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Antioxidant and Antibacterial Activity of Selected Indonesian Honey against Bacteria of Acne

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Abstract

Honey has natural bacteriostatic and bactericide activities; thus, it could be active compounds as an antibacterial against *Propionibacterium acnes,* the main bacteria in acne forming state. Our study aims to determine phenolic and flavonoid contents (qualitative and quantitative), antioxidants, along with antibacterial activity against *P. acnes* of selected Indonesian honey. Honey used in this study, including honeybees and stingless bees, were collected from Sumbawa, Riau, Belitung, Sukabumi, Banyuwangi, Bogor, and South Sulawesi. In the method, the quantitative content of phenols and flavonoid were measured using a spectrophotometer. Further, DPPH (2, 2'-diphenylpicryl hydrazyl) and ABTS (2'-azino-bis (3- ethylbenzothiazoline-6-sulfonic acid) assays were used to measure the antioxidant activities, and subsequently microdilution method was applied to determine antibacterial activity. The results showed that Indonesian honey contains phenolics and flavonoids in the range of 49.5-67.3 µg Gallic Acid Equivalent (GAE)/g and 766.5-1509.5 µg Quercetin Equivalent (QE)/g, respectively. All honey samples also show antioxidant and antibacterial activities. Of note, *Apis cerana* honey from Banyuwangi has the highest antioxidant and antibacterial activity compared to others. It has the Inhibition Concentration (IC_{50}), Trolox Equivalent Antioxidant Capacity, Minimum Inhibitory Concentration (MIC) and Minimum Bactericide Concentration (MBC) values of 59.85 mg/L, 122 µg Trolox/g, 125 mg/mL, and 500 mg/mL respectively. Our study reveals that Indonesian honey can inhibit and kill *P. acnes* as well. Nonetheless, the phenolic content of honey is not always linear with antioxidant and antibacterial activities.

1. Introduction

Acne is an inflammation of the facial skin that is very common, caused by excess oil secretion [1]. Acne formation occurs due to the follicle blockages by dead skin cells, which can be caused by several things, including the activity of hormones, genetic factors, and infection by the acne bacteria *Propionibacterium acnes* [2]. One approach to overcome acne is to kill or inhibit the growth of bacteria that cause acne with antibiotics. However, excessive use of antibiotics can promote bacteria that were originally sensitive to become resistant [3]. Therefore, it is necessary to search for natural antibacterial compounds. One of the prospective substances derived from natural ingredients that have the potential for antibacterial and the growth inhibition of acne-bacteria is honey. This particular substance is a natural ingredient produced by flower nectar, processed in the digestive tract of bees, and formed through dehydration in the honeycomb [4]. Interestingly, honey can play a significant role in bactericide and bacteriostatic like antibiotics. Antibacterial power of honey was reported to originate from the high flavonoid content in honey, which has an antibacterial mechanism using honey osmosis pressure, acidity, and the presence of flavonoid inhibiting compounds [5].

In some previous studies, honey has shown potent activity against acne bacteria. Julianti *et al.* [6] reported the antibacterial activity of Indonesian forest honey against acne bacteria with Minimum Inhibitory Concentrations (MICs) of 50% (v/v) for both *P. acnes* and

Staphylococcus epidermidis. Several types of Algeria honey also showed anti-acne bacteria by releasing hydrogen peroxide, an antibiotic that can remove bacteria and clear acne [7]. Also, honey bee (Apis mellifera) venom from South Korea exhibited antibacterial activity against acne bacteria, including *P. acnes*, *S. epidermidis*, and *Streptococcus pyrogenes* along with an anti-inflammatory activity by reducing the *P. acnes*-induced secretion of IL-8 and TNF-α in THP-1 cells [8]. However, recent antibacterial study against acne bacteria concerning antioxidant activities from different types of Indonesia honey is still limited. Therefore, this paper investigates the antibacterial capacity against acne bacteria, phytochemical contents, and antioxidant activities of Indonesia honey produced by various bee species in different Indonesia regions including two species of honey bees *Apis dorsata* (from Sumbawa, Riau, and Belitung), *Apis cerana* (from Sukabumi, Banyuwangi, and Bogor) and two species of stingless bees *Tetragonula sapiens* and *Wallacetrigona incisa* (from South Sulawesi). Apis bee, including *A. dorsata* and *A. cerana*, can be found in Indonesia's almost all islands except Maluku and Irian Jaya [9]. Unlike Apis bee, stingless bees, *T. sapiens* and *W. incisa* comes from the Meliponini tribe, which has more than one genera, for example, Melipona, Scaptotrigona, and Trigona [10]. Besides in Sulawesi [11], *Tetragonula sapiens* also distribute in Moluccas Islands, Solomon Islands, Australia, Philippines, and New Guinea [12], while *W. incisa* is a Sulawesi endemic species which is restricted in mountainous area [13]. Stingless bee produces honey derived from several flowers stored in small resin containers in the nest, while honey from Apis spp. stored in hexagonal honeycomb [14].

2. Methodology

2.1. Sample Preparation and Qualitative Phytochemical Test

About eight honey samples collected from a different origin in Indonesia (Table 1). The precipitation of samples based on SNI 3545: 2013 method. Water content was determined using a refractometer [15] and qualitative phytochemicals analyzed using the Harborne method [16].

2.2. Determination of Total Phenolic

Ethanol 96% and 5 mL of distilled water were added to 0.05 g of the sample, then 0.5 mL of 50% Folin-Ciocalteu reagent was added, then homogenized and allowed to stand in dark conditions for ±60 minutes. Measurement of samples was read using a wavelength of 725 nm. The absorbance value was then converted into total phenolic and expressed as equivalent gallic acid or Gallic Acid Equivalent (GAE) in mg GAE/g sample weight. The standard used in testing total phenolic was gallic acid with concentrations of 10, 30, 50, 70, and 100 mg/L [17].

2.3. Determination of Total Flavonoids

The total flavonoid content was carried out using aluminum chloride. Ten microliter samples, 60 µL methanol, 10 µL aluminum chloride (10% w/v), 10 µL potassium acetate (1M), and 120 µL distilled water were mixed and incubated at room temperature for 30 minutes. Absorbance was measured at a wavelength of 415 nm. The standard solution used was quercetin (in methanol) final concentrations of 0, 10, 25, 50, 75, and 100 µg/mL. Total flavonoid extract samples were expressed as milligrams of quercetin per gram weight extract (mg QE/g extract) [18].

2.4. Determination of Antioxidant Activity of DPPH Method

A total of 100 µL of DPPH 125 µmol/L solution was inserted into the 96 well plates in which there were 100 µL samples. The solution was incubated for 30 minutes at room temperature. The absorbance of the solution was measured at a wavelength of 517 nm using a multi-well plate reader [19]. The positive control used was ascorbic acid. The IC_{50} value was determined by varying the concentration of the sample calculated by the formula:

$$\text{Inhibition (\%)} = \left[\left(1 - \frac{(A_{sample} - A_{standard})}{(A_{blank} - A_{standard})}\right) \times 100\%\right]$$

2.5. Determination of Antioxidant Activity of the ABTS Method (2,2'-azino-bis (3- ethylbenzothiazoline-6-sulfonic acid)

The total antioxidant capacity was estimated as Trolox Equivalent Antioxidant Capacity (TEAC) assay using the method of Lee *et al.* [20]. Tests were based on each substance's ability to form radical cations $ABTS^{\bullet+}$, which was compared to the standard (Trolox). Radical cations were prepared by mixing 7 mM ABTS stock solution with 2.45 mM potassium persulfate (l/l, v/v). It could mix for 4-8 hours until the reaction ran perfectly marked by stable absorbance. The $ABTS^{\bullet+}$ solution was dissolved in ethanol until the absorbance reaches 0.700 ± 0.05 at a wavelength of 734 nm. The radical cation ABTS was blue and absorbed light at a wavelength of 734 nm. Measurements were taken by taking 0.9 mL of $ABTS^{\bullet+}$ solution and 0.1 mL extract of the sample dissolved in methanol. The mixture was shaken for 45 seconds and immediately measured to determine the absorbance at a wavelength of 734 nm after 1 minute. The standard used in determining the antioxidant value of the ABTS method was Trolox. Trolox standards were made with variations in concentrations between 0.625, 1.25, 2.5, 3.75, and 5 µM.

2.6. Determination of Antibacterial Activity

Antibacterial activity testing used microdilution with Trypticase Soy Broth (TSB) media on 96-well plates [21]. TSB media contained GAM 0.5% Nissui broth, 1.0% glucose (Japanese Wako), 0.3% yeast extract (Difco, France), nutrient broth 0.5 (Difco, France) and 0.2% tween-80 (Biomedical MP, Japan). The sample was diluted with the addition of 20% DMSO to make a concentration series of 1.56%; 3.125%; 6.25%; 12.5%; 25%; and 50%. *P.acnes* bacteria were incubated in the media for 72 hours under anaerobic conditions. A total of 100 µL samples, 95 µL TSB media, and 5 µL *P.acnes* bacterial inoculants were inserted into each well on the 96-well plate. The mixture was incubated at 37°C for 24 hours. The lowest extract concentration showed no bacterial growth was chosen as the Minimum Inhibitory Concentration (MIC). The mixture in the clear well was

transferred as much as 10 µL into the new 96-well plate. Then, it added 100 µL TSB media and re-incubated at 37°C for 24 hours. The lowest extract concentration that was still clear after the second incubation was chosen as the Minimum Bactericidal Concentration (MBC). The positive control used was tetracycline, while the negative control used was 20% DMSO.

2.7. Thin Layer Chromatography Analysis (TLC) Antibacterial Bioautography

The antibacterial activity test with TLC was measured by dissolving honey with 96% methanol so that the concentration was 100,000 µg/mL. Bioautography used was contact bioautography. The silica plate was fractioned three times and then eluted with the best eluent. The chromatogram was then dried and placed on agar media, which has been inoculated with bacteria at a concentration of 10^{-2} CFU/mL for 1 hour. The chromatogram band was marked on the outer cup portion and removed. The cup's incubation has been pasted with a chromatogram at 37°C for 24 hours [22]. The bacteria used were *P. acnes* bacteria.

Antibacterial compounds were identified by bioautography then eluted again using TLC. Each sample was bottled ten times to the silica plate and then dried. The plate was then placed into a vessel containing the eluent above, starting with a single eluent and then mixing. The plates were then dried and observed under UV lamps with wavelengths of 254 nm and 366 nm. The plate was then sprayed with reagent Dragendorff for alkaloids, ammonia for flavonoids, $FeCl_3$ for phenolic, and H_2SO_4 for terpenoids [16].

2.8. Statistical Analysis

All tests were carried out three times. The total flavonoid, total, total tannin, and antioxidant activity of DPPH and ABTS methods were presented descriptively. The data obtained were expressed as a mean value ± standard deviation (SD) of three replications. The statistical analysis used was the analysis of variance (one-way ANOVA) using SPSS 16 software with a value of α of 0.05.

2.9. Tools and Materials

The tools that used in this study were refractometer, goblet, test tube, pipette, measuring cup, stirring rod, flask, Erlenmeyer, filter paper, microplate 96 well, spatula, micropipette, incubator, vortex, aluminum foil, UV-Vis spectrophotometer, UV lamps, vessels, silica plates, G60F254 silica plates, and Petri dishes. While, the main ingredients used in the study were three types of *A. dorsata* honey, three kinds of *A. cerana* honey, and two types of stingless bee honey. The main ingredients of this study are summarized in Table 1.

Table 1. The honey sample used in this study

No.	Type of sample	Species	Origin
1.	Honey of honeybee	*Apis dorsata*	Sumbawa
2.			Riau
3.			Belitung
4.		*Apis cerana*	Sukabumi
5.			Banyuwangi
6.			Bogor
7.	Honey of stingless bee	*Tetragonula sapiens*	South Sulawesi 1
8.		*Wallacetrigona incisa*	South Sulawesi 2

Table 2. Phytochemical content of honey

No.	Type of sample	Species	Origin	Water content (%)	Phenolic	Flavonoid	Tannin
1.	Honey of honeybee	*Apis dorsata*	Sumbawa	24.60^{c}	+	+	-
2.			Riau	21.06^{a}	+	+	-
3.			Belitung	25.00^{c}	+	-	-
4.		*Apis cerana*	Sukabumi	24.53^{c}	+	+	-
5.			Banyuwangi	22.13^{c}	+	+	-
6.			Bogor	22.93^{c}	+	+	-
7.	Honey of stingless bee	*Tetragonula sapiens*	South Sulawesi 1	21.13^{c}	+	+	-
8.		*Wallacetrigona incisa*	South Sulawesi 2	28.86^{c}	+	-	-

Description: (+) = detected, (-) = undetected

Data marked with different letters in the same column indicate a significant difference by Duncan post-hoc at $p < 0.05$

Table 3. Total phenolic and flavonoids contents of honey

No.	Type of sample	Species	Origin	Total Phenolic Content* (µg GAE/g) ± SD	Total Flavonoid Content* (µg QE/g) ± SD
1.	Honey of honeybee	*Apis dorsata*	Sumbawa	57.5±1.9^{abc}	766.5±41.2^{ab}
2.			Riau	49.5±0.2^{a}	1115.7±90.3^{bcd}
3.			Belitung	57.4±0.2^{abc}	917.5±113.5^{a}
4.		*Apis cerana*	Sukabumi	64.4±0.8^{bc}	935.2±112.9^{abc}
5.			Banyuwangi	57.9±0.9^{ab}	938.8±87.6^{abc}
6.			Bogor	58.8±3.7^{ab}	1250.2±123.6^{cd}
7.	Honey of stingless bee	*Tetragonula sapiens*	South Sulawesi 1	52.8±0.5^{abc}	1509.5± 112.1^{d}
8		*Wallacetrigona incisa*	South Sulawesi 2	67.3±1.0^{c}	1218.7±190.1^{cd}

Information: (*) average ± standard deviation of 3 replications of honey samples.

Data marked with different letters in the same column indicate a significant difference by Duncan post-hoc at $p < 0.05$

Table 4. Antioxidant activity of DPPH, ABTS, and antibacterial activity of honey

No.	Type of sample	Species	Origin	Antioxidant activity		Anti-bacterial	
				DPPH* (IC_{50}) (mg/L)) ± SD	ABTS* (TEAC) (µg/g)) ± SD	MIC (mg/mL)	MBC (mg/mL)
1.	Honey of honeybee	*Apis dorsata*	Sumbawa	347.94±15.76^{d}	19.06±4.17^{ab}	125	500
2.			Riau	443.53±12.32^{g}	48.51±2.36^{b}	250	500
3.			Belitung	130.23±0.78^{c}	10.98± 3.40^{a}	250	250
4.		*Apis cerana*	Sukabumi	518.49±1.19^{h}	21.30±3.80^{ab}	250	250
5.			Banyuwangi	59.85±1.07^{b}	122.13±6.44^{c}	125	500
6.			Bogor	787.79±17.06^{i}	46.91±5.77^{b}	125	500
7.	Honey of stingless bee	*Tetragonula sapiens*	South Sulawesi 1	372.26±5.02^{e}	41.03±4.31^{ab}	125	250
8.		*Wallace trigona incisa*	South Sulawesi 2	398.57±3.47^{f}	19.83±4.17^{ab}	250	500
9	Ascorbic acid			4.72±0.11^{a}	-	-	-
10	Thiamphenicol			-	-	0.313	0.625

Information:
(*) average ± standard deviation of 3 replications of honey samples.
Data marked with different letters in the same column indicate a significant difference by Duncan post-hoc at $p < 0.05$
IC_{50} indicates the ability to scavenge DPPH radical of 50%
TEAC: Trolox Equivalent Antioxidant Capacity of using ABTS method
MIC: Minimum inhibitory concentration
MBC: Minimum Bactericidal Concentration

Table 5. Pearson correlation coefficient between phenolic levels, flavonoids, antioxidant activity and antibacterial in honey and propolis extract

	Antioxidant		Antibacterial	
	IC_{50}	TEAC	MIC	MBC
Phenolic	0.119	-0.254	0.268	-0.012
Flavonoid	0.389	0.025	-0.270	0.075

Note:
IC_{50} indicates the ability to scavenge DPPH radical of 50%
TEAC: Trolox Equivalent Antioxidant Capacity of using ABTS method
MIC: Minimum inhibitory concentration
MBC: Minimum Bactericidal Concentration

Table 6. Pearson correlation coefficient of antioxidant activity between DPPH and ABTS methods

	Antioxidant	
	IC_{50}	TEAC
IC_{50}	1	
TEAC	-0.320	1

Note:
IC_{50} indicates the ability to scavenge DPPH radical of 50%
TEAC: Trolox Equivalent Antioxidant Capacity of using ABTS method

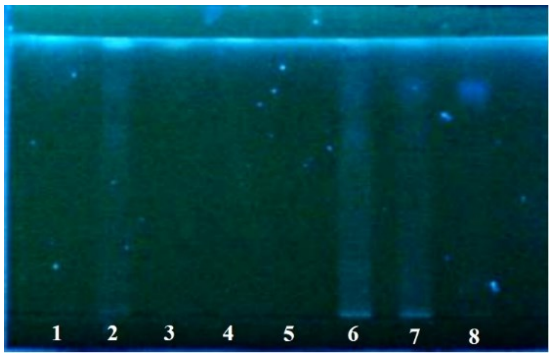

Figure 1. Chromatogram of eight types of honey with the mobile phase of ethyl acetate: methanol: acetic acid: water (5: 2: 2: 1). From left to right: 1 (Sumbawa), 2 (Riau), 3 (Belitung), 4 (Sukabumi), 5 (Banyuwangi), 6 (Bogor), 7 (South Sulawesi 1), 8 (South Sulawesi 2).

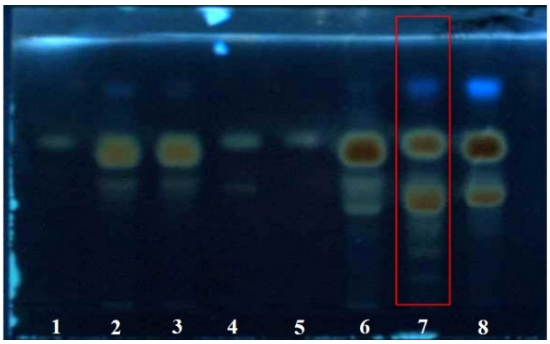

Figure 2. Chromatogram of eight types of honey after sulfuric acid staining of 10% (v/v). From left to right: 1 (Sumbawa), 2 (Riau), 3 (Belitung), 4 (Sukabumi), 5 (Banyuwangi), 6 (Bogor), 7 (South Sulawesi 1), 8 (South Sulawesi 2).

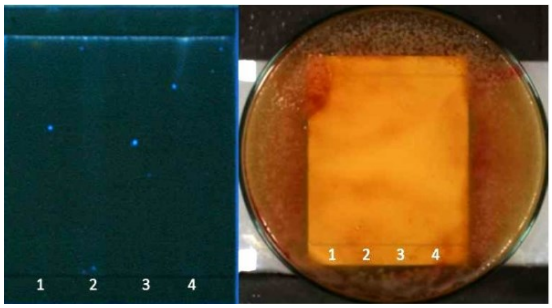

Figure 3. Chromatogram (a) and bioautogram (b) of honey, from left to right: 1 (Sumbawa), 2 (Riau), 3 (Belitung), and 4 (Sukabumi honey) against *P. acnes.*

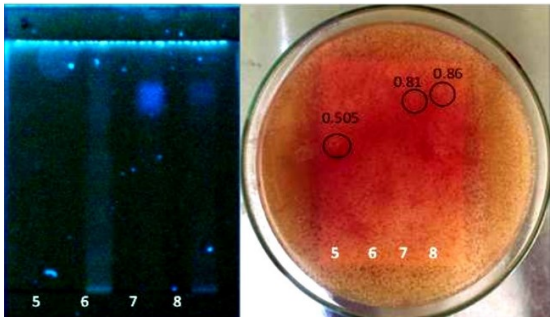

Figure 4. Chromatogram (a) and bioautogram (b) of honey, from left to right: 5 (Banyuwangi), 6 (Bogor), 7 (South Sulawesi 1), and 8 (South Sulawesi 2) against *P. acnes.*

3. Results and Discussion

3.1. Water Content

Water content function determines the quality and durability of food [23]. The testing of honey water content was repeated three times (SNI 01-3545-2013). The refractive index of honey obtained by observation with a refractometer was then converted to moisture content. The water content of eight different types of honey and the origin of honey shows varying values (Table 2). The highest water content is demonstrated in *W. incisa* South Sulawesi 2 at 28.86%, which shows a significant difference with the seven other honey samples.

In comparison, the lowest water content is shown by *A. dorsata* Riau honey of 21.06%, which is not significantly different from honey *T. sapiens* South Sulawesi 1. This indicates that differences in species of bee, source of nectar, climate, temperature, environmental conditions, and processing techniques significantly influence water content ($p < 0.05$). The standard for maximum water content for *A. mellifera* honey is 22% (SNI 01-3545-2013).

Water content in honey depends on the plant origin, processing techniques, and storage conditions. High water content can affect honey's viscosity, cause crystallization, interfere with sensory properties, and solubility. Besides, high water content causes honey to undergo fermentation and damage, while low water content keeps honey from damage and has a safe storage period for a relatively long time [24].

3.2. Phytochemical content

Honey contains various phytochemical compounds that have a function as bioactive compounds. In this study, the presence of flavonoids, phenolics, and tannins was determined. Interestingly, we identified that all the honey contains phenolic (Table 2). Positive results of phenolic testing are indicated by the occurrence of changes to the reddish color of honey.

Moreover, almost all the honey contains flavonoids, except *W. incisa* South Sulawesi 2 honey. The positive results of flavonoid testing are indicated by the occurrence of orange and yellow changes in the amyl alcohol layer. The phytochemical content identified qualitatively was then further tested quantitatively, through total flavonoid analysis and total phenolic analysis. The total quantitative content of tannins was not carried out due to all honey samples' negative results in this qualitative test.

3.2.1. Total Phenolic Content

The total phenolic content is the number of phenolic compounds found in a plant or natural substance. Total phenolic can be used to determine the potential of honey as a source of antioxidants. The analysis of total phenolic levels was established by the Folin-Ciocalteu method. Folin-Ciocalteu reagent consists of sodium tungstate, sodium molybdate, hydrochloric acid, phosphoric acid, and lithium acetate [25]. The principle of the Folin-Ciocalteu method is the occurrence of an oxidation reaction of phenolic compounds in an alkaline atmosphere by reacting to Folin-Ciocalteu, which produces a blue molybdenum-tungsten complex with a color intensity comparable to phenolic compounds. The intensity color is measured at a wavelength of 750 nm.

This research found that the average total phenolic levels in eight types of honey range from 49.5-67.3 µg GAE/g (Table 3). The highest phenolic level is exhibited by *W. incisa* honey from South Sulawesi 2, which does not show a significant difference with *A. dorsata* honey from Sumbawa and Belitung along with *A. cerana* honey from Sukabumi, Banyuwangi, and Bogor. This suggests that the different types of bees and the origin of honey are not significantly different ($p < 0.05$) to the total phenolic levels (H_o is rejected, and H_i is accepted). In another study applying the same method, the phenolic content of *A. mellifera* honey originating from Pati and Magelang ranged from 2422-4433 µg/g [26]. The difference in the value of total phenolic levels is suggested by the different types of bees and the extraction method.

In contrast, the total levels of phenolic stingless bee honey originating from Masamba, South Sulawesi, were reported to be 1900 µg/g [27]. The difference in the value of total phenolic levels in stingless bee honey samples with Usman *et al.* [27] is very significant due to different treatments. This is done by adding propolis to honey samples by 15%.

Phenolic compounds are compounds that have one or more hydroxyl groups that bind directly to an aromatic ring. Those aromatic rings affect the stability of the bond of oxygen atoms with hydrogen atoms in the hydroxyl group. These properties cause phenolic compounds and polyphenols, including the groups of compounds that function as antioxidants. Importantly, phenolic compounds act as hydrogen donors for free radicals, which subsequently developed stable compounds [28].

3.2.2. Total Flavonoid Content

The objective of total flavonoids measurement is to identify the potential of honey as a source of antioxidants. The determination of total flavonoid content was conducted using aluminum chloride assay. The principle of quantitative analysis from the $AlCl_3$ method is the formation of complexes between aluminum chloride and neighboring ketone and ortho hydroxy groups from flavones and flavonols [29]. Flavonoid levels were calculated as the equivalent number of quercetins.

The research found that the average total flavonoid levels in eight types of honey differ from 766.5 to 1509.5 µg QE/g (Table 3). The highest flavonoid levels are shown by *T. sapiens* honey from South Sulawesi 1, which does not significantly differ with honey sample *A. dorsata* honey from Riau, *W. incisa* South Sulawesi 2, and *A. cerana* honey from Bogor. The lowest flavonoid levels are shown by *A. dorsata* honey from Sumbawa samples, which is not significantly different from *A. dorsata* honey from Belitung, *A. cerana* honey from Sukabumi, and *A. cerana* honey from Banyuwangi. This indicates that differences in species of bee, type of nectar, the origin of honey, processing technique, and harvest time are not significantly different ($p < 0.05$) on the total levels of flavonoids production. Previous reports showed that *A.*

mellifera honey originating from the Pasuruan area, East Java, had a flavonoid level of 472.5 µg/g and *A. cerana* honey from Kediri, East Java at 1562.7 µg/g [30]. Bee types can cause a difference in the value of flavonoid levels obtained. The study of Ustadi *et al.* [30] showed the regional origin, harvested time, and honey processing techniques affect the levels of flavonoids.

Further, polyphenol compounds can indicate the potential as an antioxidant because they can contribute H^+ ions to free radical compounds. However, the differences in qualitative and quantitative total flavonoid results might be caused by the difference in limit detection. Too low a concentration of qualitative tests gives negative results. In case, the data in quantitative analysis of *W. incisa* honey from South Sulawesi 2 is 1,218.7 µg QE/g or 1.2 mg QE/g (Table 3) samples indicating the low concentration and possible to be not detected on the qualitative analysis.

3.3. Antioxidant Activity of DPPH Method

An analysis of antioxidant activity is conducted to determine the presence of the inhibition of free radical activity. The analysis method uses DPPH (2,2'-diphenylpicryl hydrazyl) by working to reduce free radicals. The free radical activity is obtained from the regression equation derived from the relationship between sample concentration and percent inhibition of free radical activity. The positive control used in testing antioxidant activity is ascorbic acid with a 10 mg/L concentration. Notably, antioxidant activity is the ability of a compound or extracts to inhibit an oxidation reaction, expressed in percent inhibition. The parameters used to show antioxidant activity are obtained from inhibition concentration (IC_{50}), which is the concentration of an antioxidant, which gives a percentage of 50% [31]. The source of free radicals from the method applying in this study is the DPPH compound. The principle of this test is the donation of hydrogen atoms from the substance tested on DPPH radicals into diphenyl-picryl hydrazyl non-radical compounds due to the electrons become paired and color changes from purple to yellow [31]. Based on the research, IC_{50} values obtained ranged from 59.85 to 787.79 mg/L (Table 4). The lowest IC_{50} value is indicated by *A. cerana* honey from Banyuwangi, which is not significantly different from other honey.

In contrast, the highest IC_{50} value is shown by *A. cerana* honey from Bogor samples, which are substantially different from other honey. We suggest that the type of bee does not affect the honey content but is caused by the honeybee's location in searching for nectar. The composition of honey varies depending upon the geographical and the nectar source of the region.

Ascorbic acid as positive control shows IC_{50} results of 4.72 mg/L. Nevertheless, the antioxidant activity obtained for honey has a higher IC_{50} value than positive controls indicating the lowest activity than the positive control. Of note, the IC_{50} value of antioxidant activity ranges from 0-50 ppm, which is among the most powerful antioxidants. The value between 50-100; 100-150; 150-200 and more than 200 ppm, indicating antioxidant capacity are strong, medium, weak, and very weak, respectively [31]. Honey samples have feeble antioxidant activity except in *A. cerana* honey from Banyuwangi. High flavonoid levels influence the strength of antioxidant activity indicated by the low IC_{50} value. The correlation between phenolic levels and antioxidant activity is shown in Table 4.

Based on the Pearson correlation using SPSS software, our DPPH antioxidant activity is positively correlated with phenolic and flavonoid contents in the samples tested. It has an r-value of 0.119 and flavonoid content of 0.389 (Table 5). Notably, Sarwono [32] mentioned that coefficient correlation value (r) between 0.00 – 0.25 showed a weak correlation, whereas 0.25-0.50; 0.50-0.75; 0.75-0.99 and 1.00 values indicated a sufficient, reliable, robust, and perfect correlation, respectively. In our study, the correlation between phenolic and DPPH IC_{50} values shows a weak correlation, whereas between levels of flavonoids and the DPPH IC_{50} values show a sufficient correlation. Positive values indicate that the correlation is directly proportional, where the higher the phenolic level, the higher the DPPH IC_{50} value.

3.4. Antioxidant Activity of the ABTS Method

Antioxidant activity on honey was also determined using the ABTS method. The ABTS test method measures the ability of relative antioxidants to capture ABTS radical production. ABTS radicals are produced by reacting potent oxidizing agents such as potassium persulfate with ABTS salts. Further, relative radical capture activity is expressed as a percentage of radical inhibition. The mechanism of antioxidants in counteracting free radicals ABTS is a reaction of hydrogen transfer or donation [33].

In the present study, the ability of the ABTS antioxidant activity is expressed in TEAC (Trolox Equivalent Antioxidant Capacity). The Trolox standard regression equation is obtained from the curve of the relationship between concentration and absorbance value, $Y = -0.027x + 0.2101$. The higher the TEAC value shows the more equivalent Trolox in the sample, which means that the antioxidant activity gets stronger. We found that the highest TEAC value is indicated by *A. cerana* honey from Banyuwangi samples (Table 5), which are significantly different from other samples. Whereas, the lowest activity is shown by *A. dorsata* honey from Belitung samples, which are not substantially different from *A. dorsata* honey from Sumbawa samples, *A. cerana* honey from Sukabumi, *T. sapiens* honey from South Sulawesi 1, and *W. incisa* honey South Sulawesi 2. This indicates that the origin of regions, types of plant sources, and different species of bees do not significantly affect the antioxidant activity production. The TEAC's value is based on the report of Alzahrani *et al.* [34], originating from England honey ranged from 43 to 202 µg/g. Honey tested in this study has a higher TEAC value compared to the corresponding study. Notably, the differences in origin, climate, flowering time, temperature, type of flower, and different species of bee affect honey production quality.

Moreover, the Pearson correlation between DPPH (IC_{50}) and ABTS (TEAC) antioxidant values using SPSS

software has an R-value of -0.320, which indicates a relatively sufficient correlation (Table 6). The negative values indicate an inversely proportional relationship, which means that the higher the TEAC value, the lower the IC_{50} value. On the other hand, the TEAC value correlation with phenolic and flavonoid content has R-values of -0.254 and 0.025, respectively (Table 5). Negative values on the correlation between phenolic levels and TEAC values show an inverse correlation, which is the higher the phenolic level. The lower the antioxidant activity of the ABTS method. The positive values on the correlation between the levels of flavonoids with TEAC values show a straight correlation, even though it is in a very weak correlation (r of 0.025). The higher levels of flavonoids, the more significant antioxidant activity of the ABTS method.

3.5. Antibacterial Activity

The Minimum Inhibitory Concentration (MIC) is defined as a concentration that indicates no bacterial growth. The minimum bactericidal concentration (MBC) is defined as the concentration of the sample, which shows no bacterial growth and shows no increase in MIC after the inoculation of the two new media [21]. MIC and MBC values are determined at concentrations that provide clear zones without microbial growth in the agar media by visual observation. Interestingly, we obtained that all types of honey tested were proven to inhibit and kill *P. acnes* bacteria (Table 4). The inhibiting activity of *P. acnes* with the lowest concentration is shown in *A. dorsata* honey from Sumbawa samples, *A. cerana* honey from Banyuwangi, *A. cerana* honey from Bogor, and *T. sapiens* South Sulawesi 1 with the same MBC value of 125 mg/mL. The lowest concentration of honey to be able to kill all bacteria, which is equal to 250 mg/mL indicated by *A. dorsata* honey from Belitung, *A. cerana* honey from Sukabumi, and *W. incisa* from South Sulawesi 2.

Meanwhile, thiamphenicol, which is used as a positive control, can inhibit and kill *P. acnes* simultaneously with MIC and MBC values of 0.31 and 0.625 mg/mL, respectively. All honey samples have higher MIC and MBC values than positive controls. In general, if compared to one honey sample with the other sample, *T. sapiens* has the best antibacterial activity. *A. cerana* honey from Banyuwangi has the highest antioxidant and antibacterial activity compared to other samples. The similarity of MIC and MBC values in some of the honey tested shows that honey has the same antibacterial activity even though it has different antioxidant activities.

All types of honey tested have the potential as an antibacterial against *P. acnes*. This is consistent with the study of Zainol *et al.* [14], that honey can be antibacterial in both gram-positive and gram-negative bacteria. The antibacterial power of honey can be caused by the flavonoid content in honey, which has an antibacterial mechanism using honey osmosis pressure, acidity, and the presence of inhibiting compounds [5]. The difference in antibacterial activity in honey depends on the nectar collected by bees to produce honey [3]. Gram-negative bacteria tend to be more resistant than gram-positive bacteria, so higher concentrations are needed to inhibit or kill these bacteria. The correlation between phenolic content with MIC and MBC values has r values of 0.268 and -0.012. While the correlation between flavonoids content with MIC and MBC values has an r-value of -0.270 and 0.075 (Table 5). Positive values indicate a directly proportional correlation. The higher the phenolic level, the higher the MIC value, and the higher level of flavonoids, the higher the MBC value. As for negative values show an inverse correlation, which is, the higher the phenolic level, the higher the value of the MBC, and the higher the level of flavonoids, the lower the MIC value. Supposedly, the higher the phenolic level, the lower the concentration needed to inhibit and kill the bacteria. This difference in results can be caused by a very weak correlation between phenolic levels and antibacterial activity. In general, the antioxidant and antibacterial of honey have a many times higher value than the positive control, Ascorbic acid, and thiamphenicol, indicating lower activity than its positive controls. It can happen since honey is still a multicomponent mixture, unlike ascorbic acid and thiamphenicol as a single compound. However, as a mixture, honey is potentially antibacterial and antioxidant compared with other extracts from plants [21]. To get a higher activity on the honey component, separation to get a single active component is needed.

3.6. Honey Thin Layer Chromatography

Thin Layer Chromatography was conducted to determine the chromatogram pattern in the eight honey samples. The mobile phase used in the separation of honey component is ethyl acetate: methanol: acetic acid: water with a ratio of 5: 2: 2: 1. The concentration of honey used is 2% (b/v). The TLC plate after development is then detected under 366 nm UV light. The eight honey comes from different types of bees. The types of bee inline 1-3 are *A. dorsata* honey, line 4-6 *A. cerana* honey, and 7-8 *T. sapiens* and *W. incisa.* The chromatogram pattern produced from the eight honey samples can be seen in Figure 1.

The chromatogram pattern on lines 1-6 spot is not detected, while on lines 7 and 8, there is one spot. Therefore, staining with 10% sulfuric acid has a function to show some spots that could not be detected. Sulfuric acid is a reagent which commonly used for coloring. This solution can identify many components, flavonoids, for example. The TLC plate that was given coloring was heated at 105°C for 5 minutes. Then the chromatogram was observed at 366 nm UV light. Based on Figure 2, the chromatogram produced on the plate after staining results in a higher number of bands than without staining. Most ribbons are generated on line 7 by six bands. This shows the highest compound content in South Sulawesi honey 1 of *T. sapiens*.

3.7. Thin Layer Chromatography (TLC) Antibacterial Bioautography

Antibacterial activity tested with TLC bioautography was applied to *P. acnes* bacteria. Antibacterial activity characterized by the presence of clear zones on bioautography is only found in lines 5, 7, and 8 (Figure 4).

The clear zone is formed when sprayed with tetrazolium chloride salt with a concentration of 2 mg/mL. This indicates that fraction derived from *A. cerana* honey from Banyuwangi, *T. sapiens* honey from South Sulawesi 1 and *W. incisa* honey South Sulawesi 2 can inhibit the growth of *P. acnes* bacteria. Antibacterial activity with the TLC bioautography method can be seen in Figures 3 and 4. Clear zones on bioautography show antibacterial activity. The clear zone produced in bioautography shows that the antibacterial activity compounds are Rf of 0.50, 0.81, and 0.86. The chromatograms of lines 7 and 8 (Figure 2) show blue spots suspected of being flavonoid compounds. Therefore, we strongly suggest that both south Sulawesi honey (1 and 2) contain flavonoids because they show blue spots.

The different types of bees can affect the components contained in honey samples. However, Sumbawa, Riau, Belitung, and Sukabumi honey samples do not show a clear zone after tetrazolium chloride salt is sprayed. Bioautogram samples that are not active as antibacterial turn red due to formazan compounds result from the reaction indicator of tetrazolium with bacteria [35]. These results can be caused by the concentration of honey samples that are too low. This very little concentration causes bacteria in the media not to be inhibited by honey. We suggested that compounds in honey that are predicted to inhibit bacterial growth are flavonoids. Flavonoids are related to various kinds of sugar [36]. The presence of sugar in honey causes bacterial growth to increase so that bacteria get food intake from sugar. To get better results, the sugar must be removed first from the honey matrix to detect antibacterial compounds (flavonoids) easier.

4. Conclusion

The Indonesian honey contained phenolics and flavonoids in the range of 49.5-67.3 µg Gallic Acid Equivalent (GAE)/g and 766.5-1509.5 µg Quercetin Equivalent (QE)/g, respectively. All honey samples also show antioxidants and antibacterial activities. Particularly, *Apis cerana* honey from Banyuwangi has the highest antioxidant and antibacterial activity compared with others. It has the inhibition concentration (IC_{50}), Trolox Equivalent Antioxidant Capacity, and Minimum Inhibition Concentration (MIC) and Minimum Bactericidal Concentration (MBC) values of 59.85 mg/L, 122 µg Trolox/g, 125 mg/mL, and 500 mg/mL respectively. All the Indonesian honey used in this study can inhibit and kill *P. acnes* as well.

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