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Original Research Article

Evaluation of Antimalarial Activity of Combination Extract of *Citrus aurantifolia* and Honey against *Plasmodium berghei*–İnfected Mice

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ABSTRACT

Previous studies have reported artemisinin resistance. Drugs for malaria treatment have been developed from plants. *Citrus aurantifolia* was traditionally used to treat ailments. This study aimed to evaluate an antimalarial effect from *Citrus aurantifolia*, honey and a combination of *Citrus aurantifolia* and honey against *Plasmodium berghei* and analyze phytochemicals from the formula. Thirty mice were randomized into five groups. A four-day suppressive test was performed to evaluate the antimalarial activity. Data were analyzed using one-way ANOVA followed by *Tukey's post hoc-test* to compare between and within groups. Test results which show a p-value of <0.05 were considered statistically significant. The suppressive model showed a percentage parasite suppression of 67.5, 87.5, 78.8, and 86.2 for *Citrus aurantifolia*, stingless bee honey, a combination of *Citrus aurantifolia*-honey, and DHP, respectively. The maximum parasite suppression (87.5%) was exerted by the stingless bee honey group. There were no significant differences in parasite suppression in the DHP group, honey, a combination of honey and *Citrus aurantifolia*. Phytochemical analysis revealed that stingless bee honey contains saponins, flavonoids, tannins, phenols, and alkaloids, while lime contains several saponins, flavonoids, tannins, phenols, alkaloids, and steroids. A combination of *Citrus aurantifolia* and honey has potential to produce an antimalarial activity in vivo. However, the dose of *Citrus aurantifolia* in this study was relatively high and the effect of its extract on internal organs was not yet observed. Further research is required to study to what extent lower doses of *Citrus aurantifolia* produce an antimalarial activity.

Keywords: Antimalaria, *Citrus aurantifolia*, plasmodium, parasitemia Antimalaria, *Citrus aurantifolia*, plasmodium, parasitemia.

Introduction

Malaria remains a global health problem,[1] and interventions against malaria have been performed in the last 50 years. [2] It is complicated to produce an effective malaria vaccine because it requires a multi-component vaccine that can work at various stages of plasmodium development.[3]

Artemisinin-based combination therapies (ACTs) are currently the first drug used for malaria.[2] The discovery of artemisinin-based combination therapies (ACTs) provides a way to reduce the incidence of malaria.[4] However, several countries in the world have reported resistance to artemisinin-based combination therapies (ACTs). As a result, resistance to ACTs results in fatal effects, reducing treatment efficacy and effectiveness.[5]

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The discovery of new antimalarials drugs from natural plant products has been widely studied to overcome drug resistance that threatens the control of malaria. [6] Eradication of malaria requires strong biological agents that are easy to get. Plants and their active metabolites are potential candidates to be one of the agents.[7]

Traditional medicinal plants are sources of active ingredients that have the potential to be used as anti-malarial agents.[8] Phytoconstituents that have been shown to have an antimalarial activity are flavonoids, alkaloids, steroids, and terpenes.[9]

Lime or *Citrus aurantifolia* is used traditionally to treat coughs, acne, and influenza. The Basic Health Research from the Indonesian Ministry of Health reported that lime can be used to treat bacterial, viral, fungal, and worm infections.[10] *Citrus aurantifolia* has secondary metabolites including flavonoids, alkaloids, carotenoids, phenolic acids, triterpenoids, and quercetin.[11] The antimalarial activities of *Citrus aurantifolia* aqueous extract in combination with other plants and *Citrus limon* L. alone has been tested in vivo.[12] Research has proven that giving low or high doses of orange juice could cause enlargement in gastric gland cells.[13] To avoid some health problems such as wound healing, ulcers, cancer, tuberculosis infection, and asthma, honey is commonly recommended to add to herbal formula. [14] People of South Sulawesi use traditional medicine with honey, betel fruit, Miyana leaves, and egg yolks to treat malaria. Previous research has proven that the formula can produce an antimalarial activity.[15]

None of previous studies explored *Citrus aurantifolia* extract as an antimalaria. Meanwhile, *Citrus aurantifolia* is known as an antioxidant.[16] Apart from being an antioxidant, *Citrus aurantifolia* has

an antibacterial effect. Traditionally, *Citrus aurantifolia* is used to treat sore throat, pain relief, cataracts, gastric complaints, antiseptic, and anthelmintic.[17]

In addition to *Citrus aurantifolia*, honey is a food ingredient used for treating various infectious diseases, such as tuberculosis, throat infections, and hepatitis. It is even used as medicine for asthma, eye disorders, wound healing, and constipation.[14] Its benefits have been known to overcome inflammation, cancer, proliferation, and metastasis. Previous research affirms that honey can control diabetes mellitus and cardiovascular, neurological, nervous, and gastrointestinal diseases. The important bioactive chemicals in honey are flavonoids and polyphenols.[14]

The bee species that are used for general honey production are grouped into three: the giant honeybees (*Apis dorsata*), beekeeping honeybees (*Apis cerana* and *Apis mellifera*), and stingless bees, the newest type.[18] Honey produced from stingless bees is commercially available and easy to obtain in the market in Bali. Stingless bees are a group of eusocial insects which belong to five different genera. *Melipona*, *Trigona*, *Meliponula*, *Dectylurina*, and *Lestrimelitta* are stingless bees that significantly contribute topollination.[19] A study in India proved that propolis from stingless 69bees (*Trigona sp.)* has a strong broad-spectrum antimicrobial activity.[19] Honey has been used for long time ago by humans both as food and medicine.

Honey is known to have an inhibitory effect on bacteria, viruses, and fungi. [20] The traditional belief mentions that mild attacks of honey bee can be useful against malarial fever.[21] However, there has been no research using honey as an antimalarial agent. Based on this description, honey and *Citrus aurantifolia* may be used as an alternative herbal therapy. Therefore, this study aimed to discover an antimalarial activity from the combination of *Citrus aurantifolia* and honey produced by stingless bees.

Materials and Methods

Malaria parasite

Plasmodium berghei ANKA strain was obtained from the Department of Parasitology of the Faculty of Medicine, Gadjah Mada University, Central Java. It was propagated at the Integrated Biomedical Laboratory of the Faculty of Medicine, Udayana University. Parasites stored in liquid nitrogen were thawed, added with DMSO, and injected intraperitoneally into donor mice. Plasmodium was propagated and harvested repeatedly in naive mice as donors.

Propagation of Plasmodium berghei

Bloodstock from frozen storage containing *Plasmodium berghei* was thawed at 37ºC and then injected into donor mice with a volume of 0.1–0.2 ml. A small amount of blood was taken from the tail of the mice daily. A thin smear was made on an object glass, fixed with methanol, and stained with Giemsa; then, the parasitemia level of the donor mice was checked. After the parasitemia level reached ±20%, the donor mice were surgically removed; then, the blood was taken for intraperitoneal infection testing in treatment mice. Parasitemia levels in mice were observed daily by making blood smears. Antimalarial activity tests were carried out after parasitemia had reached 5-10%.

Preparation of extracts

Citrus aurantifolia were obtained from traditional markets, and species identification had been done at the Food Analysis Laboratory of Faculty of Agriculture, Udayana University, Bali, Indonesia. The extraction process began with washing the fruit with running water, then cutting, blending, and weighing as much as 500 grams. The next stage of maceration was carried out by dissolving the samples with solvent, taking sample and solvent at a ratio of 1:2. The extract samples were then stirred and left to stand for 24 hours in a closed state. The crude extract was filtered to separate the extract from the dregs. The next step was evaporation. This process involved rotating the crude extract at 40^0Celsius and a pressure of 175 atm for 1.5 hours to obtain a thick extract. Condensed extracts were stored in a dark and closed container. Storage was carried out at cold temperatures (a refrigerator or freezer) until the samples were about to be used for research. The dosage of *Citrus aurantifolia* used in this study was 100mg/kg BW.

Experimental animals

Thirty male BALB/C mice, ranging between 8-10 weeks old and 25-30 grams were selected as the experimental animals. They were acclimatized to conditions of the Integrated Biomedical Laboratory of the Faculty of Medicine, Udayana University for five days on a 12-hour light/12-hour dark cycle and at room temperature of 22–27°C. All animals were housed in polycarbonate cages and provided with food and water ad libitum. Cages were regularly cleaned, and the straws were changed every two days. The detail of the procedures was reviewed and accepted by the Research and Ethical Committee of Faculty of Medicine, Udayana University with approval number (2021.03.1.0980).

Antimalarial activity test

The antimalarial activity was tested using the modified Peter test method and four-day suppressive test of blood schizonticidal activity. To perform the tests, this study required materials including honey lime extract, lime extract, and DHP Frimal which are anti-malarial drugs classified as ACTs. The honey was derived from *Trigona Sp.* Bees. The concentration of the honey was 1:1 with aquabidest as the solvent and *Citrus aurantifolia* at a dose of 100mg/kgBB. The positive control group (PC) was given 4 mL/KgBW of DHP solution, 2-4 mg/kg BW of dihydroartemisinin, and 16-32 mg/kg BW of piperaquine, while the negative control group received 4ml/Kg of DMSO. The treatment groups were P1-P4; P1 was given *Citrus aurantifolia*; P2 was only given honey; P3 was given *C. Aurantifolia* and honey, and *C. Aurantifolia*-honey-DHP was administered to P4. The extract was given for five days (D0–D4) until the eighth day (D7).

Evaluation of antimalarial test results

Blood was taken from the tail of each mouse to make a thin blood smear. The smear was made on a glass object, fixed with methanol, stained with Giemsa, and then read under a light microscope with 1,000 times magnification to count the number of parasites. The data obtained from the in vivo antimalarial activity tests presented the number of erythrocytes infected with the parasites, which were calculated from 1,000 erythrocytes, after conversion into the level of parasitemia and inhibition of the test substance on parasite growth.

Separation and purification

Extracts of *C. aurantifolia* and honey were separated using thin layer chromatography before column chromatography to find the best eluent for separation. The extracts were then separated using a column chromatography technique.

Identification of active fraction

The relatively pure active fraction was obtained from separation and purification using thin layer chromatography and column chromatography and then identified using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Compounds in the extracts of *C. aurantifolia* and honey were identified using LC-MC/MS. The solid phase was separated using chromatography that used octadecyl silica (C18) for the stationary phase, while water + 5Mm ammonium formate (a) and acetonitrile + 0.05% formic acid were used for the mobile phase (b). Identification of active fraction was done by comparing the spectrum of the compounds in the extracts with the spectrum of the standard compounds in the database.[22]

Statistical analysis

Data were expressed in mean ± standard deviation (SD). Data analysis was performed using the Windows statistical package for social sciences (SPSS). One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was performed.

Results and Discussion

The chemo suppressive effects of *Citrus aurantifolia*, honey, and the combination of these substances were summarized in Table 1. Negative control (NC), positive control (PC), *Citrus aurantifolia*, honey, and a combination of *C. aurantifolia*, honey, and DHP resulted in parasitemia percentage of 38.5%, 5.3%, 12.5%, 4.8%, and 8.17%, respectively. All experiment groups exhibited a reduction of parasite load compared to the negative control group. The result shows no significant difference

in parasite suppressions between the DHP group, honey group, or a combination group of honey and *Citrus aurantifolia*. It indicates that *C. aurantifolia*, honey, or the combination of *C. aurantifolia* and honey have potential antimalarial activity.

Phytochemical screening

A preliminary phytochemical screening test was done on the extracts of *Citrus aurantifolia*, honey, and the combination of *C. aurantifolia* and honey. The qualitative and quantitative phytochemical analysis results are shown in Table 2 and Table 3.

The administration of *C. aurantifolia*-honey combination was able to reduce the parasite burden in the experiment groups compared to a negative control. However, other research states that an extract can be said to have antiplasmodial properties if it can reduce the level of parasitemia by more than 30%.[23] This current study found the antimalarial effect of *C. Aurantifolia*-honey combination was almost similar to the standard drug, dihydroartemisinin-piperaquine (DHP). In contrast to the standard dose, the current dose was quite high at 100 mg/kg BW.

Other studies have previously found that *C. aurantifolia* was practically non-toxic with LD of 16 g/kg BW.[23] In contrast to this current study, previous research in Ghana found that *Citrus aurantifolia* has mild toxicity at a dose of about 100 mg/kg and 500 mg/kg at the subchronic stage, resulting in mild hematotoxic, nephrotoxic, and hepatotoxic effects [25]

Intradermal bee stings given to mice have an antiplasmodial effect. Bee stings can cause 56.6% of chemo suppression and extend life up to 20 days.[21] A study on Phosphofructokinase (PFK-1) mouse infection shows that a dose of 10 ug/l of honey from *Apis florea* and *Apis andreniformis* bees did not result in an antimalarial activity, while propolis had an antimalarial activity with an IC50 value of 4.48 g/ml. *P. berghei* infection, however, can be treated using propolis at a dose of 600 mg/kg because it has an antimalarial activity [21]

Propolis has been widely studied for its antimalarial activity, but research investigating an antimalarial activity in honey is still limited. The trend of the research seems to use honey in combination with herbs or other supplements. Stingless bee honey used in this study contains unstable saponins, flavonoids, tannins, phenols, and alkaloids while standard honey in this current study was commercial honey derived from the giant honeybees (*Apis dorsata*) and honeybees (*Apis cerana* and *Apis mellifera*). Standard honey contains flavonoids, tannins, phenols, and alkaloids.

Meanwhile, polyphenols, flavonoids, and flavonols were found in honey produced by five bee species from Indian including *Apis cerana indica* F., *Apis mellifera* L., *Apis dorsata* F., *Apis Florea* F., and *Trigona iridipennis* S.; the major phytochemicals screened produced by stingless bees (*Trigona* spp.) from Kalimantan, Indonesia vary.[26] The phytochemicals in honey include tannin, alkaloid, flavonoid, triterpenoid, carotenoid, coumarin, saponin, and carbohydrate.[27]

The results of a quantitative phytochemical analysis are presented in Table 2. Honey used in this study did not contain stable saponins when compared to standard honey. In Table 3, the currently used honey contains lower flavonoid, phenol, and tannin than standard honey produced by the giant honeybees (*Apis dorsata*) and honeybees (*Apis cerana* and *Apis mellifera*). Samples of different honey in this study taken from different bee species and different geographies may contain different phytochemicals. Lime or *Citrus aurantifolia* that were currently studied contains many saponins, flavonoids, tannins, phenols, alkaloids, and steroids. Evidence-based medical research states that alkaloids, terpenes, steroids, and flavonoids are examples of phytoconstituents that act as antimalarial agents. Alkaloids have diverse biological activities in treating malaria.[9] Flavonoid is hypothesized to have an antimalarial activity individually; besides, it synergistically can increase an antimalarial activity when combined with artemisinin.[28]

Other studies have also stated that plants have secondary metabolites including alkaloids, flavonoids, xanthones, quassinoids, triterpenes, and sesquiterpenes that play a role in an antimalarial activity.[29] However, the exact mechanism of these phytoconstituents cannot be certainly explained. Therefore, further research should be conducted to discover the roles of the phytoconstituents.

Conclusion

The administration of *C. aurantifolia*, honey, and the combination of both substances provides an antimalarial effect in vivo. Different types of honey from different bee species in different geographies may produce different phytochemicals. The assigned dose of *Citrus aurantifolia* in this study was relatively high; however, its effect on mice organs was not observed yet. Studies using smaller doses and monitoring toxicity levels of the lime on mice are still required.

Table 1. Activity of *Citrus aurantifolia* and honey against *P. berghei*-infected mice

Extract	Antimalarial Activity	
	% Parasitemia ± SEM	% Suppression
NC	38,5 ± 0,31	0.0
PC	5,3 ± 0,01	86.2*
CA	12,5 ± 0,3	67.5*
H	4,8 ± 1.1	87.5*
CA+H	8.17 ± 0.3	78.8*

Key: * = P < 0.05 All tests were compared to the negative control (NC)
NC: group that has been infected by *P. berghei*; PC: group that has been infected by *P. berghei* and given DHP (Dihydroartemisinine Piperaquine); CA: group that has been infected by *P. berghei* and given *C. aurantifolia*; H: group that has been infected *P. berghei* and given honey; CA+H: group that has been infected *P. berghei* and given *C. aurantifolia* and honey.

Table 2. Qualitative phytochemical analysis of *C. aurantifolia* and honey

No	Samples	Saponin	Flavonoid	Tannin	Phenol	Alkaloid	Terpenoid	Steroid
1	Standard honey	+ (unstable)	+	+	+	+	-	-
2	Stingless bee honey	-	+	+	+	+	-	-
3	*C. aurantifolia*	+	+	+	+	+	-	+

Table 3. Quantitative phytochemical analysis of *C. aurantifolia* and honey

No	Sample	Flavonoid (mg QE/100)	Phenol (mgGAE/100 g)	Tannin (mg TAE/100 g)
1	Standard honey	3.94	90.69	200.00
2	Stingless bee honey	2.72	18.71	126.92
3	*C. aurantifolia*	15.70	38.04	160.00

TAE (Tannic acid equivalent), QE (Quercetin equivalent), & GAE (Gallic acid equivalent)

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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