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Research Article

Phytochemical Analysis and Anti-Inflammatory Activity of The Combination of *Trigona apicalis* propolis Extract and Honey

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Abstract

Chronic inflammation is common in infectious diseases, rheumatoid arthritis, gout, and autoimmune diseases. However, using non-steroidal anti-inflammatory drugs (NSAIDs) is accompanied by dangerous side effects. Therefore, searching for safer alternative therapies without side effects is very important. A natural blend of ingredients produced by stingless bees from plants was potential as a remedy. Meanwhile, the potential of kelulut bee products from East Kalimantan as an anti-inflammatory is still unknown. This study aimed to compare the chemical composition of kelulut bee (*Trigona apicalis*) products and evaluate the anti-inflammatory effect of honey, propolis, and their combination. Propolis extract and honey were determined as secondary metabolites. An anti-inflammatory *in vivo* assay triggered the edema using carrageenan on male mice and measured its anti-inflammatory power value. Propolis extract and honey from *T. apicalis* have a promising anti-inflammatory effect and are significantly higher than the positive control. Meanwhile, combining propolis extract and honey did not enhance the anti-inflammatory effect. In addition, combining honey and propolis preparations with a ratio of 75 : 25 has a better effect on reducing edema volume than the other two combinations. Still, it is not better than the treatment with propolis extract or honey alone. The content of polyphenol compounds found in honey and propolis preparations is thought to have an important role in reducing edema volume.

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INTRODUCTION

Inflammation is an essential process for our body to defend against infection. However, excessive inflammation can lead to pain and chronic diseases such as rheumatoid arthritis and damage body tissues. Limitation of oxidative stress is one way to control inflammation. Natural ingredients are a source of antioxidants and can reduce aspects of the inflammatory response[1].

Indonesia is rich in natural resources that are potentially used as medicine[2]. One of the natural resources found in Indonesia, especially in East Kalimantan, is stingless bee or kelulut bee (*Trigona apicalis*) products[3]. Among 500 known stingless bee species, 40 of them have the potential to produce honey[4]. The bee products from *T. apicalis*, such as propolis, honey, and bee pollen, have been known to have health benefits[5]. Propolis is a resin-like product produced by bees and exhibits biological activity, for instance, antioxidant, antibacterial, and empirically used as an immunomodulator. Propolis is an essential component of bee hives. It is a hive's defense against bacteria, fungi, and viruses[6]. Propolis is produced by bees using its

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surrounding natural resources. Therefore, various chemical compositions are contained in propolis, such as flavonoids, tannins, phenolic compounds, terpenes, and several antioxidant compounds[7]. Interestingly, the surrounding environment also gives the diversity of propolis composition, and this difference in chemical composition affects its biological activity. In addition, Mulyati *et al*.[8] stated that the content of flavonoids produced by *T. apicalis* bee propolis is higher than *Apis* spp.
In East Kalimantan, the *T. apicalis* species is one of the stingless bee species commonly cultivated by the community[9]. Its propolis production is higher than stinging honey bees (*Apis* spp.). This species is also cultivated because of its ability to produce a distinctive honey taste (sour) and is liked by consumers. The most prominent chemical composition of *T. apicalis* produced honey is phenolic compounds such as p-coumaric acid, naringenin, caffeic acid, quercetin, and taxifolin[10].
The uniqueness of *T. apicalis* products, which have the most colonies, has encouraged further research on honey and propolis, a type of *T. apicalis* native to East Kalimantan, as potential natural ingredients to be developed as an anti-inflammatory agent. This study aims to evaluate the chemical constituent of *T. apicalis* propolis and honey extract native to East Kalimantan using qualitative phytochemical determination and compound profiles. It was continued to determine the bioactivity of propolis extract, honey, and their combination as anti-inflammatory using carrageenan-induced edema in male mice.

MATERIALS AND METHODS

Materials

The sample used was *T. apicalis* propolis and honey obtained from kelulut bee farmers in Tanah Merah, Samarinda, East Kalimantan, provided by Rendri Arista Avimaro as an apiary. Identification of bee species was carried out at the Forest Protection Laboratory of Universitas Mulawarman in April 2021. Based on the bee specimens and the characterization of the hives, it was inevitable that the sample used in this study was *Tetrigona apicalis*, better known as *Trigona apicalis*. The characterization of the *T. apicalis* nest entrance is shown in **Figure 1**.

Figure 1. *Trigona apicalis* nest. Characterizing the *T. apicalis* nest entrance differs from other stingless bee nest entrances. Nest characterization was used for bee species identification.

Methods

Extraction and fractionation

Methanol was used as a solvent to extract *T. apicalis* bee propolis samples, and used the maceration method to obtain crude propolis methanol extract. After obtaining the concentrated extract of *T. apicalis* bee propolis, liquid partition was carried out using a solvent with a ratio of ethyl acetate : *n*-hexane (1 : 1). Then it was shaken and allowed to stand until it formed two phases. The results of the ethyl acetate filtrate from the partition were collected and evaporated to obtain the ethyl acetate fraction of propolis.

Phytochemical determination

Alkaloids: As much as 5 mL of the ethyl acetate fraction was put into a test tube, 2 mL of concentrated HCl, and 1 mL of Dragendorff's reagent was added. A change in color to red or orange indicates alkaloid content.

Flavonoids: As much as 1 mL of the ethyl acetate fraction was put into a test tube, then a few drops of ferric chloride solution were added. A change in color to yellow, red or brown after exposure to light indicates the presence of flavonoids.

Triterpenoids/steroids: As much as 1 mL of the ethyl acetate fraction was put into a test tube, then 0.5 mL of chloroform and a few drops of hydrogen peroxide were added. A color change to a reddish-brown intersurface indicates the presence of triterpenoids, while the red top layer and yellow H_2SO_4 layer indicate the presence of steroids.

Saponins: As much as 1 mL of the ethyl acetate fraction was put into a test tube, then 2 mL of distilled water was added, then the mixture was heated on a hotplate for 10 minutes, filtered, and the filtrate was put into a test tube which had been added 10 mL of distilled water and shaken for two minutes. The presence of stable foam on the surface of the mixture indicates the presence of saponins.

Tannins: As much as 3 mL of the ethyl acetate fraction was put into a test tube, then two drops of 1% ferric chloride were added. A change in the solution from yellow to a greenish-black color indicates the presence of tannins.

Preparation of animals' test

This research was approved by the Health Research Ethics Committee Health Polytechnic of Ministry of Health East Kalimantan, Indonesia, with ethical clearance statement number DL.02.03/4.3/18854/2022. The test animals were male mice (*Mus musculus*) with a body weight of 20-30 g quarantined to adapt to the laboratory environment. There were 33 mice as test animals divided into 11 groups; each group contained three mice. One group served as positive control, which was treated with sodium diclofenac orally. The negative control group was treated with a blank. Nine groups serve as the treatment groups. Three groups were treated with 30, 60, and 120 mg/kg honey, another three groups were treated with 30, 60, and 120 mg/kg propolis extract, and the last three groups were treated with the combination of honey and propolis with the composition of 25 : 75; 50 : 50; and 75 : 25.

Preparation of 1% carrageenan suspension

As much as 100 mg of carrageenan was weighed then homogenized using 0.9% NaCl solution and then put into a volumetric flask and added 0.9% NaCl solution up to 10 mL[11].

Preparation of diclofenac sodium

A total of 100 mg of diclofenac sodium powder was weighed and then put into a 100 mL beaker glass and added water little by little while stirring with a stir bar until homogeneous, then put into a volumetric flask and made up to 100 mL.

Anti-inflammatory test

The anti-inflammatory test was done using carrageenan-induced paw edema[12]. The edema volume was measured using a mercury plethysmometer. The initial stage of the study was to measure each test animal's body weight and foot volume with a scale and a plethysmometer as initial data (baseline). After that, each mouse was given treatment according to its group. Each mouse was injected with carrageenan solution in the sole of its right foot as much as 0.1 mL. Carrageenan injection was carried out subplantar. After being injected with carrageenan, the mice were given the test solution according to the group orally. Thirty minutes later, the foot volume was measured using a mercury plethysmometer by dipping the sole of the rat's right foot until the mark, and then the edema volume was recorded. Measurements were made every 30

minutes until 150 minute after the treatment. Inflammation volume was the difference in the volume of the paws of the rats after and before being injected with carrageenan. At the time of measurement, the volume of the liquid in the plethysmometer must be kept at the same level each time the measurement, and the mark on the rat's foot must be visible. The measurement was done by submerging the rat's foot until the level of the mark on the foot.

Data analysis

The results of anti-inflammatory measurements were summarized using mean and standard error. The edema data of each group was plotted in a time course graph. The area under the curve (AUC) was calculated using the trapezoid method from the graph. The anti-inflammatory effects were measured using the following **Equation 1**. The statistical comparison between groups was analyzed using the ANOVA method and continued with a post hoc test.

$$Anti - inflammatory\ effect: \frac{AUCn - AUCt}{AUCn} \times 100\% \qquad [1]$$

AUC_n: Area under curve of negative control group
AUC_t: Area under curve of treatment group

RESULTS AND DISCUSSION

Phytochemical determination

The phytochemical determination of the secondary metabolite of *T. apicalis* propolis and honey was examined by reagent, while their secondary metabolite demonstrated color change. **Table I** compares the phytochemical analysis of *T. apicalis* propolis and honey. Fadzilah *et al*.[13] reported the activity of antioxidants in propolis from Malaysia of type *Heterotrigona itama*, *T. apicalis*, *T. thoracica*, and others. Our previous study[14] also showed that *Trigona* bee species originating from East Kalimantan have antioxidant and antibacterial activity. The activity is also influenced by the compounds contained therein. Propolis extract has positive phytochemical test results for alkaloid compounds. Alkaloid compounds have anti-malarial, anti-cancer, antioxidant, antimicrobial, anti-inflammatory, anti-obesity, and anti-HBV properties[15].

Flavonoid compounds are generally found in every part of the plant, such as seeds, fruit, stamens, roots, and stems. *Trigona apicalis* propolis extract and honey also showed positive test results for containing flavonoids. Flavonoid compounds have antioxidant activity[16]. Propolis and honey by *T. apicalis* are obtained from plants. Triterpenoid constituents from plants act as protective agents against insect and bacterial attacks[17]. Terpenoids were detected in both *T. apicalis* propolis and honey. Tannins are polyphenolic compounds that are naturally found in vegetables. Polyphenols comprise a large family of secondary metabolites stored in plant cell vacuoles, such as esters or glycosides. Tannins are considered high molecular-weight polyphenols[18].

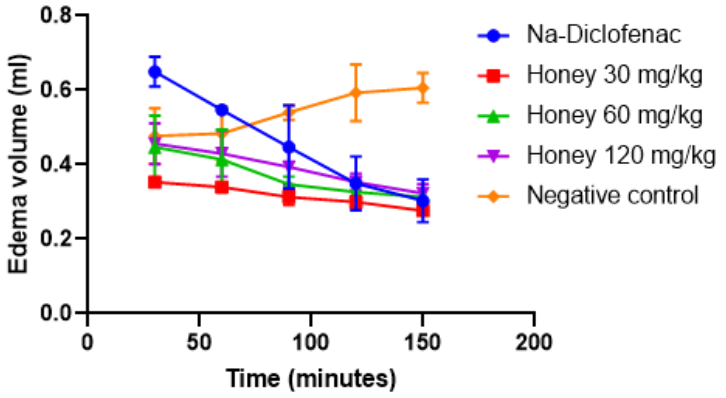
Table I. Phytochemical analysis of *T. apicalis* propolis and honey

Phytochemical content	Propolis	Honey
Alkaloid	+	-
Flavanoid	+	+
Triterpenoids/steroids	+/-	+/+
Saponin	+	+
Tannin	+	+

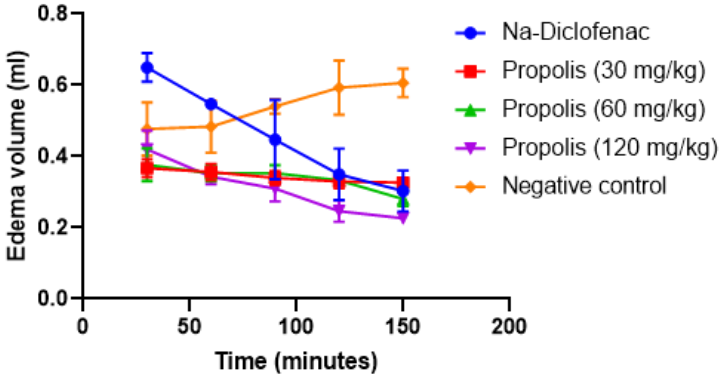
Anti-inflammation determination

Figure 2 shows the time course change of the edema volume in mice paws induced by carrageenan. Sodium diclofenac was a positive control for the assay, and the negative control group was treated with a vehicle. The treatment of honey and propolis suppresses the edema volume for 30 minutes after treatment. Meanwhile, in the positive control group, the edema volume is higher compared to the other group, including the negative control. The late onset of sodium diclofenac could cause this effect as the edema volume dropped drastically after 60 minutes. The negative control group showed increased edema volume and peaked after 150 minutes. The honey, propolis, and a combination treatment showed lower edema volume 30 minutes after treatment than the positive control. The different compositions of honey and propolis combination do not affect the anti-inflammatory effect, as seen in **Figure 2C**. All the compositions have a similar slope for reducing edema

volume. The treatment of 30 mg/kg honey gives better inflammation inhibition than the other concentrations; meanwhile, all propolis doses provide a similar anti-inflammation effect.



a



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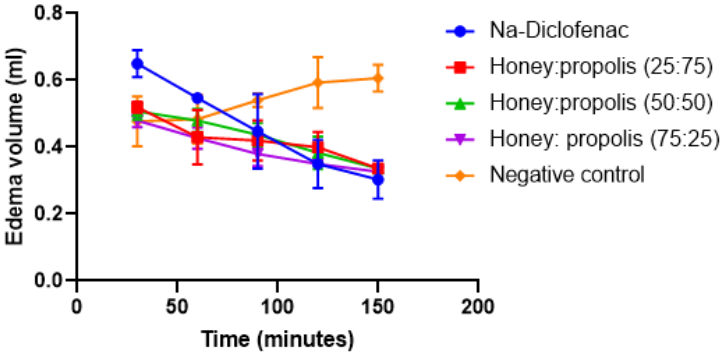

c

Figure 2. The time course change of rat paw edema volume after treatment with (**a**) honey, (**b**) propolis, and (**c**) a combination of honey and propolis. Sodium diclofenac 10 mg/kg served as a positive control, and the negative control group was treated with blank.

The anti-inflammatory effect was obtained by calculating the AUC from each group and compare to the AUC of the negative control group. The percentage of anti-inflammatory effects is shown in **Figure 3**. The result showed that the treatment of ethyl acetate fraction of propolis at doses 30, 60, and 120 mg/kg had a significant difference compared to sodium diclofenac. In contrast, treatment with honey only showed a significant difference at 30 mg/kg. Combining honey and ethyl acetate fraction of propolis did not enhance the anti-inflammatory activity. All honey and ethyl acetate fraction combinations of propolis (25 : 75; 50 : 50; and 75 : 25) have no significant difference compared to the sodium diclofenac group. The highest anti-inflammatory effect was obtained from the ethyl acetate fraction of propolis treatment at 120 mg/kg.

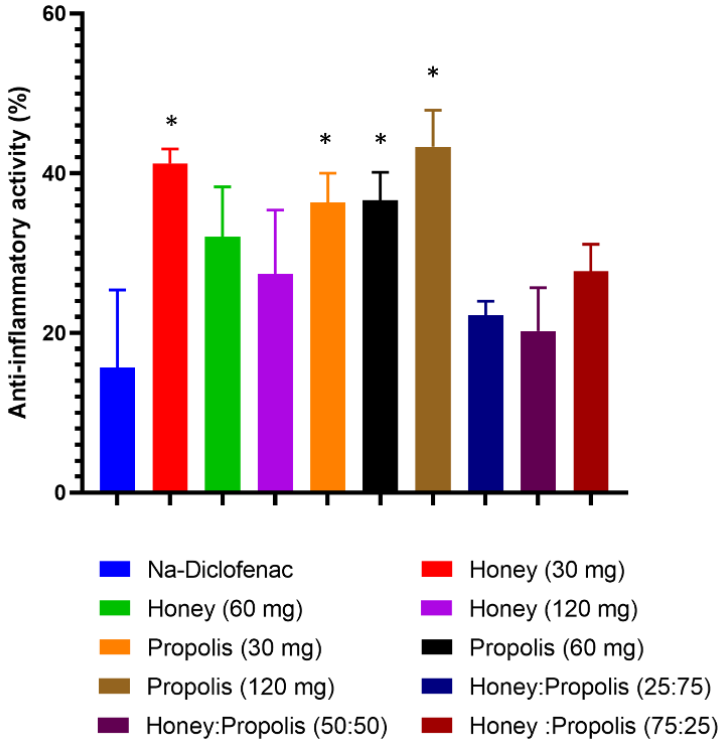

Figure 3. Anti-inflammatory effect of honey, propolis, and the combination of honey and propolis given to the mice induced with carrageenan compared to sodium diclofenac treatment (*p <0.05).

Carrageenan-induced edema is the most used method to generate acute inflammation in vivo and is suitable for evaluating the effect of oral non-steroidal anti-inflammatory agents. The injection of carrageenan elevates inflammatory mediators such as bradykinin, histamine, prostaglandins, and reactive oxygen species[19]. This study showed that treating the ethyl acetate fraction of propolis and honey exhibits anti-inflammatory activity. One of the most important compositions of propolis is caffeic acid phenethyl ester (CAPE). The CAPE has exhibited anti-inflammatory properties by reducing the expression of cyclooxygenase (COX) enzyme and blocking the release of arachidonic acid, decreasing prostaglandins and leukotrienes synthesis. At the molecular level, CAPE is known to reduce the activity of nuclear factor-kappa B (NF-kb) and reduce various inflammatory mediator cytokines such as IL-8[20]. Furthermore, the flavonoid contents of propolis also enhance the anti-inflammatory effect by inhibiting the cyclooxygenase activity[21].

Honey contains various phenolic compounds due to the natural diet of the bees. The phenolic compounds such as quercetin, chrysin, ferulic acid, hesperetin, and ellagic acid are responsible for the anti-inflammatory effect of honey by modulating the activity of cyclooxygenase 2 as well as nitric oxide production by inhibiting inducible nitric oxide synthase (iNOS)[22-24]. In addition, several compounds found in stingless bee honey, for instance, kaempferol and caffeic acid, have been shown to have an anti-inflammatory effect on ear edema-induced mice[25]. Interestingly, the combination of propolis and honey in all compositions showed a lesser effect on anti-inflammatory activity than treatment with propolis and honey alone. This effect can be caused by the chemical interaction that occurs when honey and propolis are mixed. However, the anti-inflammatory effect of this combination is still slightly higher than the positive control group but not significantly different.

CONCLUSION

Administration of honey and propolis had a better effect on reducing edema volume than the positive control. The combination of honey at a dose of 30 mg and ethyl acetate fraction of propolis at a dose of 120 mg gave the best effect as an anti-inflammatory in experimental animals. Combining honey and propolis preparations with a ratio of 75 : 25 has a better effect on reducing edema volume than the other two combinations. However, this combination was not significantly different than positive control.

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AUTHORS' CONTRIBUTION

Conceptualization and Methodology: Paula Mariana Kustiawan, Sinta Ratna Dewi, Chaerul Fadly Mochtar Luthfi M. **Extraction and phytochemical determination**: Paula Mariana Kustiawan. **Anti-inflammatory test**: Jati Pratiwi, Chaerul Fadly Mochtar Luthfi M, Alfin Syahrian Dwi Nugraha, Novia Misnawati Aisyiyah. **Data analysis and statistics**: Irfan Muris Setiawan, Paula Mariana Kustiawan. **Writing, review, and editing of manuscript**: Paula Mariana Kustiawan, Irfan Muris Setiawan.

DATA AVAILABILITY

None.

CONFLICT OF INTEREST

All authors declare no conflict of interest.

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