Evaluation of Antibacterial and Antioxidant Activities of Stingless Bee (*Tetragonula Laeviceps*) Hive from Purwodadi Botanical Garden and LC/MS Profiles of Dichloromethane Fraction

Praptiwi Praptiwi(✉), Ahmad Fathoni, Oscar Efendy, Dewi Wulansari, and Andria Agusta

Research Center for Pharmaceutical Ingredients and Traditional Medicine, The National Research and Innovation Agency (BRIN), Bogor, Indonesia
praptiwip@yahoo.com

Abstract. This study evaluates the potential of stingless bee (*Tetragonula laeviceps*) methanol extract and its fractions (hexane, dichloromethane, ethyl acetate, methanol, and acetone-water) as an antibacterial, antioxidant, phenolics content, and LC/MS profiles of dichloromethane fraction. The test was carried out by thin-layer chromatography (TLC) - bioautography (dot-blot and elution), then determined the MIC and IC_{50} values and Antioxidant Activity Index (AAI). Antibacterial tests were carried out against *Staphylococcus aureus* and *Escherichia coli*, and antioxidant potential was carried out by the DPPH method. Total phenolic content was evaluated by the spectrophotometry method. The results showed that crude fraction, hexane, and dichloromethane fractions had moderate antibacterial activity against *S. aureus* (MIC values: 128–256 μg/mL) and weak antibacterial activity against *E. coli.* Fractions of dichloromethane, methanol, and acetone water had a very strong antioxidant activity with an AAI of 5.28, 2.17, 3.00, and 3.56, respectively. The LC/MS profiles showed that the dichloromethane fraction contained 5.7-dihydroxychromone, cnidimon C, puerarin, and irisflorentin. Fractions of dichloromethane, methanol, and acetone-water fraction are potential antioxidant and antibacterial sources.

Keywords: Stingless bee · antibacterial · antioxidant

1 Introduction

Stingless bees (Hymenoptera: Apidae: Meliponini) are highly social bees in the tropics and subtropics [1]. In Indonesia, the stingless bee is known by several local names, including teuweul (West Java), klanceng (Central Java and East Java), and galo-galo (West Sumatra) [2]. Worldwide, 374 stingless bee species are distributed in the tropics and southern subtropics [3]. It is estimated that 40–50 stingless bee species exist in

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Indonesia [4, 5]. The most common species in Indonesia is *Tetragonula laeviceps* (Smith) [2, 5].

The stingless bee is a major visitor to many tropical flowering plants. They produce propolis which is well known for its therapeutic properties, including antimicrobial, antitumor, and antioxidant activity [6]. Stingless bees store their honey in pots made of cerumen, a mixture of wax, and propolis [7]. Propolis is a resinous material that bees collect from various plant exudates. Bees use propolis to narrow nest entrances, seal cracks and embalm dead organisms in the hive. The chemical composition of propolis depends on the collection location, available plant sources, and bee species [8, 9]. Several bees produce propolis, including *Apis mellifera* and the stingless bee (Meliponini) [10, 11].

Several previous studies revealed the potential of propolis from stingless bee nests. The ethanolic fraction of propolis from *Trigona* spp. inhibit the growth of *Campylobacter* spp. [12] and *Staphylococcus epidermidis* [13]. It also possesses antioxidant, antihemolytic, and anti-inflammatory properties [14]. Honey from stingless bees is used to treat coughs, stomachaches, and sore throats [15].

Studies on the antimicrobial and antioxidant activities and the chemical compounds of indigenous Indonesian bee propolis are still limited. Therefore, this study aims to determine the antibacterial and antioxidant activities of the native Indonesian wild stingless bee species, *Tetragonula laeviceps* using different solvents and to determine the chemical compounds of the potential fraction by LC-MS analysis.

2 Materials and Methods

2.1 Chemicals and Equipment

The chemicals used in this research are methanol, dichloromethane, ethyl acetate, Thin Layer Chromatography (Silica gel F254, Merck) plates, Cerium sulfate, vanillin sulfate, DPPH, iodonitrotetrazolium (Sigma), Equipment used in this study was glassware, UV cabinet, UV-Vis spectrophotometer (Shimadzu, Japan), microplate reader (Varioscan Flash, Thermo Scientific).

2.2 Sample Preparation

A Stingless bee (*Tetragonula laeviceps*) and (12.74 g) was collected from the Purwodadi Botanical Gardens. Fractionated with methanol for 24 h, repeated 3 times. The filtrate was concentrated with a rotary evaporator (Heidolph VV 2000) to obtain a methanol fraction. The methanol fraction was fractionated with hexane, dichloromethane, ethyl acetate, methanol, and acetone-water, respectively, and all fractions were stored at 4 °C for further analysis.

2.3 Fractionation and Analysis of Secondary Metabolites of Methanol Fraction of Stingless Bee Hives

Stingless bee hives (5.38 g) were fractionated using column chromatography and sea sands stationary phase. Analysis of secondary metabolites of the stingless bee hive

fraction was carried out using the thin layer chromatography (TLC) method (Silica gel F254, Merck). Ten microliters of the fraction were spotted on the TLC plate. First, the hexane fraction was eluted with hexane developer solution: ethyl acetate (4:1). Next, the dichloromethane and ethyl acetate fractions were eluted with dichloromethane: methanol (10:1), while the methanol fraction, acetone-water fraction, was eluted with chloroform: methanol: water (6:4:1). The eluted plates were observed under UV light at wavelengths 254 nm and 366 nm; then, the plates were sprayed with stain-visible reagents (Cerium sulfate and vanillin sulfate).

2.4 Determination of Total Phenolic Content (TPC)

A sample of 0.2 mL with a concentration of 10 mg/mL in ethanol p.a was added with 0.2 mL of 50% Folin-Ciocalteu reagent and then homogenized using vortex for 1 min. Next, the mixture was added with 4 mL of 2% sodium carbonate (Na_2CO_3) solution and kept in the dark for 30 min. Finally, the absorbance of the fraction solution was read at a wavelength of 750 nm with a UV-Vis spectrophotometer (Shimadzu, Japan). The results are expressed as mg gallic acid equivalent/g sample [16].

2.5 Determination of Total Flavonoid Content (TFC)

Fraction as much as 0.5 mL with a concentration of 10 mg/mL in ethanol p.a was added with 2 mL aquabidest, 0.15 mL $NaNO_2$ 5% and then incubated for 6 min, then added 0.15 mL $AlCl_3$ 10%, vortexed and incubated again for 6 min. After incubation, the mixture was added with 2 mL of 1 M NaOH and aquabidest to a total volume of 5 mL and incubated for 15 min in a dark room. Finally, absorbance was measured using a UV-Vis spectrophotometer at a wavelength of 510 nm [17].

2.6 Antibacterial Activity Test: TLC-Bioautography

The antibacterial activity was carried out against *Staphylococcus aureus* and *Escherichia coli.* Ten microliters of the fraction were transferred on a TLC plate (Silica gel F254, Merck) and then air-dried. Then the plate was immersed in the bacterial suspension (10^8 CFU/mL). The plate immersed in bacterial suspension was placed in a humidified sterile Petri dish. Then, the plate was incubated at 37 °C for 18 h. After incubation, the plates were sprayed with p-violet iodonitrotetrazolium (INT). Fractions or chemical components that are active in inhibiting bacterial growth are indicated by the formation of a white area around the fraction. Furthermore, fractions and fractions from the stingless bee hives were determined for the Minimum Inhibitory Concentration (MIC) using the microdilution method on a 96-well microplate. The lowest concentration that shows a clear colour because no bacteria grows is the minimum inhibitory concentration (MIC) [18].

2.7 Free Radical Scavenging Activity Assay Using DPPH Method: TLC-Bioautography

The free antiradical activity of DPPH using the TLC-bioautography method was carried out by transferring 10 l of the fraction to a TLC plate. After the TLC plate was dry, it

was sprayed with 2,2-diphenyl-1-picrylhydrazyl 0.2% in methanol (DPPH). Scavenging activity was observed 30 min after DPPH spraying. The fraction which had the activity to scavenge free radicals was indicated by the change of colour to yellowish-white on the purple background of the plate. The fraction was then eluted to determine the active chemical components as antioxidants. After eluting, the plates were sprayed with DPPH and observed 30 min after spraying. The formation of a yellowish-white band indicates the active component. Further tests were conducted to determine the IC_{50} value by microdilution and the value of the antioxidant activity index (AAI).

2.8 Determination of IC50 and Antioxidant Activity Index (AAI) Values

Determining the IC_{50} value of antioxidant activity using the DPPH method was carried out by a two-fold serial dilution method in 96 wells microplate. First, 100 µl of methanol p.a was filled into each well, except wells in the first row (A) were filled with 195 µl. The well in row A was added with 5 µl of the sample with a concentration of 10,240 g/mL in Dimethyl sulfoxide (DMSO) and then homogenized. Next, two-fold serial dilutions were done by transferring 100 µl of the mixture from row A to row B in the same column. The transfer was carried out until the last row, and 100 µL of the mixture was discarded in the last row. After dilution, 100 µL of DPPH was added to each well with a concentration of 61.50 g/mL and then incubated for 90 min in a dark room at room temperature. After incubation, the absorbance of the fraction was determined using a microplate reader (Varioscan Flash, Thermo Scientific) at a wavelength of 517 nm. The inhibitory concentration was calculated using the following equation:

$$IC(\%) = (A_{DPPH\ 100\%} - A_{SAMPLE}) * 100/A_{SAMPLE}$$

IC: Inhibitory Concentration, $A_{DPPH\ 100\%}$: Absorbance of DPPH, A_{SAMPLE}: Absorbance of sample.

A linear curve determined the concentration capable of inhibiting 50% of free radicals [19].

Antioxidant Activity Index (AAI) can be obtained using the following equation:

$$AAI = (DPPH)/IC_{50}\text{value}$$

(DPPH): DPPH final concentration, IC_{50}: Inhibitory Concentration of 50% DPPH.

Determination of the minimum inhibitory concentration (MIC) of antibacterial

The minimum inhibitory concentration (MIC) of antibacterial was determined using a two-fold serial dilution method in 96 wells microplate. The well on the 1st row was filled with 100 µl of Mueller Hinton Broth (MHB) medium and 90 µL of sterile distilled water and then added with 10 µL (10,240 g/mL in DMSO) (Merck, Germany) and homogenized. Serial dilutions were performed by transferring 100 µl of the mixture from rows A to B in the same column. The transfer was carried out until the last row, and 100 µL of the mixture was discarded in the last row. After completion of dilution, 100 µL of bacterial suspension was added to each well (5 x 105 CFU/mL) and then incubated at 37 °C in humid conditions for 18–20 h. After incubation, 10 µL of iodonitrotetrazolium chloride (INT) was added to each well and incubated for 15–30 min. The wells that did not change color to red indicated the presence of antibacterial activity [20].

Table 1. Minimum Inhibitory Concentration (MIC) of stingless bee fraction against *S. aureus* and *E. coli*

Sample	*E.coli* (μg/mL)	Category	*S.aureus* (μg/mL)	Category
n-Hexane Fraction	>256	Weak-Moderate	128	Moderate
Dichloromethane Fraction	>256	Weak-Moderate	256	Moderate
Ethyl acetate Fraction	>256	Weak-Moderate	>256	Weak-Moderate
Methanol Fraction	>256	Weak-Moderate	>256	Weak-Moderate
Acetone-water Fraction	>256	Weak-Moderate	>256	Weak-Moderate
Chloramphenicol (positive control)	4	Strong	4	Strong

2.9 Identification of Compounds of the Fractions Using LC-MS-MS Analysis

The fractions were diluted with methanol. The soluble fraction was filtered using a 0.45-micron Millipore filter. Five μL filtrate of the sample was injected into the LC-ESI-QTOF system (XEVO G2-XS QToF). LC-MS analysis was performed using UPLC-MS (Waters Acquity UPLC I-Class) equipped with a binary pump. The LC is connected to QTOF mass spectrometer coupled to ESI. The MS was used with positive ionization mode. The ESI parameters are source temperature 120 °C, gas atomizer 50 L/h, and source voltage +2.0 kV. Full scan mode was from 100 *m/z* to 1200 *m/z*. UPLC column was Acquity UPLC® BEH C8 1. 7 μm 2.1 x100 mm, column temperature 40.0 °C. Solvent A was 0,1% Formic Acid (FA) in H_2O; solvent B was 0,1% FA in acetonitrile. Solvents were set at a total flow rate of 0.300 mL/min. Isocratic elution system was run at 0–1.0 min with a ratio of 95:5; linear gradient of solvent A was from 95% to 60% at 1.0–11.0 min; isocratic elution system was run at 11.0–13.0 min with a ratio 0:100; linear gradient of solvent A was from 0% to 95% at 16 min.

3 Results

3.1 Antibacterial Activity and the MIC Value of Stingless Bee Fractions

The TLC-bioautography results showed several compounds in the fractions or fractions had antibacterial properties. It is indicated by the formation of a halo area around the fractions (Fig. 1). The result of TLC-Bioautography for antibacterial activity showed the presence of active antibacterial compounds indicated by a white area.

The antibacterial activity of stingless bee hive fractions was categorized as a weak antibacterial against *E. coli*. However, Hexane and dichloromethane fractions were moderate antibacterials against *S. aureus* (Table 1).

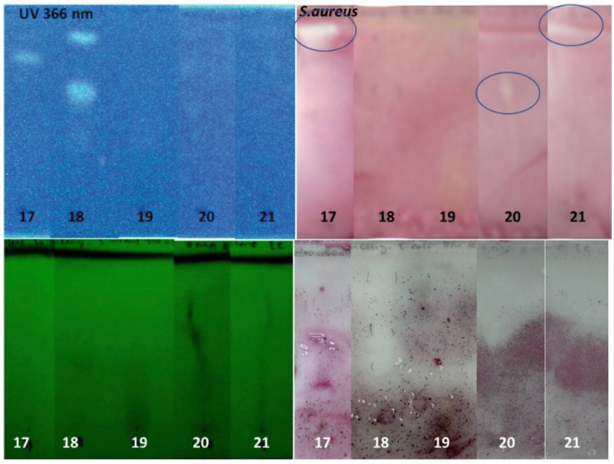

Fig. 1. Bioautogram of antibacterial activity of stingless bee hives against *S.aureus* (upper, right) and *E. coli* (lower, right). Note: hexane [17], dichloromethane [18], ethyl acetate [19], methanol [20], acetone-water [21]. Upper, left: observed under 366 nm, Lower, left: observed under 254 nm. Halo area indicated antibacterial compound

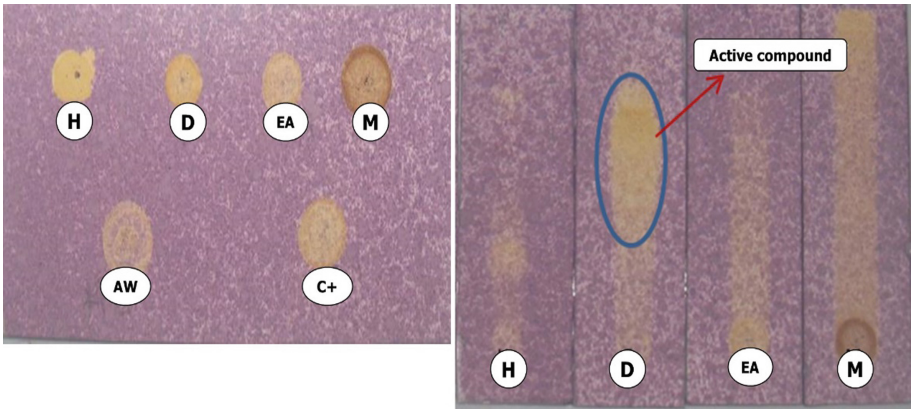

Fig. 2. Bioautogram of free radical scavenging activity of stingless beehive fraction. Remark: H: n-Hexane fraction, D: Dichloromethane Fraction, EA: Ethyl acetate Fraction, M: Methanol Fraction, AW: Acetone-water Fraction, C+: Chloramphenicol, Left: TLC-dot blot, right: TLC bioautography.

3.2 Screening of Antioxidant Activity and Determination of IC_{50} Value and AAI of Stingless Bee Fractions

TLC-Bioautography to determine the antioxidant compound was carried out by the DPPH method. The bioautogram of the antioxidant assay showed the presence of several antioxidant compounds in the fraction of stingless beehive, indicated by a yellowish-white spot or band. Dichloromethane fractions have active antioxidant compounds that

Table 2. The IC_{50} value dan AAI of stingless bee fractions

Sample	IC_{50} (μg/mL)	AAI	Category
n-Hexane Fraction	>128	-	Weak
Dichloromethane Fraction	14,15	2,17	Very strong
Ethyl acetate Fraction	47,44	0,64	Strong
Methanol Fraction	10,22	3,00	Very strong
Acetone-water Fraction	8,63	3,56	Very strong

Table 3. Total phenolic dan flavonoid content of dichloromethane and methanol fractions of stingless bee

Sample	Total phenolic (mg GAE/g fraction)	Total flavonoid (mg QE/g fraction)
Dichloromethane fraction	101.65 ± 2.02	218.36 ± 5.42
Methanol fraction	256.33 ± 3.55	321.85 ± 7.65

might be higher than other fractions (Fig. 2). Determining the IC_{50} value indicated that dichloromethane had a very strong antioxidant activity. Methanol and Acetone-water fractions also showed very strong antioxidant activity (Table 2).

Determining the IC_{50} value indicated that dichloromethane had a very strong antioxidant activity. In addition, methanol and Aceton-water fractions also showed very strong antioxidant activity.

Table 3 shows that the TPC and TFC of the methanol fraction were higher than that of the dichloromethane fraction. Therefore, The IC_{50} of methanol fraction was smaller, while the AAI was higher. Therefore, it might indicate that the methanol fraction had better antioxidant activity than the dichloromethane fraction, although both fractions were categorized as having very strong antioxidant activity.

3.3 Identification of Chemical Compounds of Dichloromethane Fraction by LC-MS/MS Analysis

Based on the MS/MS library data shows that the dichloromethane fraction contains several bioactive metabolites, namely 5.7-dihydroxychromone (retention time (RT): 4.50 min), cnidimol C (RT: 4.60 min), puerarin (RT: 6.70 min), irisflorentin (RT: 6.80 min) and some compounds that have not been identified (Fig. 3).

4 Discussion

Beehive of stingless bee contains geopropolis that possess a therapeutic effect [21]. However, the therapeutic effect of propolis depends on its chemical compounds. The

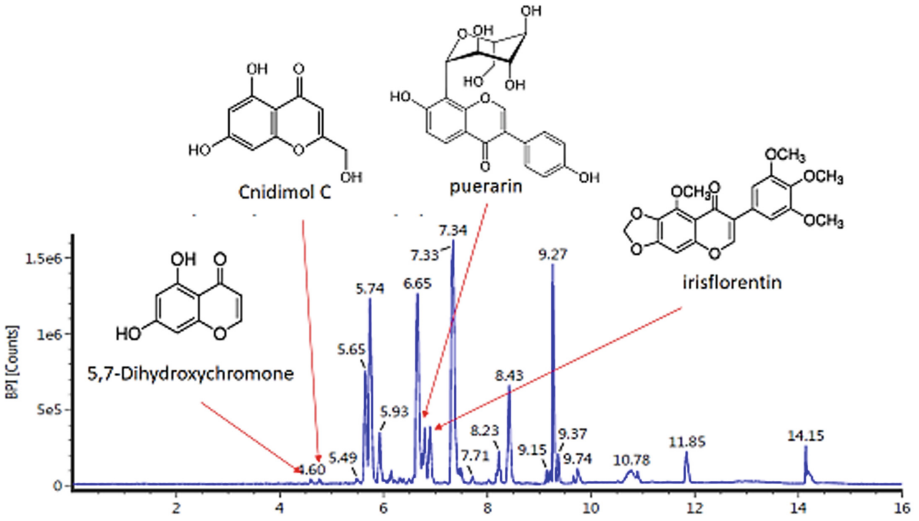

Fig. 3. LC-MS/MS Chromatogram of dichloromethane fraction of *T. laeviceps*

chemical compounds of propolis depend on the plant sources, geographical zone, season [22], and bee species [9].

In this study, we investigate the antibacterial and antioxidant activity of the hives of *Tetragonula laeviceps* collected from Purwodadi Botanical Gardens, East Java. The antibacterial and antioxidant activity of beehive fractions carried out by TLC-bioautography showed the presence of active compounds. The antibacterial compound was represented by a white area on the TLC plate, indicating there is no reduction of tetrazolium salt to colored formazan [23]. INT interacts with viable bacteria that produce dehydrogenase, which causes a colour change to purple [24].

Based on the results of the MIC values, there is a difference in the sensitivity of antibacterial activities against *E. coli* and *S. aureus.* It may be due to differences in cell wall composition between *E. coli* (Gram-negative) and *S. aureus* (Gram-positive). The cell walls of gram-negative bacteria have the periplasmic space and lipopolysaccharide layer, so they are more resistant to antibacterial compounds [25]. Hexane fraction and dichloromethane fraction have moderate antibacterial activity against *S.aureus*, so the antibacterial compound that might contribute to antibacterial activity is categorized as non-polar and semi-polar compounds that can be extracted in hexane and dichloromethane. From the identified compound, puerarin may contribute to antibacterial activity [26].

The screening of antioxidant activity of stingless beehives was performed by the DPPH method. DPPH method was widely used and is considered an accurate method to evaluate antioxidant activity [27]. The antioxidant compound was indicated by the yellowish-white area on the purple background. In the presence of the compound in the fraction that can donate electrons or hydrogens, DPPH reacts with this compound and convert the purple DPPH into a colorless non-radical form of 1, 1-diphenyl-2-picrylhydrazine [28]. Dichloromethane fractions have active antioxidant compounds

that might be higher than other fractions (Fig. 2). Determining the IC_{50} value indicated that dichloromethane had a very strong antioxidant activity. Methanol and Acetone-water fractions also showed very strong antioxidant activity.

The potential antioxidant activity of the stingless bee fractions might relate to their total phenolics content and total flavonoid content. According to dos Santos [21], the geopropolis of stingless beehives contains phenolics, flavonoids, and hydrolyzable tannins. Phenolics and flavonoids are important compounds beneficial for health and capable of curing and preventing many diseases [29]. In addition, phenolic and flavonoid content significantly contribute to antioxidant activity [30] due to the redox properties of flavonoids and phenolics [31].

The results of LC-MS analysis showed three identified compounds, i.e., 5,7-Dihydroxychromone, Cnidimol C, puerarin, and irisflorentin. The natural compound, 5,7-Dihydroxychromone, is a natural product found in plants such as *Calluna vulgaris, Viscum coloratum*, and other organisms (https://pubchem.ncbi.nlm.nih.gov/). This metabolite has neuroprotective activity in human SH-SY5Y cells with an EC value of 1.9 μM [32]. Cnidimol C is also found in *Cnidium monnieri* fruits that activity as anti-adipogenic chromone glycosides in 3T3-L1 cells [33]. Puerarin is a hydroxyisoflavone substituted by hydroxy groups at positions 7 and 4′ and a beta-D-glucopyranosyl residue at position 8 via a C-glycosidic linkage. Puerarin is a natural product also found in *Neustanthus phaseoloides*, *Clematis hexapetala*, and other organisms (https://pubchem.ncbi.nlm.nih.gov/). This metabolite has a potential therapeutic for SARS-CoV-2, Hantavirus co-infection, and colon adenocarcinoma [34, 35]. In contrast, irisflorentin is a member of 4′-methoxyisoflavones. This metabolite is a natural product also found in *Iris tectorum, Iris leptophylla*, and other organisms (https://pubchem.ncbi.nlm.nih.gov/). This metabolite is from the ethyl acetate fraction of *Belamcanda chinensis* (L.) DC roots have antitumor activities [36]. Stingless bee (*T. laeviceps*) hive fractions showed strong antioxidant activity. Therefore, *T. laeviceps* serve better as an antioxidant than an antibacterial source. The LCMS/MS analysis of the dichloromethane fraction showed the presence of 5.7-dihydroxychromone, cnidimon C, puerarin, and irisflorentin.

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