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Phytochemical Analysis of Ethanol Extract from Stingless Bee (*Tetragonula laeviceps* Smith) Honey and Its Anti-Acnes Activity

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Abstract

Ethanol extract of stingless bee (*Tetragonula laeviceps*) honey were conducted for its phytochemical analysis and anti-acnes potential by using agar well diffusion and micro-dilution methods to conduct diameter of inhibition zone and minimum inhibitory concentration (MIC) value against *Propionibacterium acnes*, respectively. The result of phytochemical analysis demonstrated that ethanol extract of the stingless bee honey contains secondary metabolites namely alkaloids, steroids, triterpenoids, phenolics, saponins, tannins and quinones. Moreover, the ethanol extract displayed anti-acnes activity with diameter inhibition zone of 17.3 mm against *P. acnes* while MIC value at 0.78 µg/mL.

Keywords: *Tetragonula laeviceps*, phytochemical analysis, anti-acnes activity.

1 Introduction

Skin with acne condition is not annoying like a chronic *disease*. However, human judgment against its condition causes uncomfortable as the effect of unpleasant odor, pricking pain, and unflattering appearance (Omar et al, 2019). Skin acne is occurred by the accumulation of non-lipid-soluble in sebum and is metabolized by *P. acne* into fatty acids that have affected inflammation in the sebaceous glands (Choi et al., 2011; Webster et al., 1995). Some antibiotics have been prescribed occasionally against inflammation as the effect of acne nevertheless, the issues take a place all over the world which the chronic wound has been resistant to commercial antibiotics (Omar et al., 2019; Choi et al., 2011). Consequently, to deal with resistance issues are needed widely exploration of natural products as an alternative to natural antibiotics, particularly for natural anti-acne.

Honey, particularly from Stingless Bees Honey, was declared to have potency as natural antibiotics by some report studies (Beluca et al., 2021; Suntiparapop et al., 2011; Avila et al., 2019). Bees collect and chemically modify plant nectars from rich vegetation and native environment which have specific organic substances for instance, in saliva secretion from abdomens glands and enzymes from cephalic glands (Avila et al., 2018). Honey from the stingless bee has a liquid texture and low crystallization ability (Abd. Jalil et al., 2017) which

is stored and left to mature inside colonies thus producing unique teste, uncommon acidity levels, sweetness, and medicinal value (Abd. Jalil et al., 2017; Chuttong et al., 2016; Avila et al., 2019). Syafrizal et al. (2019) reported that *Tetragonula leaviceps* Honey from Samarinda, East Kalimantan has a brown color with sweet and less sour taste which are influenced by several factors including ash content (more ash made the color amber-brown), heat, light exposure, duration of storage, enzymatic reaction, and presence of various compounds (Rao et al, 2016).

Physicochemical characteristics declare that the honey contains low moisture content, low pH, and less ash (Syafrizal et al., 2020; Sungtiprapop et al., 2012). The compositions of honey consist of protein, amino acids, fructose, glucose, sucrose, trace amounts of enzymes, vitamins, Minerals and other substances including phenolic compounds and other secondary metabolic compounds (Muruke, 2014; Beluca et al., 2016; Beluca et al., 2017; Beluca et al., 2019; Beluca et al., 2020; Syafrizal et al., 2019; Khongkwanmueang et al., 2020). These components are likely to play an important role in a number of biological activities of honey. Traditionally in addition to being used as a flavor enhancer, honey has been used by the ancient Chinese, Egyptians, Greeks, Syrians, and Romans to treat various diseases (Rao, at al., 2016). Chancao (2009 & 2013) reported that stingless bees from Thailand produced honey with antibacterial activity. Not only as an anti-bacterial, but honey also has the potential as an antioxidant preventing eye diseases, especially cataracts and glaucoma (Rao et al., 2016). Several researchers described the potency of honey to inhibit the bacterial activity of both gram-negative and gram-positive bacteria nevertheless, inhibition to P. acne bacteria has not been exposed yet, and thus the study aims are to analyze the phytochemicals properties and anti-acne activity of ethanol extract of stingless bee (*Tetragonula leavicept* Smith) from East Kalimantan.

2 Methodology

2.1 Materials

Honey Bees *T. leaviceps* were obtained from Honey Woody Park that is located in KM. 26 Balikpapan, East Kalimantan. The honey was collected from the honeycomb using an injection syringe and extracted using ethanol solvent then concentrated using a rotary evaporator. The crude extract was continued test for phytochemicals properties and anti-acne activity.

Propionibacterium acnes KCCM 41747 were sub cultured and maintained in nutrient agar (NA) media periodically under suitable conditions. Pure bacterial cultures were first regenerated into liquid medium (nutrient broth, NB) for 18-24 hours at 37^{o}C. All chemicals and reagents for phytochemical analysis also for anti-acne activity are analytical grade and were prepared freshly just before used.

2.2 Phytochemical Analysis

Phytochemicals analysis was carried out to screen the presence of secondary metabolites specifically alkaloids, steroids, triterpenoids, phenolics, saponins, tannins, and quinones. The standard procedures were described by Marliana et al. (2005) with minor modifications

2.3 Determination of Inhibition Zone

Determination of Inhibition zone follows the standard procedures by Clinical Laboratory Standards Institute (CLSI, 2012) with slight modification. The cultured test bacteria were each inoculated on NA media using the swab technique. A sterile cotton swab was dipped into the NB medium containing the test bacteria, then swab on the NA medium until evenly distributed, then a well was made using a drill with a diameter of 6 mm. Samples with a predetermined concentration are added to the well in the NA medium. The solvent to dissolve the extract was used as a negative control and chloramphenicol was used as a positive control. All treatments were incubated for 24 hours at 37oC. The clear area around the stew shows a positive test based on the following criteria: zone of inhibition>15.0: excellent; 13.1-15.0: very good, 10.1-13.0: good, 8.1-10.0: moderate, 6.1-8.0: less active, 6.0: inactive (Ruga & Chavasiri, 2019).

2.4 Determination of Minimum Inhibitory Concentration

Minimum Inhibitory Concentration (MIC) was determined using a micro-dilution broth according to the Clinical and Laboratory Standards Institute protocol (CLSI, 2012) and using a resazurin colorimetric assay on a 96-well microplate (Sarker et al., 2007; Cuah et al., 2014). The MIC value is defined as the lowest concentration at which the test sample still has activity. *T. leaviceps* honey sample (200 g/ml) as the main concentration was dissolved ethanol which was then varied in concentration by dilution twice in NB media. Next, NB was added to a 96-well microplate, then test samples with different concentrations were added. After that, the bacterial suspension was added and incubated at 37^{o}C for 24 hours. For the colorimetric test, resazurin (0.01%) was added to the microplate and incubated again for 10-30 minutes. The lowest concentration that does not change from blue to pink is indicated as the MIC value. The test was carried out simultaneously with the use of chloramphenicol as a positive control and negative control for wells that were not treated with bacteria.

2.5 Statistical Analysis

The result of phytochemicals analysis are expressed in symbols positive (available) and negative (unavailable). The Inhibition zone and MIC are revealed by mean ± standard deviation of three parallel measurement

3 Results and Discussion

Phytochemical analysis was carried out, which provided information about the secondary metabolite content of the ethanol extract of *T. leaviceps* honey (TLH). Qualitative phytochemicals analysis of stingless bee honey revealed a wide range of secondary metabolites components (Table 1.), which have various groups of substances and that had obtained such as alkaloids, steroids, triterpenoids, saponins, quinones, and phenolics. The presence of the components may play an essential role in the bioactivity and showed the honey properties of *T. leaviceps*.

Table 1. The result of phytochemicals analysis from ethanol extract of *T. leaviceps* Smith honey

Phytochemicals	Existence
Alkaloids	+
Steroids	+
Triterpenoids	+
Flavonoids	-
Saponins	+
Quinone	+
Phenolic	+

Note: + (detected), - (no detected)

Syafrizal et al. (2020) reported the slightly different result of the phytochemical component from TLH in Samarinda which contained alkaloids, tannins, flavonoids, and triterpenoids, however, the presence of saponins and steroids was undetected otherwise, flavonoids were not found in this study. It may be because of the small quantity so that was invisible when analysis. Several species of stingless bees scattered in several cultivated areas in East Kalimantan show the content of alkaloids nevertheless none of them contain steroids (Syafrizal et al., 2020). The number of honey constituents may be influenced by multiple factors, including environmental aspects such as the availability of flowers around the bees and the climate properties (Avila et al., 2018). Alkaloids have the function to inhibit enzymes glucose 6-phosphatase, fructose, 1, 6-biophosphatase, thereby stimulating the inhibition of glucose synthesis which results in a decrease in glucose levels and can increase the oxidation of glucose glycogen (Wild et al., 2004).Triterpenoids are volatile compounds that produce the odor or aroma given off by honey. It also plays tremendous part in the biological activities of the honey essential oil extract, such as antimicrobial and anti-inflammatory activities (Bankova et al., 2014). Moreover, phenolics are reputable as a class of bioactive compounds that shows strong in vitro and in vivo antioxidant effects (Avila et al., 2016).

Determination of antibacterial activity was carried out by the agar diffusion method. This method is very simple and easier to measure the clear zone or inhibition zone formed. The results of the antibacterial test of the stingless bee honey and chloramphenicol (positive control) with the agar diffusion method showed very good inhibition. Based on these results, the MIC test was performed to determine the minimum inhibitory concentration of *P. acne*. The inhibition and MIC values are shown in table 2.

Table 2. The Inhibition Zone and Minimum Inhibitory Concentration (MIC) values of ethanol Extract of *T. leviceps* Smith Honey and Chloramphenicol

Zone Inhibition	**Inhibition Values**
Sample of 100 µg/ml	17.3±0.9
Chloramphenicol of 0.5 µg/ml	25.0 ± 0.0
Minimun inhibitory Concentration	**MIC Value (µg/ml)**

Sample	0.78
Chloramphenicol	0.039

According to Ruga and Chavasiri (2019), the criteria or provisions for antibacterial strength can be determined by identification of the clear zone or inhibition zone formed where if the inhibition zone is >15mm it can be declared that the antibacterial ability of a sample is excellent, the inhibition zone is from 13.1 to 15.0 mm is very good while 10.1-13.0 mm shows good inhibition, the zone of inhibition is 8.1-10.0 mm is moderate and 6.1-8.0 mm indicates the antibacterial strength is not good. In addition, the inhibition zone below 6.0 mm means that it does not have antibacterial activity. The zone of inhibition (ZOI) of TLH demonstrated 17,3±0,9 mm at a concentration of 100 ppm, which was categorized as excellent. It indicated that bacteria have a susceptible response toward the honey. Continuously, the diluted sample showed a lower MIC value of 0.78 µg/mL. Compared with the TLH from Thailand, TLH from Balikpapan is most active against the activity of *P. acne* bacteria which is not detected with TLH from Chantaburi and Trat Province in Thailand (Suntiparapop et al., 2012). The complexity of physicochemical properties, phytochemical compounds, and other major compounds, that are influenced by the flower resins, may have synergetic action for increased the bioactivity of TLH. The antimicrobial capacity of honey, in this case, is anti-acne, maybe because of a synergistic combination among low pH, high osmolarity, and certain molecules such as hydrogen peroxide, peptides, alkaloids, volatile compounds including triterpenoids, saponins, and other components. Even though honey has been diluted and its hyperosmolarity is reduced, honey is still able to inhibit bacterial activity due to the acids produced through the oxidation of glucose by the enzyme glucose oxidase to form high gluconic acid with hydrogen peroxide as a byproduct (Chancao et al., 2009; Chancao et al., 2013)). The results of this study indicate that TLH has potential as an antibacterial agent, especially against bacteria that cause skin infections with MIC values below 10 µg/mL, comparable to chloramphenicol which was used as a standard or positive control in this study showing the same MIC value against the test bacteria, namely 0.039 µg/mL.

4 Conclusions

The results of the phytochemical analysis obtained on the ethanolic extract of *T. laeviceps* honey revealed many things that were similar to the results of other previous studies. Inhibitory activity against P. acne bacteria showed excellent inhibition with a MIC value of 0.78 g/mL. Based on the results of the phytochemical analysis and anti-acne activity, it is feasible to consider that *T. leaviceps* honey may have some therapeutic potentials as well as skincare. However, clinical studies have to be evaluated to investigate the effects in-vivo.

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