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In Vitro Antimalarial and Cytotoxic Activities of *Sauropus androgynus* Leaves Extracts

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ABSTRACT

Malaria is one of the tropical diseases that have worldwide implications, especially in developing countries. Medicinal plants have served as a potential source for the future of antimalarial drugs. The leaves of *Sauropus androgynus* is known for its antibacterial, analgesic, anti-inflammatory, antihypertensive, and wound healing effects. The leaves of *S. androgynus* have been consumed as food for its nutritive values. However, the antimalarial compound(s) from its leaves has not been reported before. This research aims to investigate the *in vitro* antimalarial activities of the n-hexane, chloroform, and 96% ethanol extracts of *S. androgynus* leaves, as well as to study the cytotoxic activity of the extracts. The leaves of *S. androgynus* were successively extracted with n-hexane, chloroform, and 96% ethanol in order of increasing polarity. The antimalarial activity used *Plasmodium falciparum* 3D7 strain (chloroquine-sensitive). The cytotoxicity assay used Huh7it cells with tetrazolium based colorimetric method. After cell viability was performed, the cytotoxicity value can be determined. The chloroform, n-hexane, and 96% ethanol extracts showed antimalarial activity with IC_{50} values of 0.85 µg/mL, 1.23 µg/mL, and 1.88 µg/mL, respectively. On the other hand, the cytotoxic results of chloroform, n-hexane, and 96% ethanol extracts were 136.00 µg/mL, 766.56 µg/mL, and 896.07 µg/mL, respectively. In conclusion, the research indicates that the chloroform extract of *S. androgynus* leaves has potential for use as antimalarial agents.

Keywords: *Sauropus androgynus, Plasmodium falciparum* 3D7, Antimalarial, Cytotoxic

Introduction

Malaria is a tropical disease caused by protozoan parasites of the genus *Plasmodium*.[1] The parasites infect the human host through feeding by female *Anopheles* mosquito.[2] Malaria in humans is caused by five species of the parasites which are *P. falciparum, P. vivax, P. ovale, P. malariae* and *P. knowlesi*. The most dangerous *Plasmodium* species that infect humans is *Plasmodium falciparum*.[3,4] Malaria received important attention because of the morbidity and mortality in children and adults in tropical countries. WHO reported that there are 3.3 billion people at risk of malaria with the highest population in Africa. In Indonesia, it is estimated that 35% of the population lives in areas at risk of contracting malaria, especially in endemic areas. The prevention, diagnosis, and treatment of malaria has been widely implemented.[5-7] Currently, mortality has risen in recent years, probably due to the increasing resistance of the parasite to the various antimalarial medicines.[8,9] Until nowadays Artemisinin-based combination therapies (ACTs) are now generally considered as the best current treatment for uncomplicated falciparum malaria.[10,11] The crucial problem in malaria control programs is the parasite resistance to most commercially available antimalarial drugs and vector (mosquitoes) resistance to insecticides.[12] As a result, the discovery of new antimalarial drugs from the natural product can be a potential source to open new ways in the field of antimalaria therapy.[13-17] In addition, such compounds can be modified to get potential and safe antimalarial drugs. Some of the antimalarial compounds from plants are alkaloids, terpenes, flavonoids, and xanthones.[14]

The leaves of *Sauropus androgynus* from Euphorbiaceae family have been consumed as a highly nutritive food. *S. androgynus* leaves have been known to enhance breast milk production. They are also reported to function as antibacterial, analgesic, anti-inflammatory, antihypertensive, and wound healer.[18] Preliminary phytochemical analysis showed the presence of components such as tannins, flavonoids, alkaloids, and phenols in *Sauropus androgynus*.[19] Despite that, the antimalarial compound from the leaves of *S. androgynus* has not been reported before.

This study focuses on the investigation of the *in vitro* antimalarial and cytotoxic activities of n-hexane, chloroform, and 96% ethanol extracts of *S. androgynus* leaves. Furthermore, the various extracts were screened for the presence of plant secondary metabolites.

Materials and Methods

Plant materials

Leaves of *S. androgynus* were collected by Dr. Husin Rayesh Mallaleng in January 2018 and identified by Materia Medica Batu, East Java. The voucher specimen was deposited at Faculty of Pharmacy, Universitas Airlangga with herbarium number 074/68/102.7/2018.

Extraction

Dried leaves of *S. androgynus* (960 g) were extracted with 4.8 L n-hexane by maceration (3x 24 hours) at room temperature. Subsequently, the marc was successively extracted with 4.8 L chloroform, and 4.8 L 96% ethanol. All filtrates from each solvent were evaporated separately with a rotary evaporator to obtain the crude extracts and the yields were calculated.[20,21]

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Phytochemical screening

Phytochemical screening was carried out using Thin Layer Chromatography (TLC) method.[22,23] The samples were applied on to the TLC plate, developed with a suitable mobile phase, and then stained with Dragendorf reagent (alkaloids), anisaldehyde sulfuric acid (terpenes), and concentrated NH_4OH (flavonoids). The spots were identified under UV 254 nm and 366 nm.

In vitro antimalarial assay

P. falciparum 3D7 strain (chloroquine-sensitive) was cultivated by using Trager and Jensen method.[24] Human O+ red blood was collected from Indonesian Red Cross Society (Palang Merah Indonesia/PMI) Surabaya and was cultivated in the Faculty of Pharmacy, Universitas Airlangga**.** Cultures were cultivated in human O+ red blood cells with 5% hematocrit in RPMI 1640 medium (Gibco BRL, USA) supplemented with 22.3 mM HEPES (Sigma), hypoxanthine, sodium bicarbonate, and 10% human O+ plasma. Serial concentrations of the extracts (100; 10; 1.0; 0.1; and 0.01 μg/mL) were used. The assay was done in a 24 microwell plate with 1% initial and experimental parasitemia (1 mL/well of suspension), then inserted into the candle-jar, and incubated in a CO_2 incubator at 37°C for 24 and 48 hours. Then, a thin blood smear was made on a glass slide, fixated in methanol, and stained with 10% Giemsa for 10 minutes.[25]

The level of parasitemia was observed under a microscope on 3000's erythrocytes with 1000x magnification. Next, the number of parasites in the ring, trophozoite, and schizont stages was compared with those at 24 and 48 hours incubation. Then, the percentage of parasites growth was calculated by comparing it with the negative control. Finally, fifty percent inhibitory concentration (IC_{50}) of each extract were determined to express the antimalarial activity. IC_{50} was defined as the concentration of the compound causing 50% inhibition of parasite growth relative to untreated control.[26]

% parasitemia = (number of infected RBC/3000 RBC's) x 100%

The percentage of the growth inhibition of the parasites was calculated using the formula below.[27]

% growth = % parasitemia - D_0

% inhibition = 100% - (X_s/X_c x 100%)

Where; X_s = parasitemia in the treated group, X_c = parasitemia in the negative control group, D_0 = parasitemia in the first day

Cytotoxic assay and selectivity index value

Cytotoxic assay was done by using Huh7it cells (hepatoma cancer) collected and cultured in the Laboratory of Science and Technology Research Partnership for Sustainable Development Institute of Tropical Diseases (SATREPS ITD) Universitas Airlangga, Surabaya.

Huh7it cell culture was grown on DMEM media in 96 well micro plates with a density of 2.3 x 10^4 cells/well in 100 μL/well. The condition of incubation was 5% CO_2, 37°C for 24 hours. Serial concentrations of the extracts (1000; 800; 600; 400; and 100 μg/mL) were used. Huh7it cells in 96 well plates were treated with different concentrations of the samples or control. The culture plates were observed using ELISA reader at 560 nm and 750 nm wave lengths. The 50% cytotoxic concentrations (CC_{50}) was determined by plotting concentrations of extract on x-axis and percentage of cell viability on y-axis to obtain a dose response curve.[28] The percentage of viability was measured with the formula below:

Percentage Toxicity = (Absorbance of control-absorbance of sample)/absorbance of control) x 100%

Percentage Viability = (Absorbance of sample/absorbance of control) x 100%

Cell viability assay was performed to assess the fifty percent cytotoxic concentration (CC_{50}) of extracts against Huh7it cells. Then, the SI value was calculated from the ratio of cytotoxicity to biological activity[29,30] (SI = CC_{50}/IC_{50}).

Statistical analysis

Data are expressed as mean ± standard deviation (SD). IC_{50} and CC_{50} value were calculated using Probit Analysis.

Results and Discussion

Antimalarial and study of parasite morphology

The antimalarial activity of the extracts can be shown as a change in the morphology and number of the parasites. The study of parasite morphology in the thin blood smears highlights the fact that extracts from different solvents of the plant have different antimalarial activities. The specific changes in morphology produced by a particular extract may predict different modes of action of the active compounds in the extracts. The parasitemia decreases with increasing concentrations of the extracts, reflecting an inhibitory activity on the parasite replication.[31] Protein synthesis in the parasite begins soon after the merozoite invasion has reached its peak in ~24 hours, and persists at about this level for another 24 hours, thus spanning the entire parasite erythrocytic life cycle of 48 hours.[31] The results of parasite count at different morphological stages after 24 and 48 hours of incubation are shown in Tables 1 and 2, respectively. The Tables also show the percentage parasitemia and percentage inhibition of parasiteamia by *S. androgynus* leaves extracts against *P. falciparum* 3D7. Parasite stages of the specific development were checked at the beginning of incubation (0 hours), 24, and 48 hours. The parasite was classified into three stages: Ring (R), Trophozoites (T), and Schizonts (S).

After 24 hours incubation, the culture that was given the chloroform extract showed a lower number of ring stages than the negative controls (Table 1). Whereas the trophozoite and schizont stages were relatively similar. It means that the number of merozoites in the ring stage that invade new erythrocytes from the previous schizont stages were reduced compared to the negative controls. Although the parasite morphology was checked every 24 hours until 48 hours of incubation, the IC_{50} values were determined at 48 hours of incubation to account for potential effects on biochemical pathways of the parasite.

Cytotoxic assay and selectivity index value

Huh7it cell line was used to investigate the cytotoxicity of the extracts, this is because the metabolism of most drugs and chemical compounds are carried out in the liver. In addition, malaria can cause damage to liver cells. IC_{50} and CC_{50} values of *S. androgynus* leaves extracts are shown in Table 3 and Table 4. Antimalarial activity of the chloroform, n-hexane, and 96% ethanol extracts was shown by IC_{50} values of 0.85 μg/mL, 1.23 μg/mL, and 1.88 μg/mL, respectively while the cytotoxic results of the chloroform, n-hexane, and ethanol 96% extracts were 136.00 μg/mL, 766.56 μg/mL, and 896.07 μg/mL, respectively.

The principle of the MTT method is the reduction of MTT tetrazolium yellow salt by a reductase system. Tetrazolium succinate which belongs to the respiration chain in the mitochondria of living cells forms purple formazan crystals and is insoluble in water. Addition of stopper reagent dissolves these coloured crystals and then absorbance is measured using an ELISA reader. The intensity of the purple colour formed is directly proportional to the number of living cells. So that if the intensity of the purple colour is greater, it means there are more living cells and thus decrease activity of plant material.[32]

The purple colour intensity would only be possible if only the extracts tested did not kill the cells. The ability of the cells to survive suggests that the compounds in the plant extracts protect the cells against cell-mediated damage. The result of MTT assay suggests that the extracts contains intrinsic biological components that can protect the cells against *P. falciparum* mediated damage. Selectivity index (SI) is defined as the ratio of the cytotoxic on the human cells to the antimalarial activity.[33] It is used to estimate the potential of molecules or extracts to inhibit parasite growth without toxicity. Low SI indicates that the antimalarial activity is probably due to cytotoxic activity that is higher than antimalarial activity against the parasite themselves. Meanwhile, higher SI value offers the potential for safer therapy.[34]

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Based on the criteria of antimalarial activity, extract exhibiting IC_{50} >100 µg/mL is considered inactive. Extract showing IC_{50} <100 µg/mL can be continued as a potential antimalarial agent. Active extract showing IC_{50} <10 µg/mL should be selected for the next purification based on the bioassay-guided isolation method. *In vitro* antimalaria screening that showed *P. falciparum* inhibitory activity of the extract with IC_{50} value less than 10 µg/mL is a primary step in the development of new antimalarial agent. To obtain safer antimalarial drug, the development procedure can be monitored with the determination of CC_{50} and SI values. All of the extracts were essentially non-toxic to Huh7it cells (CC_{50} >50 µg/mL).[35] Based on SI value, extract with SI < 4 can be classified as marginally active, SI 4-10 are considered partially active, and active when SI > 10, and can actively functioned as antimalarial.[36] From the results of the study, all extracts tested possessed good potentials for antimalarial because of their selectivity indices (>10).

Table 1: Differential Count of Parasite Stages and Percentage of Parasitemia of *S.androgynus* Leaves Extracts after 24 hours of Incubation

Sample	Concentration (µg/mL)	% Parasitemia			Mean % Parasitemia	Mean % Inhibition after 24 hours
		R	T	S		
D_o		0.60	-	-	0.60	
n-hexana	100.00	0.52	0.03	0.04	0.59	54.58 ± 14.33
	10.00	0.56	0.40	0.06	1.02	0
	1.00	0.42	0.59	0.12	1.13	0
	0.10	0.45	0.42	0.14	1.01	0
	0.01	0.43	0.46	0.11	1.00	0
Chloroform	100.00	0.59	0.04	0.04	0.67	44.96 ± 2.97
	10.00	0.73	0.35	0.04	1.12	0
	1.00	0.30	0.41	0.08	0.79	0
	0.10	0.34	0.40	0.09	0.83	0
	0.01	0.56	0.34	0.13	1.03	0
Ethanol 96%	100.00	0.59	0.07	0.04	0.70	53.33 ± 18.86
	10.00	0.49	0.15	0.06	0.70	53.33 ± 18.86
	1.00	0.40	0.58	0.03	1.01	0
	0.10	0.39	0.39	0.03	0.81	0
	0.01	0.43	0.61	0.21	1.25	0

Note : Ring (R), Trophozoites (T), and Schizonts (S)

Table 2: Differential Count of Parasite Stages and Percentage of Parasitemia of *S.androgynus* Leaves Extracs after 48 hours of Incubation

Sample	Concentration (µg/mL)	% Parasitemia			Mean % Parasitemia	Mean % Inhibition after 48 hours
		R	T	S		
D_o		0.60	-	-	0.60	
n-hexane	100.00	0.10	0.73	0.06	0.89	87.12 ± 2.02
	10.00	0.89	0.48	0.29	1.66	52.26 ± 14.32
	1.00	1.16	0.24	0.44	1.84	44.53 ± 7.52
	0.10	1.46	0.38	0.29	2.13	30.45 ± 7.18
	0.01	1.91	0.40	0.23	2.54	13.24 ± 8.78
Chloroform	100.00	0.26	0.45	0.06	0.77	92.22 ± 2.21
	10.00	0.12	0.55	0.48	1.15	74.83 ± 0.97
	1.00	0.89	0.29	0.50	1.68	50.80 ± 2.10
	0.10	1.35	0.20	0.30	1.85	47.87 ± 1.25
	0.01	1.19	1.37	1.37	1.93	36.62 ± 0.72
96% Ethanol	100.00	0.55	0.13	0.04	0.72	93.88 ± 2.09
	10.00	0.71	0.36	0.57	1.64	48.10 ± 16.68
	1.00	0.93	0.34	0.57	1.84	38.88 ± 9.28
	0.10	1.29	0.40	0.40	2.09	25.67 ± 2.30
	0.01	1.45	0.20	0.54	2.19	20.72 ± 5.04

Note : Ring (R), Trophozoites (T), and Schizonts (S)

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Phytochemical analysis of S. androgynus extracts

The results of the phytochemical screening indicated the presence of terpenes in all extracts, however alkaloids and flavonoids were not detected (Table 5). These results differ from the literature that states it contains tannins, flavonoids, alkaloids, and phenols.[19] This can be due to differences in plant sources.

The antimalarial compounds in *S. androgynus* leaves could be predicted to be terpenenoids. Some plants from the family of Euphorbiaceae are known to have antimalarial activity. Ethanol and n-hexane extracts from the root bark of *Uapaca nitida* are known to contain terpenoid compounds (betulinic acid and triterpene).[37,38] Meanwhile, the ethanol extract of the leaves of *Croton steenkampianus* contains diterpenoids and is active as antimalarial.[39] Whereas, in the Sauropus genus, it is has been reported that a 90% methanol fraction of *S. spatulifolius* leaves has antimalarial activity against *P. falciparum* K1 strains with IC_{50} of 6.10 μg/mL.[40] Terpenes as antimalarial inhibit the growth stages of the plasmodium parasite from ring form to trophozoites. Beside that, terpenes inhibit nutrient uptake by the parasites by inhibiting the permeation pathway.[41,42] From the phytochemical screening study, all extracts of *S. androgynus* contain terpenes. Moreover, from the antimalarial activity observed, the chloroform extract has better antimalarial potential.

Table 3: Percentage of Viability and CC_{50} Value of *S. androgynus* leaves extracts

Concetration (μg/mL)	% Viability Cell		
	n-hexane	Chloroform	96% Ethanol
1000	4.33	0	36.15
800	45.80	0	64.11
600	67.72	1.84	73.36
400	78.15	7.22	86.75
200	91.01	43.77	95.87
100	96.39	53.87	100.00
CC_{50} (μg/mL)	766.56	136.00	896.07

Table 4: Antimalarial and Cytotoxic Activities of *S. androgynus* leaves extracts

Extracts	IC_{50} in 48 hours (μg/mL)	CC_{50} (μg/mL)	SI
n-hexane	1.23	766.56	625.77
Chloroform	0.85	136.00	160.19
Ethanol 96%	1.88	896.07	476.89

Selectivity Index = CC_{50} / IC_{50}

Table 5: Phytochemical constituents of extracts

Extracts	Yield (%)	Phytochemical constituents		
		Alkaloids	Terpenes	Flavonoids
n-hexane	3.02	-	+	-
Chloroform	4.06	-	+	-
96% Ethanol	1.76	-	+	-

Key: (+) = present, (-) = absent

Conclusion

Based on the results, the chloroform extract of *S. androgynus* leaves has good *in vitro* antimalarial activity against *P. falciparum* 3D7 strain. The chloroform extract exhibits the highest *in vitro* antimalarial activity with an IC_{50}, CC_{50}, and SI values of 0.85 μg/mL, 136.00 μg/mL, and 160.19, respectively. Terpenoids in the chloroform extract may be responsible for its antimalarial activity.

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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