at room temperature. The absorbance of was measured at λ 415 nm using the microplate reader96 well™ (Versa Max ELISA Microplate Reader, USA).

Quercetin ethanolic solution (5–200 mgL^{-1}) was used as standards. The calibration curve of standards (quercetin) was measured by the absorbance from microplate reader instrument and was calculated using SoftMax 6.5.1 software. The equation formula was y = 0.0366x - 0.0146 and R^2 = 0.998, where Y is the yield of QE (total flavonoid content) and X is the absorbance of quercetin or samples. All determinations were carried out in triplicate.

Measurement of Antioxidant Activity with DPPH Assay

The microplate antioxidant activity with DPPH assay was based on the method described by Bobo Garcia (2015)[14] with some modifications. DPPH method procedure of antioxidant activity assay is in Table 2. The absorbance was recorded using a microplate reader 96 well™ (Versa Max ELISA Microplate Reader, USA). The % DPPH quenched was calculated using Equation 2,

$$\text{\% DPPH quenched} = \left[1 - \left(\frac{\text{A sample} - \text{A blank}}{\text{A control} - \text{A blank}}\right)\right] \times 100$$

The concentration of samples resulting in 50% inhibition on DPPH was calculated, expressed as anEC_{50}value (µg/ml), and obtained by using SoftMax Pro6.5.1 software. All determinations were carried out in triplicate.

Measurement of Antioxidant Activity with FRAP Assay

The microplate FRAP assay was based on the method described by Bolanos De La Torre (2014)[15] and Shinta Marlin (2017)[16] with some modifications. The FRAP reagent solution contains 10:1:1 of acetate buffer (300 mM, pH 3.6), TPTZ (40 mM dissolved with 40 mM HCl) and ferric chloride (20 mM in water). The procedure of FRAP method assay is in Table 3. The absorbance was measured at λ 595 nm using a microplate reader (VersaMax; Molecular Devices, USA). The percentage of reducing power capacity can be calculated using the Equation 3,

$$\text{\% Capacity} = (1 - \text{Ts}) \times 100\% \quad (3)$$

Ts = Transmittan

As = – log Ts

As = As positive control – As blank FRAP solution

The result was expressed as EC_{50} (µg/ml), calculated using the equation of nonlinear regression by Microsoft Office Excel and SoftMax Pro6.5.1 software. EC_{50}. The analysis was done in triplicate.

HPLC-ESI-MS/MS Analysis

The analytical LC-MS/MS experiments were performed using ACQUITY UPLC I-Class System connected through a split to the mass spectrometer the Xevo G2-XS Q-tof Mass Spectrometer (Waters, USA) in Research Centre for Chemistry, Indonesian Institute of Science. The column temperature was set at 40°C. The flow rate was 0.30 mL/min. The HPLC analyses were performed using a linear gradient solvent system consisting of A: B (0.1% formic acid in H_2O: 0.1% formic acid in methanol) as follows: t= 0 min 95% A; t= 3min75% A; t= 7 min 40% A; t= 10 min 20% A; t =13 min 100% B; t = 15 min 5% B. The injection volume was 1 µL. The total run time was 15 min. Sample manager temperature was 20°C. Wash solvent was ACN: IPA: Me OH: H_2O (1:1:1:1). Wash solvent pre inject 10% MeOH in H_2O. Data acquisition was using MS^E function ESI ionization type under positive electrospray. Acquisition range was 100-1200 *m/z*. Capillary and cone voltage was 0.8 kV. and 30 V, respectively.

The source temperature was at 120°C, desolation gas flow was 1000 L/hr, and cone gas flow 50 L/hr. Scan times was 0.250 s. Data acquisition and data processing use UNIFI® Software.

RESULTS

Determination of Percentage Yield (%)

The extractant from three samples of rough propolis, smooth propolis, and mix propolis, then process using rotary evaporator and oven vacuum to get dry extract and wax free of propolis ethanolic extracts. As can be seen in Table 5 obtained highest yield at Mix Propolis Ethanolic Extract (MPEE), PEE from mix propolis, equal to 20.21% with the content of propolis dry matter in PEE equal to 40.33 mg / mL.

Phytochemical Screening

Phytochemical screening of PEE showed the presence of alkaloids, flavonoids, phenolics, glycosides, tannins, terpenes and saponins and negative to anthraquinone. Here is the test results data shown in Table 4.

Determination of Total Phenolic and Flavonoid Content

The TPC and TFC from three types of ethanolic extract propolis as shown in Figure 1. The yields of total phenolic content from smooth, rough and mix propolis were 426.91 ±61.08 mg GAE/g extract; 269.57 ± 20.37 mg GAE/g extract; and 319.51 ± 6.37 mg GAE/g extract.

Table 1: Phytochemical Screening of Propolis Ethanolic Extract.

Phytochemical Contents	Methods
Alkaloid	Mayer, Dragendorff, and Bouchardat reagents
Flavonoid	Shinoda and Wilson Toubock reaction
Phenolics	Folin–Ciocalteu method
Terpenoid	Liebermann- Burchard reagent
Tannin	Gelatin test, Gelatin-salt test, ferrous (III) chloride test
Glycoside	Molisch reaction
Anthraquinone	Borntrager reaction

Table 2: Composition of the solution to test the antioxidant activity with DPPH methods.

Material	Volume (µL)		
	Blank	Control	Sample
Gallic acid / PEE	-	-	20
DPPH 150 µmol/L	-	180	180
Ethanol p.a	200	20	-

were shaken for 60 seconds and incubated for 40 minutes in the dark and measured absorbance at λ 516 nm

Based on the measurement results, the yields of total flavonoid content from smooth, rough and mix propolis were 791.06 ±13.06 mg QE/g extract; 324.43 ±11.84 mg QE/g extract; and 530.86 ±31.43mg QE/g extract.

Measurement of Antioxidant Activity

The antioxidant activity test was performed using DPPH and FRAP assay. Antioxidant capacity was expressed as EC_{50} (µg/mL) of DPPH scavenging activity and FRAP capacity. The following results are obtained as shown in Table 5. The best antioxidant activity using DPPH method was smooth propolis ethanolic extract (SPEE), PEE from smooth propolis, with the EC_{50} value of 25.53µg/mL. The DPPH scavenging activity of the PEE in descending order of potency was gallic acid >smooth propolis> mix propolis> rough propolis.

From the experiments, SPEE also showed the best reducing power capacity with the EC_{50} value of 26.41 µg/mL. The descending order of the FRAP reducing power of the PEE was gallic acid > smooth propolis> mix propolis> rough propolis.

Table 3: Composition of the solution to test the antioxidant activity with FRAP methods.

Material	Volume (µL)		
	Blank	Control Blank	Positive Control / Sample
FRAP reagent solution	-	270	270
Gallic acid / PEE	-	-	30
Ethanol p.a	300	30	-

were incubated for 30 minutes at a temperature of 37°C and measured absorbance at λ 595 nm

Table 4. Phytochemical Screening of Propolis Ethanolic Extract

Phytochemical Contents	Smooth Propolis	Rough Propolis	Mix Propolis
Alkaloid	+	+	+
Flavonoid	+	+	+
Phenolics	+	+	+
Terpenoid	+	+	+
Tanin	+	+	+
Glycoside	+	+	+
Anthraquinone	-	-	-

Note. + : detected, - : No detected

Table 5: Extraction yield, content of phenolics and flavonoids, and antioxidant activity of PEE.

Sample Name	Extraction yields (% w/w)	Dry matter in PEE (mg/mL)	TPC	TFC	EC_{50} DPPH Inhibition (µg/mL)	EC_{50} FRAP Capacity (µg/mL)
			(mg GAE/g)	(mg QE/g)		
SPEE	17.06 + 0.23	36.07 + 0.77	426.91 + 61.08	791.06 + 13.06	25.53	26.41
RPEE	18.03 + 0.38	34.12 + 0.45	269.57 + 20.37	324.43 + 11.84	31.66	34.62
MPEE	20.21 + 0.14	40.43 + 0.27	319.51 + 6.37	530.86 + 31.43	69.96	32.1

Note: SPEE, Smooth Propolis; RPEE, Rough Propolis; MPEE, Propolis Mix; TP, total phenolics content; GAE, gallic acid equivalents; TF, total flavonoids content; QE, quercetin equivalents; DPPH, 2,2-Diphenyl-1-picrylhydrazyl; EC_{50}, Effective Concentration 50% antioxidant capacity; FRAP, ferric reducing antioxidant power. Data shown in the table are expressed as mean ± standard deviation (=3).

A clear picture in Figure 2-5 is obtained when the TPC, TFC and antioxidant assays are compared. From Figure 2-3, It was found that TPC and TFC with EC_{50} values for DPPH had weak correlation (r^2 = 0.6776 and r^2 = 0.8009, respectively). Then from Figure 4-5, It was found that TPC and TFC with EC_{50} values for FRAP had high correlation (r^2 = 0.9999 and r^2 = 0.9769, respectively).

HPLC-ESI-MS/MS Analysis

The composition of a propolis ethanolic extract of *Tetragonula sp* was examined on Aquity UPLC and the LC-MS/MS chromatogram was shown in Figure 6-7. Tentative identification of phytochemicals in smooth propolis extract and rough propolis extract were presented in Table 6 and 7, respectively. As shown in Figure 6-7, a total of various compounds were identified by comparing molecular weight (M), and m/z fragment with the UNIFI literature data and the results were shown in Table 6-7.

DISCUSSION

The yields of propolis extract from this study seen in Table 5 are lower than extraction of Bolivian Propolis that obtained by Nélida Nina (2016)[17] ranged from 45.76 to 59.68 % w/w and yield of propolis *T. incisa, T. fuscibisca* and *T. fuscobalteata* maceration use methanol described by Paula M. Kustiawan (2014).[18] But it yields is higher than the extraction conducted by Hasan, A. E.Z (2014).[19] He obtained from five regions in Indonesia were different, yields of propolis from Makassar 1.85±0.51% w/w, Pekanbaru 19.97±2.19 (%) w/w, Kendal 7.28±1.59% w/w, Pandeglang 11.05±3.20% w/w, and Banjarmasin 8.38±0.70% w/w.

The propolis extraction that modified by Sahlan *et al.*[9] suitable used for propolis that is not heat resistant. In this research, the solvent used is ethanol 96%, which is semi-polar so that the active compounds with the different polarity is expected to be extracted perfectly. The active compounds are obtained while doing the stirring (mixing) a lot faster. Ethanol as organic solvents is commonly used than methanol in the pharmaceutical industry as reaction media in natural products extraction and for cleaning of equipment.

The content of phenolic and flavonoid compounds in the different extracts obtained from Propolis *Tetragonula spp* in Table 5 shows that SPEE, smooth propolis, presented the highest TPC 426.91 ± 61.08 mg GAE/g extract and TFC 791.06 ± 13.06 mg QE/g extract.

The results of TPC are superior to Algerian Propolis those reported by Zina Mouhoubi Tafinine (2016)[20] ranged from 1.71 to 53.51 mg GAE g^{-1}, Chinese Propolis those reported by Kai Wang (2014)[21] ranged from 145.54+75.89 to 233.98+70.84 mg GAE g^{-1}, Bolivian Propolis those

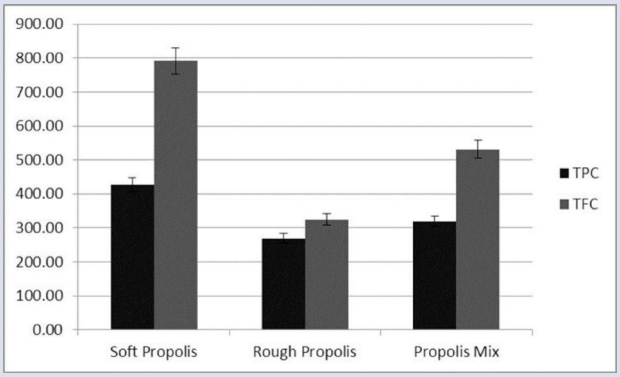

Figure 1: Total phenolic and flavonoid content from propolis ethanolic extract.

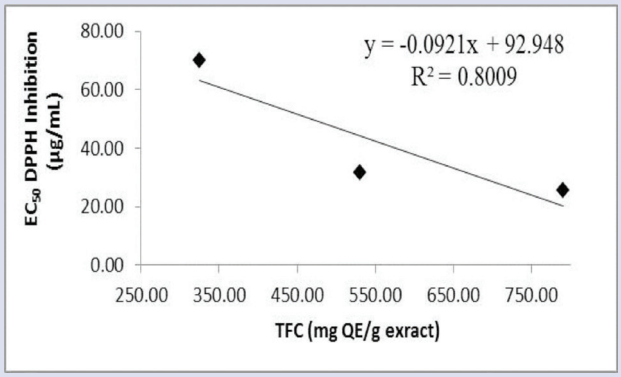

Figure 3: Regression Equation between TFC and Antioxidant Activity by DPPH assay.

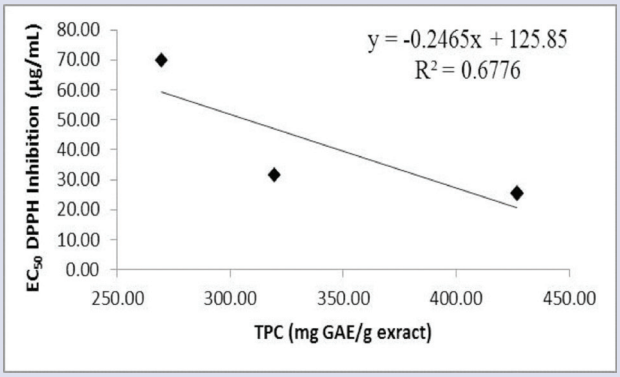

Figure 2: Regression Equation between TPC and Antioxidant Activity by DPPH assay.

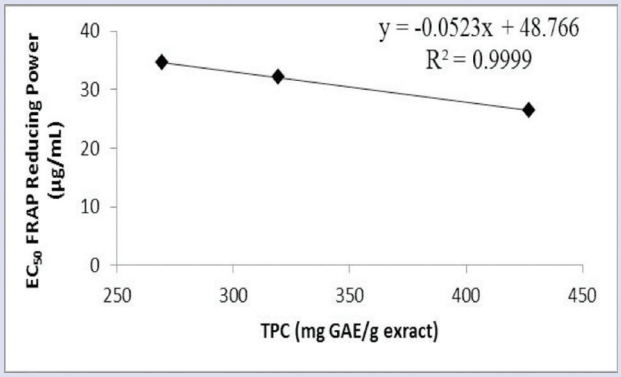

Figure 4: Regression Equation between TPC and antioxidant capacity by FRAP assay.

Table 6: **Identification of chemical compounds in smooth propolis by HPLC-ESI-MS/MS data.**

No	Tentative Component Identification	Observed m/z	Observed RT (min)	m/z Fragment	Formula
1	Kushenol-F	425.1952	9.87	257, 42	$C_{25}H_{28}O_6$
2	8-epi-Helenalin	263.1279	10.04	263, 507	$C_{15}H_{18}O_4$
3	(-)-Sesamin	355.1172	10.76	355, 487	$C_{20}H_{18}O_6$
4	(-)-Sesamin	377.0989	10.76	355, 453	$C_{20}H_{18}O_6$
5	Curcumin	369.1328	11.04	299, 369	$C_{21}H_{20}O_6$

Table 7: **Identification of chemical compounds in rough propolis by HPLC-ESI-MS/MS data.**

No	Tentative Component Identification	Observed m/z	Observed RT (min)	m/z Fragment	Formula
1	Kushenol F	425.1954	9.88	425, 465	$C_{25}H_{28}O_6$
2	8-epi-Helenalin	263.1281	10.04	263, 507	$C_{15}H_{18}O_4$
3	(-)-Sesamin	355.1176	10.75	355, 453	$C_{20}H_{18}O_6$
4	Curcumin	369.1326	11.04	369, 423	$C_{21}H_{20}O_6$
5	Curcumin	391.1149	11.04	369, 437	$C_{21}H_{20}O_6$

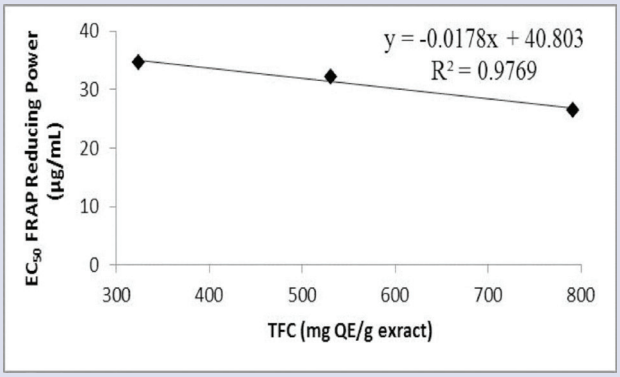

Figure 5: Regression Equation between TFC and antioxidant capacity by FRAP assay.

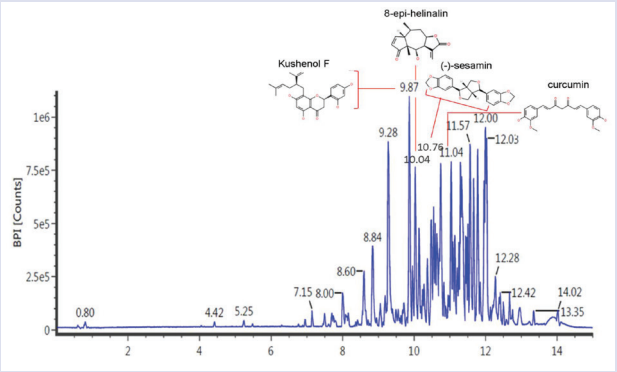

Figure 6: UPLC TOF MS^E (100-1200) 6eV ESI+ - Low CE (BPI) Profile of Smooth Propolis.

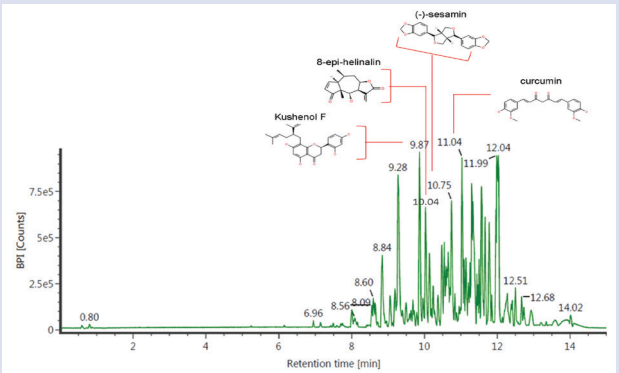

Figure 7: UPLC TOF MS^E (100-1200) 6eV ESI+ - Low CE (BPI) Profile of Rough Propolis.

reported by Nélida Nina (2016)[17] ranged from 43.0±0.3 to 176.0±4.8 mg GAE g^{-1}, and Brazilian Propolis those reported by Mara L F Bittencourt (2015)[22] ranged from 31.88±0.61 to 204.30±3.80 mg GAE g^{-1} of dry weight. But the results of TPC are inferior to Propolis Stingless Bees (Meliponinae) those reported by Karuane Saturnino (2016)[4] ranged from 620.01 ± 6.45 to 631.29 ± 4.22 mg GAE g^{-1} and Argentina Propolis those reported by Ana Lilia Salas (2016)[23] ranged from 587 ± 20 to 593 ± 15 mg GAE/g.

The results of TFC are superior to Algerian Propolis those reported by Zina Mouhoubi Tafinine (2016)[20] ranged from 1.25 to 49.46 mg QE g^{-1}, Chinese Propolis those reported by Kai Wang (2014)[21] ranged from 124.92+79.74 to 126.23+78.46 mg QE g^{-1}, Argentina Propolis those reported by Ana Lilia Salas (2016)[23] ranged from 165 ± 12 to 185 ± 15 mg QE g^{-1}, and Bolivian Propolis those reported by Nélida Nina (2016)[17]ranged from 5.5±0.6 to 57.1±2.8 mg QE g^{-1}. Overall, the total flavonoid contents of propolis ethanol extract from *Tetragonula sp* South Sulawesi Indonesia are significantly superior to another country. Flavo-

noids, one of the secondary metabolites belongs to a polyphenolic class, are commonly found in different parts of the plant sources, propolis and honey.[24]

The strongest antioxidant activities (lower EC_{50}) were found in SPEE, 25.54 µg/mL. SPEE samples, with highest TPC and TFC were also the high active towards DPPH, with values ranging of regression equation between TPC and TFC with AA by DPPH assay was Y = -0.2465X + 125.85; R^2 = 0.6776, and Y = -0.0921X + 92.948; R^2 = 0.8009, respectively.

The DPPH assay is based on the reaction of the DPPH radical with the hydrogen-donors molecules from PEE. Phytochemical content in PEE inhibits the oxidation of other molecules, provided depends on its concentration, and reactivity towards the reactive oxygen species. A lower EC_{50} correlate better with higher DPPH radical scavenging activity, which represents the concentration of the extract to decrease 50% of the DPPH solution initial absorbance. Antioxidant potency is usually associated with the content of phenolic compounds due to their extensive conjugated π-electron systems that facilitate the donation of electrons from the hydroxyl moieties to oxidizingradical species.[22]

From the calculation data, it is known that PEE from this research are strong antioxidant, according to Jun *et al.* (2003),[25] that classification of antioxidant power are strong antioxidant with strength level (IC_{50}<50 ppm), active (IC_{50} 50-100 ppm), moderate (IC_{50} 101-250 ppm), weak (IC_{50} 250-500 ppm), and inactive (IC_{50}> 500 ppm).[25] The EC_{50} DPPH radical scavenging activity of Propolis in this research are inferior to Bolivian Propolis those reported by Nélida Nina (2016)[17] ranged from 4.54 to 48.27 µg/mL, Propolis Stingless Bees (Meliponinae) those reported by Karuane Saturnino (2016)[4] ranged from 29.81 ± 2.49 to 50.23 ± 1.60 µg/mL and Chinese Propolis those reported by Kai Wang (2014)[21] ranged from 15.49±70.59 to 28.69±71.52 µg/mL, but it superior to the Brazilian Propolis those reported by Mara L F Bittencourt (2015)[22] ranged from 21.50 to 78.77 µg/mL.

The reducing power of FRAP capacity obtained from PEE related with a lower EC_{50}, the concentration to reduce 50% of the FRAP reagent initial absorbance. The reducing power obtained for the rough propolis ethanolic extract (RPEE), PEE from rough propolis, are the lowest than the other types of propolis sample. SPEE samples gave the best reducing power. The EC_{50} reducing power capacity of PEE was inferior to *Garcinia porrecta* Laness extract those reported by Shinta Marlin (2017)[16] ranged from 1.33 to 19.96 µg/mL.

The antioxidant capacity of the PEE was determined using the FRAP method, based on the reduction of potassium ferricyanide. The reducing agents in the PEE induced reduction of the ferric ions (Fe^{+3}) to ferrous ion (Fe^{+2}). Ion Fe^{+3}chelated with nucleophilic aromatic rings as specific chelators groups present in the polyphenolic compound. An increase in absorbance indicates a high reducing power.[20] The reducing power capacity of the samples is probably due to the phytochemical components present in propolis extracts.

Tetragonula spp produces propolis that has large quantities of total flavonoid and phenolic compounds compared to other types of bees.[26] Propolis have the highest antioxidant activity compare than other bee product.[20]

HPLC-ESI-MS/MS analysis used to identify the primary compounds of PEE was carried out comparing the Rt, molecular weight and MS fragmentation patterns with UNIFI literature database. As shown in Figure 6, a total of 11 compounds were identified in smooth propolis extract, and the results were shown in Table 6. As shown in Figure 7, a total of 9 compounds were identified in rough propolis extract, and the results were shown in Table 7. The results of the LC-MS analysis provide major peaks determining the presence of phytochemical compounds, of Kushenol-F,, 8-epi-Helenalin, (-)-Sesamin, and Curcumin, but various compounds were not identified in UNIFI database, or previous research has studied chemical compound in Propolis. Tetragonula bees gather propolis from diverse resinous plant parts, and in different phytogeographic regions, its chemical composition might vary significantly.[3]

Curcumin, 8-epi-Helenalin, (-)-Sesamin, and Kushenol-F, were found in propolis extract have antioxidant activity. Curcumin, from genus *Zingiberaceae*, has a unique conjugated structure shows a typical radical trapping ability as a chain-breaking antioxidant, including two methoxylated phenols and an enol form of β-diketone.[27] Helenalin was an antioxidant potential, while widely considered not to be an attribute of sesquiterpene lactone due to their structure.[28] The presence of phenylpropanoid compound namely lignan such as sesamin provide adefense mechanism against reactive oxygen species.[29] Kushenol-F, a flavonoid compound, also indicate the complex antioxidant activity.

The content of 8-epi-helenalin compounds in this research, was the first reported to be found in propolis. While sesamin has been reported by Bankova V (2000) contained in Propolis of the Canary Islands.1 Then the content of curcumin in propolis has been reported by Li Yang (2013) who examines Chinese propolis.30 Then the compound kushenol F has been reported to be present in Libyan propolis by Siheri *et al* (2016).[31]

CONCLUSION

Smooth propolis which taken from inside the nest was the most potent antioxidant among of all the types of examined propolis. The antioxidant activity was influenced by the phenolic content of.

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CONFLICT OF INTEREST

We have no conflicts of interest to disclose and we hereby transfer, assign, or otherwise convey all copyright ownership, including all rights incidental thereto, exclusively to the journal, in the event that such work is published by the journal.

ABBREVIATIONS USED

PEE: Propolis Ethanolic Extracts; **FRAP**: Ferric Reducing Antioxidant Power; **UPLC-TOF-MS:** Ultra Performance Liquid Chromatography TOF Mass Spectrometer; **EC_{50}**: The concentration of samples resulting in 50% antioxidant capacity; **DPPH:**2,2-Diphenyl- 1-picrylhydrazyl; **TPTZ:**2,4,6-Tri(2-pyridyl)-s-triazine; **TPC**:Total Phenolic Content; **TFC**: Total Flavonoid Content; **GAE:** Gallic Acid Equivalents; **QE :** Quercetin Equivalents; **AA:** Antioxidant Activity; **MPEE:** Mix Propolis Ethanolic Extract; SPEE: Smooth Propolis Ethanolic Extract; **RPEE:** Rough Propolis Ethanolic Extract (RPEE).

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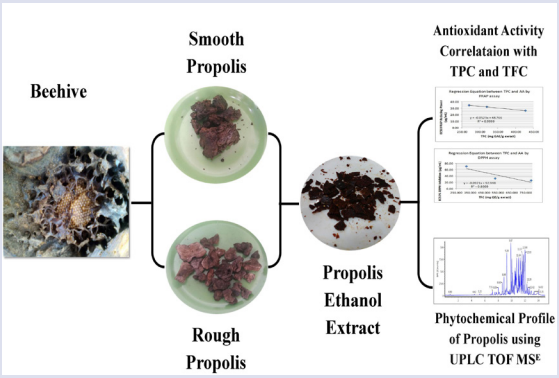
GRAPHICAL ABSTRACT



SUMMARY

- Tetragonula Bees produce propolis that contained one of the biological activity as an antioxidant, which is the most potent antioxidant of all the bee products.
- The types of beehive were smooth (taken from inside the nest), rough (taken from outside the hive) and mix (a combination of both). Smooth propolis was the most potent antioxidant among of all the types of examined propolis.
- The antioxidant activity was influenced by the polyphenol content of.
- This research was the first study reported polyphenol compound 8-epi-helenalin found in propolis.

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