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Luvunga scandens (Roxb) as the promising source of antimalarial drugs against *Plasmodium falciparum* 3D7

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ABSTRACT: *Luvunga scandens* is traditionally used for malaria, antibacterial, antifungal, antidote to snake bites, and against scorpion stings. Based on the ethnomedicine approach, we focus to explored the antimalarial effect of *L. scandens*. The stem and leaves of *L. scandens* were gradually extracted with n-hexane, dichloromethane, and methanol. All samples were examined antimalarial against *Plasmodium falciparum* 3D7 and cytotoxicities against HepG2 cell lines. The active extract was fractionated by Vacuum Liquid Chromatography (VLC) and the fractions obtained were further tested for antimalarial and cytotoxicity. The most potent extracts against *P. falciparum* were the dichloromethane extracts of *L. scandens* leaves (LS-L-D) and stem (LS-S-D), with IC_{50} values of (6.61±0.04 µg /mL) and (5.38±0.01 µg/mL), respectively. Both extracts were found to be safe and effective because their selectivity indices (SI) values were greater than 2. Based on the antimalarial activity,toxicity, and percentage of yield, we fractionated LS-L-D using VLC, and obtained 26 fractions (F1-F26). Fraction 23 (F23) was identified as the most active fraction with an IC_{50} value of 2.44±0.09 µg/mL and CC_{50} value of 46.35±0.03. The HPLC profile of F23 shows several peaks. The major peak has a UV spectrum with maximum absorption at wavelengths of 247, 257, and 322 nm, respectively, indicating the characteristics of the flavonoid compound group, especially isoflavones. This data suggested that isoflavones possibly take a role in the antimalarial activity of fraction 23. Further study needs to be conducted to isolate and identify the active compounds from this fraction.

KEYWORDS: *Luvunga scandens*; antimalarial; cytotoxicity; medicine.

1. INTRODUCTION

Malaria is still regarded as a serious worldwide health threat, almost affecting a significant portion of the entire world population. Its prediction is widespread in tropical countries. According to the latest malarial reports in 2020 World Health Organization (WHO), about 241 million malarial cases in 85 malaria-endemic countries were reported globally, showing a significant increase from 227 million cases in 2019. The spike was due to improper services, transportation, and distribution of medicines during the pandemic situations [1,2]. The plasmodium species responsible for the parasitic malarial disease is exclusively transmitted from an infected host to a healthy guest by a female mosquito name *Anopheles* belonging to the family *Culicidae* [3]. Almost all of the plasmodium species, i.e., *Plasmodium falciparum, Plasmodium vivax, Plasmodium knowlesi, Plasmodium malariae*, and *Plasmodium ovale*, can cause malaria, but of these, the most virulent in the sense of morbidity and mortality rate is *Plasmodium falciparum* [4].

The main root responsible for malaria's high morbidity and mortality ratio is the escalating malarial parasite's resistance to the already commercially available therapeutics agents. Even drug resistance to the most dangerous and virulent plasmodium strain i.e., *P. falciparum* has been reported in various endemic areas of this planet to nearly all of the currently available commercial antimalarial drugs, such as sulfadoxine/pyrimethamine, mefloquine, halofantrine, and quinine [4,5]. The first cases of *P. vivax* malaria among migrated Australians in Papua New Guinea emerged in 1989, but *P. vivax* resistance to

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chloroquine has also been reputed in Madagascar, Ethiopia, and Southeast Asia. Therefore, an urgent need arises here to find some new antimalarial moieties with good potency and sufficient effectiveness to lower morbidity and mortality rates and combat the resistance problem.

Natural products, especially plants, have played a significant role in discovering novel drugs since prehistoric times. Even the major classes of antimalarials, quinine, and artemisinin, were found to be derived from plants, specifically from the bark of *Cinchona officinalis* and the leaves of *Artemisia annua*, respectively [6]. Indonesia has a good position worldwide due to its distinguished biodiversity and many potent medicinal plants. More than 30,000 flowering plants and approximately 7,000 medicinal plant species have been proclaimed from the rich fertile soil of Indonesia. Thus, the God-gifted Indonesian land's natural resources, including its wide range of species and abundance of effective medicinal plants, must be researched and utilized solely for well-being and prosperity [7].

A never-ending supply of unique molecules is probably present in the understudied folk plant remedies, and validating this ethnopharmacological profile may lead to creative approaches for discovering new drugs [8]. Thus based on ethnopharmacological history, one of the important species from the genus Luvunga, i.e., *Luvunga scandens* (Roxb.) belonging to the family Rutaceae, was analyzed by applying a series of analyses for their scientific validation. This plant is primarily found in the tropical and humid conditions of China, Cambodia, India, Laos, Indonesia, Philippines, Malaysia, Myanmar, Thailand, and Vietnam. Traditionally, the stem decoction of *L. scandens* is more commonly used in treating malaria in Indonesia and China [9,10]. It has also documented uses in treating fungal, antibacterial, antidote to snake bites and against scorpion stings [8,9]. Three compounds have been ioslated from roots of Luvunga scandens, namely Isobutylglucosinolate, β-sitosterol, and stigmasterol. Farnesyl acetate, farnesol, flindissol carvotanacetone, methyl eugenol, caryophyllene oxide, myristicin, 1,8-cineole, carvone, a-pinene, camphene, phellandrene, terpinolene, a-terpineol, camphor, caryophyllene also isolated from the essential oil of *L.scandens* [10]. Research on *L.scandens* ability to fight malaria is limited. Therefore this study aims to evaluate the antimalarial and cytotoxicity of *L. scandens* stem and leaves.

2. RESULTS

2.1 Extraction and Fractionation of *L. scandens*

In this study, six extracts were obtained, namely 3 extracts from *L. scandens* stem (methanol extract/LS-S-M, dichloromethane extract/ LS-S-D, and n hexane extract/LS-S-H and 3 extracts from *L. scandens* leaves (methanol extract/LS-L-M, dichloromethane extract/ LS-L-D, n hexane extract/LS-L-H). The LS-L-M has the highest percentage of yield (%) compared to another extract. The weight and % yield of all extracts is shown in Table 1.

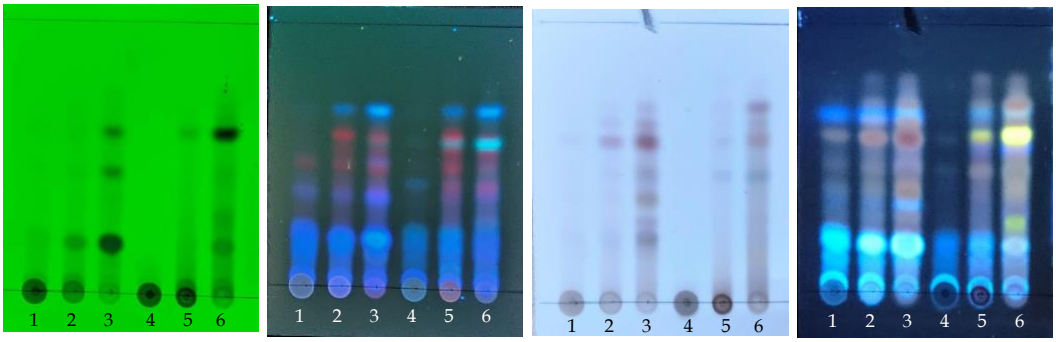
Table 1. The weight and % yıeld of all extract

Plant species	Parts	Code	Weight (g)	Yield (% w/w)
Luvunga scandens	Leaves	LS-L-H	0.27	1.08
		LS-L-D	0.71	2.84
		LS-L-M	0.99	3.96
	Stem	LS-S-H	0.22	0.88
		LS-S-D	0.23	0.92
		LS-S-M	0.77	3.08

Thin layer chromatography (TLC) profiles of all extracts were shown in Figure 1. Bioassay-guided fractionation was used to find the active antimalarial fraction. Furthermore, the active extract was fractionated and a total of 26 fractions were obtained (F1-F26).

2.2. Antimalarial Activity and Cytotoxicity of Extracts and Fractions

A total of six extracts of *L. scandens* were examined for their antimalarial activity against *P. falciparum* 3D7 and cytotoxicity against HepG2 cell lines. Among all of the extracts, the potent extracts with significant antimalarial activities were found dichloromethane extracts of leaves (LS-L-D) and stem (LS-S-D) with their IC_{50} values of 6.61±0.04 µg/mL and 5.38±0.01 µg/mL, respectively. The result of cytotoxicity assay showed that LS-L-D was nontoxic against HepG2 cells with a CC_{50} value of 50.55±0.02 µg/mL and LS-S-D was slightly toxic with a CC_{50} value of 42.49±0.09 µg/mL, respectively (**Table 2**). The cytotoxicity classification was justified according to the classification reported by Garcia-Huertas et al., in which an extract was considered very toxic with CC_{50}<10 µg/mL, moderately toxic with CC_{50} 11-30 µg/mL, slightly toxic with CC_{50} 31-50 µg/mL, and potentially nontoxic with CC_{50}>50 µg/mL (11).



(a) **(b)** **(c)** **(d)**

Figure 1. Thin Liquid Chromatgraphy (TLC) profile of LS-S-H (1), LS-S-D (2), LS-S-M (3), LS-L-H (4), LS-L-D (5), and LS-L-M (6) using silica gel as a stationary phase and hexane-ethyl acetate (60/40 v/v) as a mobile phase. The TLC spots were observed under UV 254 nm (a), UV 366 nm (b), sprayed with H_2SO_4 10% and heated 103°C for 5 min (c), UV 366 nm after sprayed with H_2SO_4 10% and heated 105 °C for 5 min (d).

Table 2. Antimalarial activity (IC_{50}), Cytotoxicity (CC_{50}), and SI of extracts from *L.scandens* leaf and stem

Sample	IC_{50} (µg/ml)	CC_{50} (µg/ml)	SI
LS-L-H	14.66±0.09	67.73±0.08	4.62
LS-L-D	6.61±0.04	50.55±0.02	7.64
LS-L-M	18.69±0.09	>100	5.35
LS-S-H	13.41±0.12	>100	7.45
LS-S-D	5.38±0.01	42.49±0.09	7.89
LS-S-M	12.77±0.11	>100	7.83

Data represent the mean ± standard error with technical triplicate at each inhibitor concentration. Data were fitted in GraphPad Prism to determine the IC_{50} and CC_{50} values.

Based on the antimalarial activity, cytotoxicity, and percentage of yield obtained, we fractionated LS-L-D. Fractionation was carried out using Vacum Liquid Cromatgraphy (VLC) and 26 fractions were obtained (F1-F26). The fractions were screened for antimalarials at 10 ug/ml concentrations, and only 9 fractions (F11, F17, F18, F19, F21, F22, F23, F24, and F25) showed inhibition of more than 60% (Figure 2). Therefore, the IC_{50} calculation is carried out on these fractions. Fraction 23 (F23) was identified as the most active fraction with an IC_{50} value of 2.44±0.09 µg/mL and slightly toxic with a CC_{50} value of 46.35±0.03 (Table 3).

2.3. HPLC Profile of F23

The primary major peak of fraction 23 in the UV spectra was discovered to exhibit maximum absorption at wavelengths of 247, 257, and 322 nm, respectively. The UV spectra of the major peak indicated the characteristics of the flavonoid compound group, especially isoflavones. The HPLC and UV-Vis spectra of F23 are shown in Figures 3 and 4.

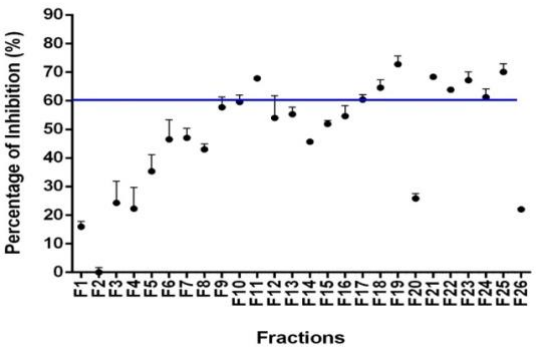


Figure 2. The percentage of inhibition for each fraction was obtained through fractionation of dichloromethane extract of *Luvunga scandens* using Vacuum Liquid Chromatography (VLC) with a gradient of n-hexane and ethyl acetate. All fractions were assessed against *P. falciparum* 3D7 at a 10 µg/mL concentration. Data from three independent experiments are reported as Mean ± SD.

Table 3. Antimalarial activity (IC_{50}), Cytotoxicity (CC_{50}), and SI of nine fractions from *L. scandens* leaf dichloromethane extract

Fractions	IC_{50} (µg/ml)	CC_{50} (µg/ml)	SI
F11	14.21±0.26	53.33±0.02	3.74
F17	9.30±0.14	48.06±0.03	5.17
F18	8.93±0.10	47.77±0.03	5.35
F19	8.89±0.12	60.27±0.02	6.78
F21	5.87±0.12	53.08±0.04	9.04
F22	3.84±0.07	65.66±0.04	17.09
F23	2.44±0.09	46.35±0.03	18.99
F24	3.84±0.07	61.80±0.04	16.09
F25	3.87±0.07	63.11±0.05	16.31

Data represent the mean ± standard error. The data were examined with technical triplicates at each inhibitor concentration. Data were fitted in GraphPad Prism to determine the IC_{50} and CC_{50} values.

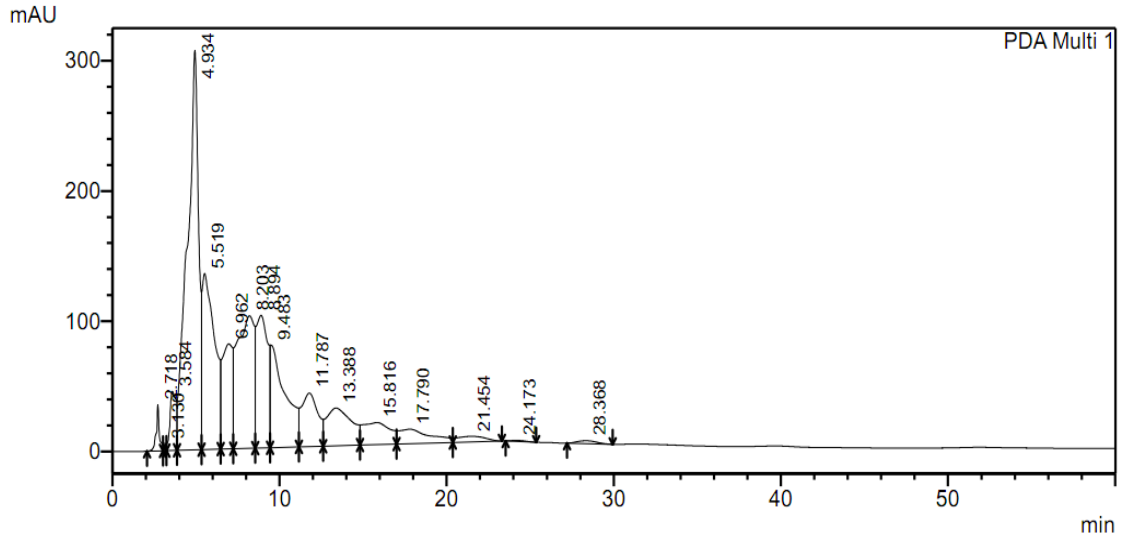


Figure 3. HPLC profile of fraction 23 (F23). The analysis using methanol: water (7.5: 2.5 v/v) mixture as a mobile phase at flow rate 0.7 mL/minutes, injection volume of 40 µL, and the major peak was observed at 254 nm.

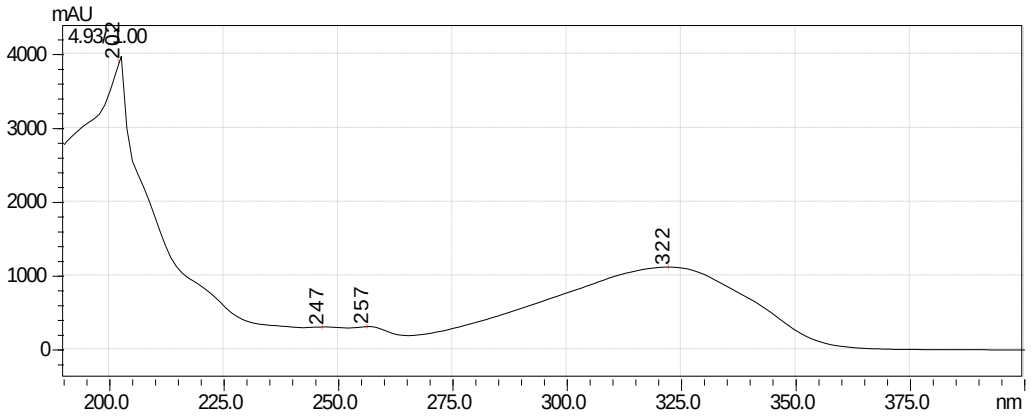


Figure 4. Uv-visible spectra of major peak of fraction 23 (F23).

3. DISCUSSION

Herbal goods are becoming increasingly popular worldwide due to their many appealing qualities, such as safety, nontoxicity, lack of side effects, and ease of accessibility at reasonable prices. Even nowadays, the industry for herbal drugs has developed as a rising segment in the global market as the main issues related to modern commercial medicines have been recognized [12]. Therefore, it is necessary to check a variety of therapeutic herbs for potential biological effects. The investigation of the area's living traditional knowledge of using plants as a source of medicines has become a recent trend in research. This is what the current study is doing. Nature, especially plants of ethnopharmacological significance, are potential repositories of novel antimalarial drugs because they contain a large number of chemical compounds with various structural and pharmacological properties [3]. Therefore, we have appraised one of the important species, from the genus Luvunga and of the family Rutaceae criteria previously reported, LS i.e., *Luvunga scandens* (Roxb), for their antimalarial activity determination based on already available ethnopharmacological use in treating malaria. Mostly Stem decoction is used in treating malaria [9,10].

This study extracted *L. scandens* stem and leaves from different solvents' polarities and obtained 6 extracts. These extracts were examined against *P. falciparum* 3D7 and HepG2 cell lines. The results revealed that 2 extracts (LS-L-D and LS-S-D) were active as antimalarial with IC_{50} values of 6.61±0.04 and 5.38±0.01 µg/mL, respectively, while the remaining extracts were found to be moderately active as antimalarial. The criteria for the antimalarial activity of the extract according to Basco et al., 1994 and Dolabela et al., 2008 were as follows: IC_{50} <10 µg/mL was the active extract, IC_{50} 10-50 µg/mL was included in the moderate criteria, IC_{50} 50-100 µg /mL extract has low activity, and IC_{50} >100 µg/mL indicates the extract is inactive [13,14]. Regarding the evaluation of *in vitro* toxicity of LS-L-D and LS-S-D against HepG2 cells showed that LS-L-D was nontoxic with CC_{50} of 50.55±0.02 µg/mL and LS-S-D was slightly toxic with CC_{50} of 42.49±0.09 µg/mL. We further fractionated the LS-L-D to obtain 26 fractions (F1-F26). The antimalarial activity showed that fraction 23 was the most active fraction with IC_{50} values of 2.44±0.09 µg/mL and slightly toxic with a CC_{50} value of 46.35±0.03 µg/mL. The selectivity index (SI) of F23 was ≥2, suggesting potential therapeutic for malaria [15]. The F23 was the potential fraction to be further studied to isolate the active antimalarial compounds

HPLC profile of F23 shows the maximum absorption at wavelengths of 247, 257, and 322 nm, respectively. The major peak indicated the characteristics of the flavonoid compound group, especially isoflavones. Most flavonoids' UV spectra consist of two major absorption maxima, one of which occurs in the range of 240-285 nm (band II) and 300-400 nm (band I). In general terms, band II absorption may be considered as having originated from the A-ring benzoyl system and the band I from the B-ring cinnamoyl system [16]. Furthermore, in isoflavones, band II appears in the 245-270 nm region, and band I shoulder in the range 300-340 nm [17]. The UV spectra profile suggested that fraction 23 contains isoflavones as major substance, possibly taking a role in its antimalarial activity.

Several studies regarding the antimalarial activity of isoflavones have been reported. Six isoflavones isolated from stems and leaves of *Andira inermis* (biochanin A, calycosin, formononetin, genistein, pratensein, and prunetin) have been shown to possess antimalarial against the *P. falciparum* poW and Dd2 [18]. Two compounds isolated from root bark of *Millettia dura* inhibit *P. falciparum* 3D7 and the Dd2 strains (70–90% inhibition at 40 µM) [19]. These previously reported studies support the possibility of isoflavones compounds in fraction 23 as an active antimalarial. Meanwhile, several compounds isolated from *L. scandens* were reported as well. Luvungaside A, 1,3-dihydroxy-2-methoxy- 10-methyl-9-acridone, 1-hydroxy-3-methoxy- 10-methyl-9-acridone, and arborinine were acridone alkaloid compounds isolated from rhizomes of *L. scandens* which expressed significant hepatoprotective effect [20]. Two triterpenes compounds, namely flindissol and 3-oxotirucalla-7,24-dien-21-oic acid have been isolated from dichloromethane extracts of *L. scandens* stem and showed potency as antitumor agents [21]. This present study provides a scientific basis for the traditional application of *L. scandens* stem decoction in malarial treatment. Furthermore, there is an opportunity for continuous study to obtain active antimalarial compounds from *L. scandens* leaves especially isoflavones from fraction 23 which will gain the value of *L. scandens* as antimalarial sources.

4. CONCLUSION

In this study, we have conducted a thorough investigation into the potential of a traditionally important medicinal plant, i.e., *Luvunga scandens*, by revealing its hidden antimalarial prowess. The LS-L-D extract stood out as a promising candidate, with an IC_{50} value of (6.10±0.04 µg/mL) against *Plasmodium falciparum* 3D7 and no cytotoxicity (50.55 ±0.02 µg/mL) against HepG2 cell lines. The lack of cytotoxicity of

LS-L-D makes it a promising extract for the development of novel antimalarial drugs, and the fractions derived from this active extract were classified based on their ability to inhibit *P. falciparum* 3D7. Fraction 23 was categorized as the most active one based on its better IC_{50} profile (2.44±0.09 µg/mL) against *P. falciparum* 3D7 strain and CC_{50} value (46.35±0.03 µg/mL) against HepG2 cell lines. The antimalarial effect of this fraction is probably due to the presence of a major peak which is predicted to be isoflavones. However, more research is needed to fully realize the therapeutic potential, including the isolation of Fraction 23 to reveal the active antimalarial compound hidden within. This research paves the way for potential advances in malaria treatment and opens up new avenues for drug development.

5. MATERIALS AND METHODS

5.1 Materials, Chemicals, and Reagents

The leaves and stem of *L. scandens* were obtained from Balikpapan Botanical Garden, East Kalimantan, Indonesia, on November 2015. A licensed botanist identified this plant at Purwodadi Botanical Garden, East Java, Indonesia (0074/IPH.06/HM/XII/2015). The specimens were stored at the herbarium of the Institute of Tropical Disease, Universitas Airlangga, Surabaya, Indonesia (specimen voucher: BP02).

The chemicals and reagents used in this study are n-hexane pro analysis (Merck), dichloromethane p.a. (Merck), methanol p.a. (JT Backer), chloroform p.a. (JT Backer), ethyl acetate p.a. ((Sigma-Aldrich, Germany), methanol pro HPLC (Merck), water pro HPLC (Merck), Silica TLC plate GF_{254} gel (Merck 0.25mm), RP-18 TLC plate (Merck 0.5 mm), Chloroquine diphosphatase (Sigma-Aldrich, Germany), Giemsa (Merck, Germany), RPMI 1640 medium (Gibco), hypoxanthine (Sigma-Aldrich), sodium bicarbonate (Sigma-Aldrich), gentamycin (Sigma-Aldrich), NBT (Sigma-Aldrich), APAD (Oriental Yeast Co., Ltd), and diaphorase (Sigma-Aldrich).

5.2 Extraction and Fractionation

The *Luvunga scandens* leaves (LS-L) and stem (LS-S) were dried and powdered using electric grinding. A total of 25 g of the powder was weighed and extracted gradually with n-hexane, dichloromethane, and methanol by ultrasound-assisted extraction. The obtained extracts were filtered and evaporated to give dry extracts. The most active extract against the chloroquine-sensitive *Plasmodium falciparum* (3D7) strain was separated into a total of 26 fractions (F1-F26) using vacuum column chromatography (VLC). The mobile phase in the separation process was a gradient of n-hexane and ethyl acetate. Initially, the n-hexane to ethyl acetate ratio was 9.5ml and 0.5ml, respectively, and it was gradually adjusted by decreasing the n-hexane ratio and increasing the ethyl acetate ratio throughout the VLC, i.e., the gradient for F23 comprised 2 ml of n-hexane and 8 ml of ethyl acetate as the mobile phase. Silica gel 60 G/F_{254} (Merck) was utilized as a stationary phase.

The six extracts were identified by thin layer chromatography (TLC) profiles using hexane-ethyl acetate (60/40 v/v) as the mobile phase and silica gel 60 F_{254} as the stationary phase. Furthermore, the plates were visualized directly after drying and with the help of UV at 254 and 366 nm in UV TLC visualizer. Ten percent of H_2SO_4 (10%) is used as a spray reagent.

5.3 *In vitro* culturing of *Plasmodium falciparum*

Chloroquine-sensitive (3D7) *P. falciparum* strains were used to determine the antimalarial activity of *L. scandens* extracts and fractions. The culture was maintained according to some modification protocols described by Trager and Jansen at the Institute of Tropical Disease, Universitas Airlangga, Surabaya, Indonesia [22]. Parasites were maintained in fresh human erythrocytes type O suspended at 2% hematocrit in RPMI 1640 (Gibco) containing hypoxanthine, sodium bicarbonate, gentamycin and incubated at 37°C under a mixed gas (5% O2, 5%CO2, and 90% N2).

5.4 Antimalarial activity

The antimalarial spectrum of the extracts and fractions of *L. scandens* were determined by *in vitro* antimalarial assay using lactate dehydrogenase (LDH). An aliquot of 5 milligrams of the sample was diluted in Dimethyl sulfoxide (stock at 10,000 µg/mL). This stock was used to prepare serial dilution at a final concentration of 50, 10, 5, 1, 0.5, 0.1, 0.05, and 0.01 µg/mL. Briefly, 1 µL of the sample was added to each well in a sterile 96-well plate in triplicate. Then 100 µL parasite (ring stage, 0.3% parasitemia, 2% hematocrit) was added. The well plate was incubated for 72 h at 37°C under 5% O_2, 5%CO_2, and 90% N_2. After incubation, the harvest plate is carried out and stored at -30°C overnight. Before the LDH assay, the plate

was thawed at room temperature. About 90 µL of the substrate (10 ml LDH buffer, 2 mg NBT (10 mg/mL), 50 µL APAD stock (10 mg/mL), and 200 µL Diaphorase stock (50 units/mL) were added in well, covered using aluminum foil, and shake for 30 minutes at room temperature. The absorbance of each well was measured at wavelength 650 nm using a multiscan sky-high microplate spectrophotometer (Thermo fisher scientific). The inhibition of the sample was calculated using this formula:

$$A = \frac{B-C}{B} \times 100\%$$

Where A= % *Inhibition*; B= *optical density of control well*; C= *optical density of sample well*

5.5 Cytotoxicity Assay

The hepatocellular carcinoma cells (HepG2) were cultured in DMEM (high glucose) supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) penicillin-streptomycin solutions in a 5% CO_2 incubator at 37°C. The HepG2 cell lines were washed with 5 ml of 1x DPBS, added with 1 ml of 0.25% Trypsin EDTA and incubated at 37°C for 5 minutes. After that, the culture plates were added with 4ml of DMEM media and were centrifuged at 4,000 rpm for 4 mins. A hemocytometer was used for counted the cells' pellets [23,24].

The serial dilution extracts and fractions in DMSO were seeded in the 96 well plates with the final concentration in 100, 50, 25, 12.5, 6.2, 3.12, 1.56, and 0.78 µg/mL with media as the negative control. The $1x10^4$ HepG2 cell lines were added to each well except the negative control and incubated for 44h at 37°C in a humidity 5% CO_2 atmosphere. Following the incubation, 10 µl of 0.5mM resazurin was added to all of the wells and continued to incubate for 4 hours. The fluorescence was measured at excision 530 nm and emission 595 nm based on resazurin's ability to be reduced to resorufin [25].

5.6 High-Performance Liquid Chromatography (HPLC)

The profile of fraction 23 (F23) was examined by using the high-performance liquid chromatography (HPLC) technique, Shimadzu's LC-06 analytical HPLC system (Kyoto, Japan), together with two pumps model LC-10AD, diode array detector (DAD) model SPD-M20A, an SCL-10A controller, a system controller, and LC Solution software. Methanol and distilled water were used as the mobile phase (MP) in the chromatographic studies conducted at a regulated temperature. Before analysis, a 0.45 µm membrane filter was used to filter both mobile phases. All these fractions were isocratically eluted in a solution of methanol and water (7.5: 2.5 v/v) at a flow rate of 0.7 ml/min and an injection volume of 40 µL.

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Conflict of interest statement: The authors declared no conflict of interest in the manuscript.

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