

In Vitro* Antimalarial Activity and Toxicity of *Helianthus annuus* L. Leaf Extract against *Plasmodium falciparum

Nuriha Marangoh¹, Suciati², Wiwied Ekasari^{2*}

¹Master Program of Pharmaceutical Sciences, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia

²Department of Pharmaceutical Sciences, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia

*Corresponding author: wiwied-e@ff.unair.ac.id

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Abstract

Background: Malaria is one of the public health problems in Indonesia. The morbidity rate is still quite high, especially in the eastern part of Indonesia. **Objective:** This study aimed to determine the inhibitory activity of the leaf extracts of *Helianthus annuus* L. against *Plasmodium falciparum* strain 3D7 as well as the cytotoxicity of the extracts. **Methods:** The leaves of *H. annuus* were extracted by the maceration method with n-hexane, chloroform, and ethanol 96% to increase polarity. The antimalarial assay was performed by using Trager and Jensen method, and the cytotoxicity test was carried out by the MTT method. **Results:** The results of antimalarial study showed that the chloroform extract had IC_{50} value of 0.002 $\mu\text{g/mL}$ and CC_{50} value of 138.03 $\mu\text{g/mL}$, 96% ethanol extract had an IC_{50} value of 0.02 $\mu\text{g/mL}$ and CC_{50} value of 617.81 $\mu\text{g/mL}$, and n-hexane extract had an IC_{50} value of 1.29 $\mu\text{g/mL}$ and CC_{50} value of 104.89 $\mu\text{g/mL}$. The selectivity index (SI) values of the chloroform, ethanolic, and n-hexane extracts were calculated and obtained 69,015.00, 30,890.50, and 81.31, respectively. **Conclusion:** To conclude, the chloroform extract of *H. annuus* L. leaf gave strong antimalarial activity against *P. falciparum* strain 3D7 without any cytotoxicity; therefore, *H. annuus* L. leaf can be a good candidate for the development of an antimalarial drug.

Keywords: antimalarial, *Helianthus annuus* L., *Plasmodium falciparum*, IC_{50} , cytotoxicity

Abstrak

Pendahuluan: Penyakit malaria merupakan salah satu masalah kesehatan masyarakat di Indonesia. Angka kesakitan penyakit ini masih cukup tinggi, terutama di daerah Indonesia bagian timur. **Tujuan:** Penelitian ini bertujuan untuk mengetahui aktivitas antimalaria terhadap *Plasmodium falciparum* strain 3D7 secara *in vitro* dan uji toksisitas dengan menggunakan ekstrak dari daun *Helianthus annuus* L. **Metode:** Daun *H. annuus* diekstraksi dengan metode maserasi bertingkat cara menggunakan pelarut n-heksana, kloroform dan etanol 96% secara berurutan. Uji antimalaria dilakukan dengan metode Trager dan Jensen, sedangkan uji toksisitas dengan metode MTT. Indeks Selektivitas (SI) dihitung berdasarkan rasion CC_{50}/IC_{50} . **Hasil:** Ketiga ekstrak menunjukkan aktivitas antimalaria, ekstrak kloroform memiliki nilai IC_{50} 0,002 $\mu\text{g/mL}$ dan CC_{50} sebesar > 100 $\mu\text{g/mL}$, ekstrak etanol 96% memiliki harga IC_{50} 0,02 $\mu\text{g/mL}$ dan CC_{50} >100 $\mu\text{g/mL}$, dan ekstrak n-heksana memiliki harga IC_{50} 1,29 $\mu\text{g/mL}$ dan CC_{50} > 100 $\mu\text{g/mL}$. Hasil perhitungan indeks selektivitas yaitu ekstrak kloroform memiliki SI 69.015,00, ekstrak etanol 96% memiliki SI sebesar 30,890,50 dan ekstrak n-heksana memiliki SI sebesar 81,31. **Kesimpulan:** Ekstrak kloroform daun *H. annuus* menunjukkan aktivitas antimalaria paling kuat diantara sampel uji dengan nilai IC_{50} sebesar 0,002 $\mu\text{g/mL}$, serta menunjukkan bahwa toksisitas yang rendah dengan harga CC_{50} sebesar > 100 $\mu\text{g/mL}$. Oleh karena itu daun *H. annuus* diharapkan dapat dikembangkan menjadi kandidat untuk pengembangan obat antimalaria yang efektif dan aman.

Kata kunci: antimalarial, *Helianthus annuus* L., *Plasmodium falciparum*, IC_{50} , cytotoxicity

INTRODUCTION

Malaria is still considered one of the significant health problems in low and middle tropical countries (WHO, 2016). Malaria is caused by the infection of protozoa from the genus *Plasmodium* which is transmitted through infected female *Anopheles* mosquito bites. This disease greatly affects mortality and health, especially in high-risk groups, such as infants, toddlers, and pregnant women, with 45% to 50% cases due to *Plasmodium falciparum* infection (WHO, 2016). As reported annually, Indonesia is one of nine malaria-endemic countries in the South-East Asia region, accounting for 21% of the region's reported cases and 16% of malaria deaths (WHO, 2019). Middle and high endemic malaria areas include Papua, West Papua, and East Nusa Tenggara malaria (Department of Disease Control, Ministry of Public Health, 2018).

Antimalarial drugs that exist today are still effective but threatened by increased resistance; for that, plants can be one of the antimalarial drugs that are clinically effective and can be well tolerated so that they can be an essential alternative treatment. One of the plants that can be used is *Helianthus annuus* L. (sunflower). This plant is from the Asteraceae family and has been scientifically proven to have antimalarial activity. The ethanolic extract of *H. annuus* root showed antimalarial activity with IC₅₀ values of $2.3 \pm 1.4 \mu\text{g/}$ (Ekasari *et al.*, 2019). However, using the root as a source of antimalarial agents has many obstacles, mainly because it can kill the plant itself. Based on this, the present study continues to examine the antimalarial activity of other parts *H. annuus*, such as the leaves, which have long been used as a traditional antimalarial drug (Muti'ah *et al.*, 2013). Triastutik (2013) reported that the ethyl acetate fraction of *H. annuus* leaf contains sesquiterpenes, and this has been proven by identification using UPLC-MS which shows the presence of artemisinin, heliangolide, linoleic acid, and eupalinolide C (Muti'ah *et al.*, 2013). Sesquiterpenes have been known to have antimalarial activity through the formation of free radicals and destroy vital components of the parasite so that the parasites die (Macias *et al.*, 2002).

Based on the background above, this study focus on the antimalarial activity of three extracts (n-hexane, chloroform, and ethanol) of *H. annuus* leaf against *P. falciparum* strain 3D7. The cytotoxicity study was also conducted to preliminary determine the safety of the extracts (Soh & Benoit-Vical, 2007).

In addition to activity, data on an ingredient's safety or cytotoxicity is an important part of the research. As a result, a cytotoxicity test was performed on each extract

of the test using the MTT assay method, yielding the CC50 value, while the SI value was calculated from the CC50 value to IC50, determining the potential criteria for safe therapeutic extracts to inhibit parasitic growth without cytotoxicity.

MATERIALS AND METHODS

Materials

The solvents used to make *H. annuus* L. leaf extracts were n-hexane (Merck), chloroform (Merck), ethanol 96% (Merck), ethyl acetate (Merck), NH₄OH, and aquadest. Materials used to make *P. falciparum* culture media and to make *in vitro* test solutions were aquadest, HEPES buffer (Sigma), RPMI 1640 (Gibco BRL., USA), sodium bicarbonate, gentamicin, plasma, and human erythrocytes, DMSO (Sigma-Aldrich), Giemsa dyes, methanol, and emersion oil.

Plant materials

The leaves of *H. annuus* L. were obtained from PT. Megastar Oro-Oro Ombo, Malang, East Java, in November 2016. Materia Medica Batu, East Java identified the sample, and the voucher specimen (number 074/008/101.8/2017) was kept at the Faculty of Pharmacy, Universitas Airlangga. The leaves were air-dried at room temperature for seven days. The dried leaves were then grounded before extraction.

Method

Extraction

Extraction was carried out by using the maceration method. Dried leaf powder of *H. annuus* (500 g) was extracted exhaustively with n-hexane, chloroform, and ethanol to increase polarity. The sample was immersed in the n-hexane for 24 h, followed by filtration to separate the extract and residue. The residue was then re-extracted with n-hexane, the procedure was repeated three times for each solvent. The residue was air-dried for 1 - 2 hours to remove any remaining solvent before extraction with the next solvent. All collected filtrates were then concentrated in vacuo to obtain the n-hexane, chloroform, and ethanolic extracts, and the weight of which were 16.45 g, 38.48 g, and 31.00 g, respectively.

Antimalarial activity

P. falciparum 3D7 strain (chloroquine-sensitive) were used and cultivated using the Trager and Jensen method (Pandey *et al.*, 1999). Serial concentrations of the extracts that were tested for antimalarial activity were 100; 10; 1.0; 0.1; and 0.01 $\mu\text{g/mL}$. The antimalarial assay was done in 24 microwell plates with 1% initial and experimental parasitemia (1 mL/well suspension). It was placed in a candy jar and CO₂ incubator at 37°C for 48 hours. The incubated materials were then collected

and used to make a thin smear in a glass slide, fixated in methanol, and stained with 10% Giemsa. The number of parasites was then observed under a microscope and compared with the negative control to determine the percentage of parasite growth inhibition. The IC₅₀ value was calculated using Probit Analysis.

Cytotoxicity assay

The cytotoxicity test was carried out by using Huh7it cells at 96 well microplates with a density of 3 x 10⁴ cells/well in 100 µL/well. The condition of incubation was 5% CO₂, 37°C for 24 hours. After the incubation, 100 µL of extracts were added to each well at concentrations 2000, 1000, 800, 600, 400, 200, 100, 10, 1, 0.1, and 0.01 µg/mL, followed by incubation for 48 hours. After that, 150 µL of MTT solution was added and then absorbance was measured at 560 nm and 750 nm (Widyawaruyanti *et al.*, 2015)

Data analysis

Calculation of inhibition concentration 50% (IC₅₀) and cytotoxic concentration (CC₅₀) was undertaken using Probit analysis. A regression curve was plotted

between the logarithm of sample concentration (x) and the percentage inhibition (y) to obtain a linear regression equation.

RESULTS AND DISCUSSION

Table 1 showed that all the leaf extracts of *H. annuus* had an inhibitory effect against *Plasmodium falciparum* strain 3D7. The percentage inhibition among the concentration tested revealed a dose dependents effect. The percentage inhibition increased in the higher concentration. The results of the antimalarial study showed that the chloroform extract had an IC₅₀ value of 0.002 µg/mL, 96% ethanol extract had an IC₅₀ value of 0.02 µg/mL, and n-hexane extract had an IC₅₀ value of 1.29 µg/mL.

Chloroform extract of *H. annuus* L. leaf showed the highest inhibitory activity against *Plasmodium falciparum* which caused malaria, compared to other extracts (Figure 1). Chloroform extract from *H. annuus* L. leaf had antimalarial activity against *P. falciparum* strain 3D7 with an IC₅₀ value of 0.002 µg/mL.

Table 1. The IC₅₀, CC₅₀, and SI Values of *H. annuus* L. leaf extracts towards *P. falciparum* strain 3D7 at 48 hours

Samples	CC ₅₀ (µg/mL)	IC ₅₀ (µg/mL)	Selectivity Index
<i>H.annuus</i> n-hexane extract	104.89 ± 1.81	1.29 ± 0.19	81.31
<i>H.annuus</i> Chloroform extract	138.03 ± 1.36	0.002 ± 0.002	69,015.00
<i>H.annuus</i> Ethanol extract	617.81 ± 4.58	0.02 ± 0.005	30,890.50

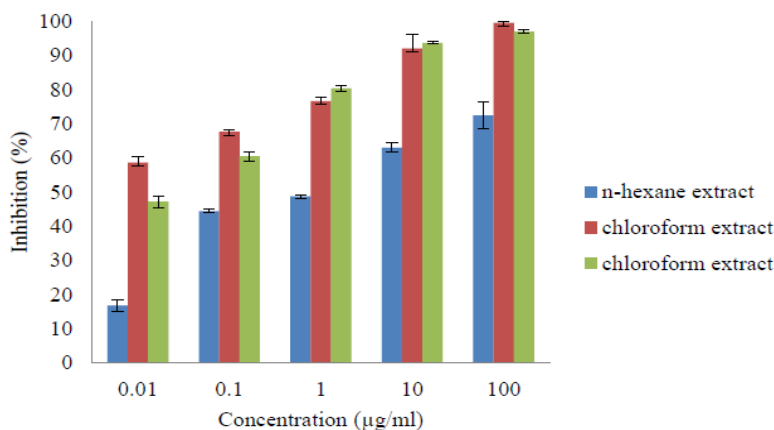


Figure 1. The percentage inhibition of *Helianthus annuus* L. leaf extract against *Plasmodium falciparum* 3D7 strain

Furthermore, to determine the level of safety or cytotoxicity of the test material, the cytotoxicity test was conducted using the MTT assay method. Hepatocyte cells that had been infected with *P. falciparum* would react with the MTT reagent to produce a purple colour which was then absorbed by absorbance at wavelengths of 560 nm and 750 nm. The cell viability was calculated based on the chloroform extract had CC₅₀ value of 138.03 µg/mL, 96% ethanol extract had CC₅₀ value of 617.81 µg/mL, and n-hexane extract had CC₅₀ value of

104.89 µg/mL, and chloroform extract had SI of 69,015.00, 96% ethanol extract had SI of 30,890.50 and n-hexane extract had viability percentage compared to the absorbance of the control. Cytotoxicity test results from *H. annuus* L. leaf extract can be seen in Figure 2. In Table 1, according to Peters *et al.* (1975) and Fadeyi *et al.* (2013), the cytotoxicity classification of all *H. annuus* L. leaf extracts was reported non-toxic to *P. falciparum* strain 3D7 with a CC₅₀ > 30 µg/mL.

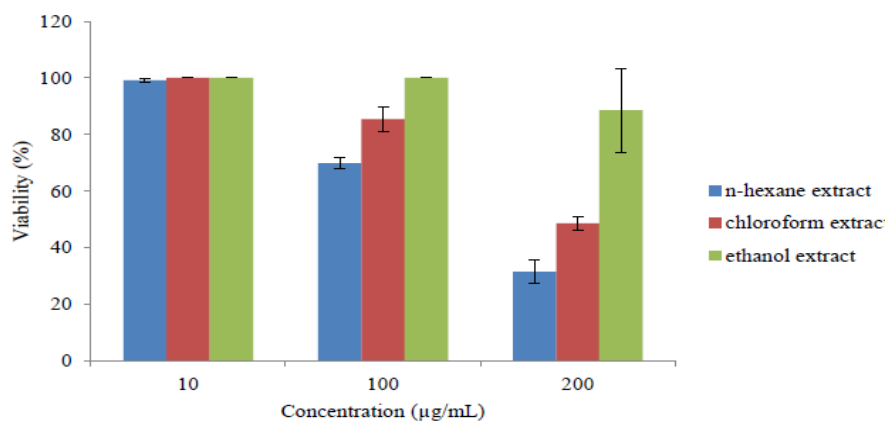


Figure 2. Cytotoxicity test results of *Helianthus annuus* L. leaf extracts

In Table 1, it could be seen that the IC_{50} value of Chloroform extract provided the highest antimalarial activity with an IC_{50} value of 0.002 µg/mL and included in the highly active category (< 0.1 µg/mL) (Noor, 2007), with CC_{50} from the chloroform extract of 138.03 µg/mL and SI 69,015.00. Whereas, the n-hexane extract gave antimalarial activity with an IC_{50} value of 1.29 µg/mL so that the active category (> 0.1 µg/mL) was included (Noor, 2007), and the CC_{50} value was 617.81 µg/mL, and in the ethanol extract the IC_{50} value was 0.02 µg/mL and was included in the very active category (Noor, 2007) and CC_{50} value was 104.89 µg/mL.

Antimalarial activity of *H. annuus* L. leaf chloroform extract may be caused by the metabolite in the extract. It was reported that *H. annuus* L. leaf extracted with dichloromethane solvent contain terpenoid class compounds, namely: lactone sesquiterpenes, annuolide E, leptocarpin, bisnorsesquiterpene, Eannuionone, and sesquiterpen L, heliannuol, helibisabonol A (Macias *et al.*, 2002). This is reinforced by the research of Triastutik (2013) that based on the identification of methanol extracts of *H. annuus* L. leaf, it was found the lactone sesquiterpenes compound, while the research of Mutiah *et al.* (2017) show that in the ethyl acetate fraction it was found artemisinin compound. These two compounds are important components to be effective compounds that can inhibit *Plasmodium falciparum* 3D7 parasites. Sesquiterpenes have been known to have antimalarial activity through the formation of free radicals and destroy vital components of the parasite so that the parasites die (Macias *et al.*, 2002).

The Selectivity Index (SI) value was calculated as the CC_{50} value to IC_{50} in Table 1 shows that all extracts have a good SI value towards *P. falciparum* 3D7. Since all the values of SI were greater than four (> 4), they are included in the active category and said that a good

value of selectivity index could be a promising drug (Badisa *et al.*, 2009). On the other hand, a high selectivity index can be a safer therapy potential because the selectivity index is introduced as potential extract to inhibit parasitic growth without cytotoxicity (Soh & Benoit-Vical, 2007).

As a result, it was proposed that the extract can be used to isolate antimalarial compounds in a safe and effective manner. (Kweyamba *et al.*, 2019).

CONCLUSION

The leaf of *Helianthus annuus* L. can be a good candidate for antimalarial drug development. The chloroform extract of the leaf possesses the strongest antimalarial activity compared to the n-hexane and ethanol extracts.

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