

Metabolite Profiling of the Extract and Antimalarial Activity of the Tablets Derived from the Cortex of *Alstonia spectabilis*

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<https://dx.doi.org/10.13005/bpj/2912>

(Received: 29 December 2023; accepted: 14 February 2024)

Malaria is a global health concern that threatens many countries. *Plasmodium sp.* may facilitate human transmission and is currently relatively resistant to chloroquine. The people in Nusa Tenggara, Indonesia, have traditionally employed the cortex of Black Pulai (*Alstonia spectabilis*) as a treatment for malaria for a considerable period. The objective of this study is to analyze the phytochemical composition of the 96% ethanol extract of *A. spectabilis* cortex (ASCE) and evaluate the antimalarial properties of tablets derived from *A. spectabilis* cortex extract (ASCT). A metabolite profile analysis was conducted on ASCE utilizing a UPLC-QToF-MS/MS method. And antimalarial test was conducted on ASCT on mice (*Mus musculus*) infected with *Plasmodium berghei*, and the blood smears of the mice were analyzed along with liver tissue damage. The results showed that administering ASCT could reduce the percentage of parasitemia in mice as well as the average liver damage score. This situation is feasible due to the presence of significant compounds in the ASCE that are anticipated to function as antiplasmodium agents, including villalstonine, vincadifformine, and pleiocarpamine. From these findings, one can infer that ASCT, which includes ASCE as its active component, has the potential to serve as a preferred antimalarial medication.

Keywords: *Alstonia Spectabilis*; Antimalarial; Metabolite Profiling; Tablet.

Malaria is a global health problem that threatens lives in more than 104 countries ¹ and it is currently estimated that there are 300-500 million cases each year ^{2,3}. Various *Plasmodium* species, such as *Plasmodium vivax*, *Plasmodium falciparum*, *Plasmodium malariae*, and *Plasmodium ovale* ⁴, are capable of causing this human transmission.

Malaria cases decreased from 238 million to 229 million in 87 malaria-endemic countries, according to the World Health Organization (WHO), with cases per 1000 population at risk falling from 80 in 2000 to 57 in 2019 ^{5,6}. In Indonesia, this disease is a very serious problem, this is proven by the high morbidity rate and causing many deaths ⁷. This

condition is because Indonesia has a heterogeneous climate and is vulnerable to regional and global climate change, thereby increasing the risk of transmission via mosquito vectors⁸.

This disease really threatens people's health, especially for poor people living in remote areas, besides that it can also reduce productivity, cause economic losses, and increase the mortality of babies, children and adults⁴. The WHO characterizes malaria elimination as the cessation of local transmission, signifying a reduction in the occurrence of malaria cases to zero in specific geographical regions³. However, over the past few decades, there has been a growing resistance of Plasmodium parasites to antimalarial quinine derivatives, particularly chloroquine, rendering the drug ineffective⁹.

The increasing resistance of Plasmodium to the chloroquine, and there are also side effects of chloroquine such as gastrointestinal disorders, headaches, blurred vision, insomnia, and exacerbation of psoriasis¹⁰, encourages researchers to continue looking for new and more effective antimalarial drugs¹¹. Plants have become one of the most important sources in the search for potential new antimalarial drugs in the traditional medicine of various ethnicities throughout the world¹². The indigenous people of Nusa Tenggara, Indonesia, traditionally employ Black Pulai (*Alstonia spectabilis*) as an antimalarial remedy¹³. This plant has demonstrated robust inhibitory activity against the *P. falciparum* 3D7 strain, as confirmed through in vitro testing in initial research⁹.

The great benefits contained in *A. spectabilis* have great potential for development to be used as a medicinal product so that it is more easily accepted by patients, but the difficulty of penetrating active substances from natural ingredients into the body due to differences in lipid solubility and molecular size is an inhibiting factor for drug molecules to pass through biological membranes thereby interfering with systemic absorption¹⁴. One solution to form a dosage form that has good stability is tablets¹⁵, therefore further investigation has been conducted to formulate tablets using a 96% ethanol extract derived from the cortex of *A. spectabilis*.

This study aims to assess the phytochemical composition of *A. spectabilis* cortex

extract (ASCE) utilizing ultra-high-performance liquid chromatography quadrupole time-of-flight mass spectrometry (UPLC-QToF-MS/MS). Additionally, it seeks to evaluate the antimalarial properties of tablets derived from *A. spectabilis* cortex extract (ASCT) by conducting experiments on mice infected with *P. berghei*.

MATERIALS

Materials

Plant material

A. spectabilis cortex was obtained from the Nusa Tenggara area of Indonesia and has been determined at the UPT Materia Medica, Batu, Indonesia.

Chemical material

Merck (Darmstadt, Germany) supplied ethanol 96%, formic acid, dichloromethane, methanol, and wright eosin, while Smart-Lab Indonesia (Tangerang, Indonesia) provided acetonitrile H-1010. Imedco (Tangerang, Indonesia) was the source for ket-A-100, xylazine 2%, and hydroxychloroquine sulfate.

ASCT

ASCT was formulated and provided to us by PT. Agaricus Sido Makmur Sentosa, Malang, Indonesia). ASCT was formulated using wet granulation, with the active ingredient being 96% ethanol extract of *A. spectabilis* cortex. Each ASCT is equivalent to 100 g of simplicia. The excipients used as constituents of the tablet formula were lactose, microcrystalline cellulose, sodium starch glycolate, silica hydrate, magnesium stearate, copovidine, and methylparaben.

Parasite culture

The *P. berghei* culture used is the ANKA strain, which was obtained from the Health Animal Clinic Research and Diagnostic Laboratory, Malang, Indonesia.

Animal subject

The test animals used were male Swiss mice (*Mus musculus*) weighing 20-25 g and 2-3 months old. These mice were obtained from the Biosciences Institute, Brawijaya University and have passed the ethical suitability test from the Brawijaya University Research Ethics Commission with ethical suitability letter No: 156-KEP-UB-2023.

METHOD

Extraction

Roughly 500 g of powdered *A. spectabilis* cortex underwent ultrasonic extraction with 96% ethanol through three ten-minute cycles, utilizing a Soltec Sonica 5300EP S3. The resulting solution was filtered and dried at 50 °C using a Heidolph G3 rotary evaporator. After this procedure, 25 g of the 96% ethanolic extract was acquired and subsequently subjected to additional testing and analysis, in this study, metabolite profiling was carried out¹⁶.

Metabolite profiling

Metabolite profiling was conducted using a UPLC-QToF-MS/MS instrument at the Forensic Laboratory Center of the Indonesian National Police Criminal Investigation Agency. A 96% ethanolic extract was generated through solid-phase extraction (SPE) using dichloromethane and methanol as solvents. Subsequently, 5 L of the extract was evenly injected into an ACQUITY UPLC® H-Class System (Waters, USA) equipped with an MS Xevo G2-S QToF detector (Waters, USA). The separation of samples occurred on an ACQUITY BEH C18 column (1.7 μm \times 2.1 mm \times 50 mm) with acetonitrile + water and 0.05% formic acid + 0.05% formic acid as mobile phases. The flow rate was maintained at 0.2 mL/minute. MassLynx 4.1 was employed to process the UPLC-QToF-MS/MS analysis results, generating chromatogram data and m/z spectra for each detected peak. The identified compounds were then validated using online databases, including MassBank (<https://massbank.eu/MassBank>), ChemSpider (<http://www.chemspider.com>), and PubChem (<https://pubchem.ncbi.nlm.nih.gov>).

Antimalarial activity test

The procedure for testing the antimalarial activity of ASCT followed the standard Peter's Test Method¹⁷. This research used a completely randomized design (CRD) through an experiment with four treatment groups, where each treatment consisted of three mice. Normal control group (without any induction), negative control group (mice induced by *P. berghei*), positive control group (mice induced by *P. berghei* + hydroxychloroquine sulfate 0.2 mg/20 gBW mice/day), and treatment group (mice induced by *P. berghei* + ASCT at a dose of 5.46 mg/20 gBW of mice/day).

Mice were acclimatized for 7 days before

the *P. berghei* inoculation process. Inoculation was carried out by administering $1 \times 10^7/0.1$ ml of suspension intraperitoneally to donor mice that had a parasitemia percentage of more than 20%. Donor mice blood was taken and infected as much as $1 \times 10^7/0.1$ ml into treated mice intraperitoneally.

Antimalarial activity test on mice according to treatment groups. Treatment is given if all test mice show parasite growth with parasitemia $\pm 1\%$ after 24 h post-infection. The test material was administered using a gastric probe once a day for 3 days.

The observation of parasitemia rates was carried out for 7 days to evaluate the effectiveness of ASCT. Thin blood smear preparations are made by dripping blood onto an object glass, then staining with Morphology of Peripheral Blood (MDT) dye. Meanwhile, liver tissue observation was carried out using liver damage scoring, necrosis, and degeneration. Blood and liver preparations were scrutinized using a light microscope (Nikon Eclipse type Ei) assisted by an Optilab microscope camera. The examination involved observing 10 different fields of view at a magnification of 1000 times for blood preparations. In the case of liver preparations, the examination was conducted in 5 fields of view with a magnification of 400 times.

RESULTS AND DISCUSSION

Metabolite profiling

In order to determine the specific compounds responsible for the antimalarial activity of ASCT, the UPLC-QToF-MS/MS instrument was utilized to identify secondary metabolite compounds. The total ion chromatogram (TIC) is visible in (Figure 1). In the interim, the percent area, m/z, retention time (RT), compound name and molecule formula values are displayed in (Table 1).

Following the metabolite profiling conducted through UPLC-QToF-MS/MS, accelerated solvent extraction produced a collection of 37 compounds. Among these, 8 were unidentified, and 29 were recognized or known compounds. In the process of metabolite profiling, not every peak in TIC can be identified among all compounds that have been detected. An indication of this is the presence of unknown compounds, which are those that are not included in the database. These compounds may consist

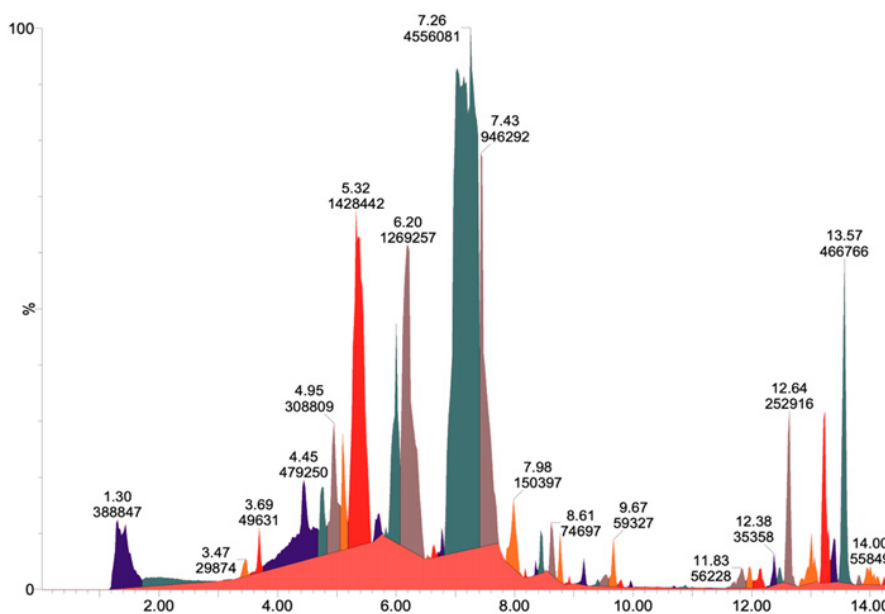


Fig. 1. TIC of ASCE

of undetected impurities or degradants by the instrument, as well as novel compounds that have not yet been added to the database. The unidentified compounds with elevated concentrations are of particular significance. Several “major” or “dominant” compounds are identified through the examination of these metabolite compounds^{18,19}. The percent area indicates that these compounds are more abundant in the sample compared to other compounds. In the ASCE, the major compounds were the alkaloid villalstonine (44.6101 %), vincadifformine (22.2509 %), and pleiocarpamine (10.2855). Every known major compound possesses antimalarial properties.

Antimalarial activity test

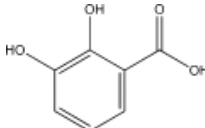
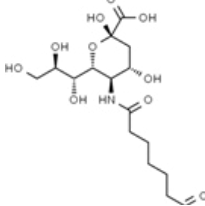
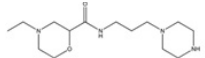
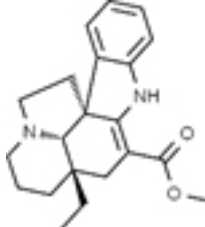
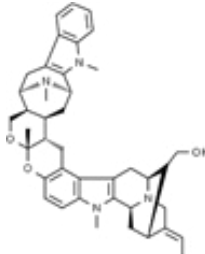
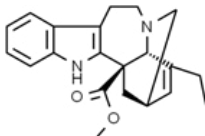
The analysis of parasitemia percentage revealed the growth of *P. berghei*, which was inoculated into mice, commencing on day 1 (D1). The assessment of parasite growth involved calculating the number of erythrocytes infected with ring, trophozoite, and schizont stages per thousand erythrocytes, multiplied by 100%. Table 2 presents the results of parasitemia growth before any treatment. It's noteworthy that malaria is transmitted by the hemoprotozoa *P. berghei* to small rodents. As an anti-malarial drug screening model, *P. berghei* is extensively utilized, owing

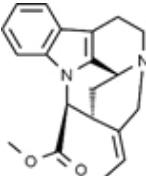
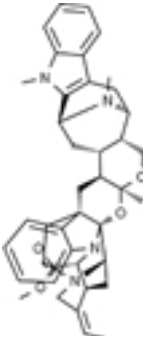

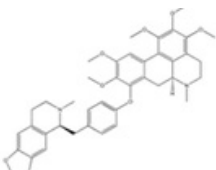

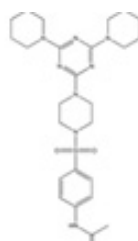
to its status as a malaria parasite that exclusively infects mammals apart from humans. The in vivo evaluation of this parasite was justified on the grounds that its structure, physiology, and life cycle are remarkably similar to those of human malaria²⁰.

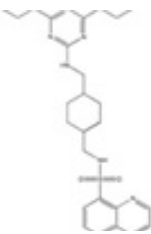
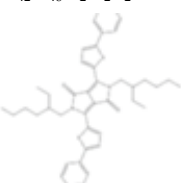
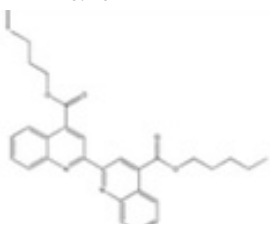
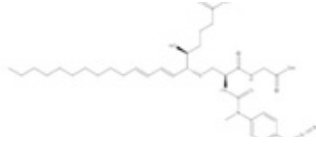
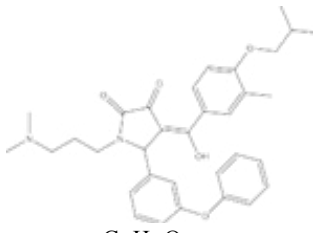
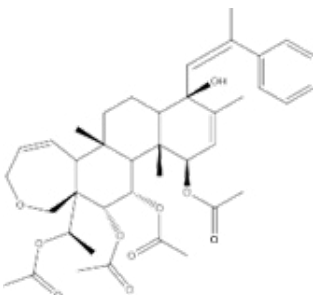
In Table 2, it is presented that the growth of parasites in the ASCT group showed that ASCT had good results in inhibiting the growth of parasitemia compared to the negative control and the chloroquine groups. In Figure 2, illustrates the distinctions in the features of regular and infected erythrocytes. Normal erythrocytes exhibit a yellowish hue and lack a nucleus. In contrast, erythrocytes infected with *P. berghei* appear paler, dotted, and larger in size compared to normal erythrocytes.²¹

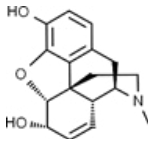
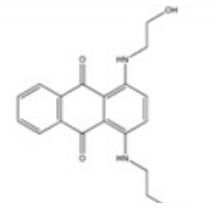
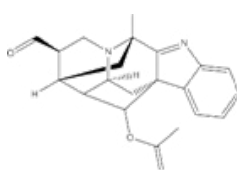
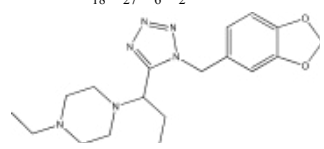
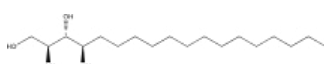
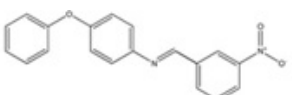
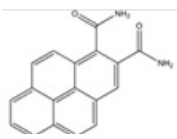
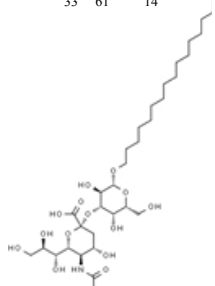
The results of Table 3 and Figure 3 show that the ASCT group gave a lower liver damage scoring value compared to the negative group, both from necrosis and degeneration scoring. Degeneration is an early sign of liver damage due to toxins, which is temporary (reversible), and cells can still recover or return to normal if toxin exposure is stopped. Degeneration is distinguished by alterations in the cell cytoplasm, marked by an augmentation in cell fluid and

Table 1. Prediction of compounds in ASCE

No.	RT (minute)	% Area	m/z	Molecular Formula and Structure	Compound Name
1	3.475	0.2421	154.0269	$C_7H_6O_4$ 	2,3-Dihydroxybenzoic acid
2	3.693	0.4022	393.1633	$C_{16}H_{27}NO_{10}$ 	6R)-3,5-Dideoxy-5-[(7-oxoheptanoyl)amino]-6-[(1R,2R)-1,2,3-trihydroxypropyl]-α-L-threo-hex-2- ulopyranosonic acid
3	4.747	1.2172	356.1741	$C_{14}H_{30}N_4O_2Cl_2$ 	4-Ethyl-N-[3-(1-piperazinyl)propyl]-2-morpholinecarboxamide dihydrochloride
4	5.325	22.2509	338.2000	$C_{21}H_{26}N_2O_2$ 	Vincadifformine
5	5.718	0.3906	644.3731	$C_{41}H_{48}N_4O_3$ 	Macralstonidine
6	6.007	3.7115	336.1836	$C_{21}H_{24}N_2O_2$ 	Catharanthine

7	6.204	10.2855	322.1686	$C_{20}H_{22}N_2O_2$	Pleiocarpamine
					
8	7.258	44.6101	660.3675	$C_{41}H_{48}N_4O_4$	Villalstonine
					
9	7.982	1.2543	674.3829	$C_{42}H_{50}N_4O_4$	1-Benzyl-3- {[4'-(4-[4-(hydroxymethyl)phenyl]-6- {[2-(1- pyrrolidinylmethyl)-1- pyrrolidinyl]methyl}-1,3-dioxan-2-yl)-3- biphenyl] methyl} urea
					
10	8.355	0.0796	680.3099	$C_{40}H_{44}N_2O_8$	(6aS)-1,2,3,9,10-Pentamethoxy-6- methyl-8- (4- {[(5S)-6-methyl -5,6,7,8- tetrahydro[1,3]dioxolo [4,5- g]isoquinoline-5- yl]methyl} phenoxy)- 5,6,6a,7-tetrahydro-4H- dibenzo[de,g]quinoline
					
11	8.439	0.3998	418.2260	$C_{26}H_{30}N_2O_3$	Bis(ù-piperidinylacetyl) dibenzofuran
					
12	8.769	0.3726	528.2628	$C_{25}H_{36}N_8O_3S$	N-(4-(4-(4,6-Di(piperidin-1-yl)-1,3,5- triazin-2- yl)piperazin-1- ylsulfonyl)phenyl)acetamide
					

13	8.924	0.0518	498.2525	$C_{24}H_{34}N_8O_2S$	N-{{[4-({[4,6-Bis(ethylamino)-1,3,5-triazin-2-yl]amino}methyl)cyclohexyl]methyl}-8-quinolinesulfonamide
					
14	9.171	0.2327	676.3161	$C_{42}H_{48}N_2O_2S_2$	2,5-Bis(2-ethylhexyl)-3,6-bis(5-phenyl-2-thienyl)-2,5-dihydropyrrolo[3,4-c]pyrrole-1,4-dione
					
15	9.410	0.0585	484.2364	$C_{30}H_{32}N_2O_4$	Dipentyl 2,2'-biquinoline-4,4'-dicarboxylate
					
16	9.669	0.9600	674.3460	$C_{33}H_{50}N_6O_7S$	N-[(4-Azidophenyl)(methyl)carbamoyl]-S-[(4S,5R,6E,8E)-1-carboxy-4-hydroxy-6,8-nonadecadien-5-yl]-L-cysteinylglycine
					
17	9.958	0.0404	542.2783	$C_{33}H_{38}N_2O_5$	1-[3-(Dimethylamino)propyl]-4-[hydroxy(4-isobutoxy-3-methylphenyl)methylene]-5-(3-phenoxyphenyl)-2,3-pyrrolidinedione
					
18	10.196	0.0059	692.3562	$C_{40}H_{52}O_{10}$	(5bS,8R,11R,11aR,12S,13R,13aR)-13a-[(1R)-1-Acetoxyethyl]-8-hydroxy-5b,9,11a-trimethyl-8-[(1Z)-2-phenyl-1-propen-1-yl]-1,3,5a,5b,6,7,7a,8,11,11a,11b,12,13,13a-tetradecahydrophenanthro[2,1-c]oxepine-11,12,13-triyl triacetate
					

19	10.401	0.0095	285.1367	$C_{17}H_{19}NO_3$	D-Morphine
					
20	10.682	0.0167	326.1263	$C_{18}H_{18}N_2O_4$	1,4-Bis[(2-hydroxyethyl)amino]-9,10-anthraquinone
					
21	10.879	0.0381	350.1628	$C_{21}H_{22}N_2O_3$	Perakine
					
22	10.992	0.0127	394.1887	$C_{18}H_{27}N_6O_2Cl$	1-{1-[1-(1,3-Benzodioxol-5-ylmethyl)-1H-tetrazol-5-yl]propyl}-4-ethylpiperazine hydrochloride
					
23	11.829	0.4556	317.2934	$C_{18}H_{39}NO_3$	Phytosphingosine
					
24	11.954	0.2622	318.1006	$C_{19}H_{14}N_2O_3$	N-[(E)-(3-Nitrophenyl)methylene]-4-phenoxyaniline
					
25	12.159	0.3477	288.0902	$C_{18}H_{12}N_2O_2$	Pyrenediamide
					
26	12.482	0.1811	695.4094	$C_{33}H_{61}NO_{14}$	Hexadecyl 3-O-((6R)-5-acetamido-3,5-dideoxy-6-[(1R,2R)-1,2,3-trihydroxypropyl]-α-L-threohex-2-ulopyranosyl)-α-D-galactopyranoside
					

27	13.009	0.8410	897.4572	$C_{47}H_{63}N_9O_7S$	Methyl (1-{2-(3-{[2-(3-{[2-(1- acetamidoethyl)-1,3-thiazol-4- yl]methyl}-2-oxo-1-imidazolidinyl)-3-methylpentanoyl]amino}-2-hydroxy-4- phenylbutyl)-2-[4-(2- pyridinyl)benzyl]hydrazino}-3,3- dimethyl-1-oxo- 2-butanyl)carbamate
28	13.390	0.5536	697.4245	$C_{46}H_{55}N_3O_3$	(2'-Cyano-4- biphenyl)methyl (1R,3aS,5aR,5bR,7aR ,8S,13aR, 13bR,15aR,15bR)-8-(hydroxymethyl)-1-isopropenyl - 5a,5b,8,13a-tetramethyl- 1,2,3,4,5,5a,5b,6,7, 7a,8,13,13a, 13b,14,15,15a,15b-octadecahydro -3aH-cy clopenta[7,8]phenanthro [1,2-g]quinoxaline -3a-carboxylate
29	13.565	3.7825	521.3480	$C_{29}H_{43}N_7O_2$	1-(4-{2-Isopropyl-6-[4-(3- methylphenyl)-1-piperazinyl]-4- pyrimidinyl}-1-piperazinyl)-3-(4- morpholinyl)-1-propanone

Table 2. % growth of parasitemia

Group	% parasitemia growth	
	Before treatment	After treatment
Normal Control	0.00	0%
Negative Control	90.00	26%
Positive Control(Chloroquine 0.2 mg/20 gBW mice/day)	92.00	15%
ASCT5.46 mg/20 gBW of mice/day	95.00	14%

swelling. However, the cell nucleus can maintain its integrity as long as the cell does not undergo severe injury²². This degeneration occurs due to oxidation disorders, which cause the buildup of protein deposits in the cytoplasm, so that the cytoplasm appears cloudy. Parenchymatous degeneration represents the least severe form of degeneration and is considered reversible. Continued parenchymatous degeneration can cause hydrophic degeneration. Hepatocytes that experience hydrophilic degeneration will swell and appear as vacuoles filled with water in the cytoplasm. The cause of persistent damage to

hepatocytes can be tearing of the plasma membrane and changes in the nucleus, so that hepatocytes experience necrosis. In addition, necrosis can occur as a result of reactive chemical metabolites binding to nucleophilic proteins within hepatocytes or the presence of metabolites with free chains capable of covalently binding to proteins and unsaturated fatty acids in cell membranes. This process leads to lipid peroxidation and membrane damage, ultimately culminating in hepatocyte death²³.

The results of blood observations and liver damage showed that the ASCT group with dose 5.46 mg/20gBW of mice/day could be an

alternative herbal antimalarial treatment. In this study, it was proven that ASCT had the ability to inhibit the growth of the *P. berghei* parasite in the blood of mice better than the positive control chloroquine. This is thought to be the way antimalarial drugs work in inhibiting the growth of parasites in the blood of mice so that they can reduce the percentage of parasitemia during the three days of observation.

The ASCE contains several antimalarial compounds, one of which is alkaloids, where

alkaloid compounds inhibit parasite growth by blocking parasite growth through intracellular transport of choline. In addition to alkaloid compounds as antimalarials, namely by inhibiting the detoxification process of parasite hemorrhoids through vacuoles, food becomes a non-toxic malaria pigment²⁴.

Villalstonine is one of the compounds contained in ASCE. This compound has demonstrated diverse biological activities, encompassing anticancer, antiameobic, and

Table 3. Average Liver Damage Scoring

Average Liver Damage Scoring Group	Necrosis	Degeneration
Normal Control	0.7	0.8
Negative Control	4.0	3.8
Positive Control(Chloroquine 0.2 mg/20 gBW mice/day)	1.3	2.0
ASCT5.46 mg/20 gBW of mice/day	2.5	2.1

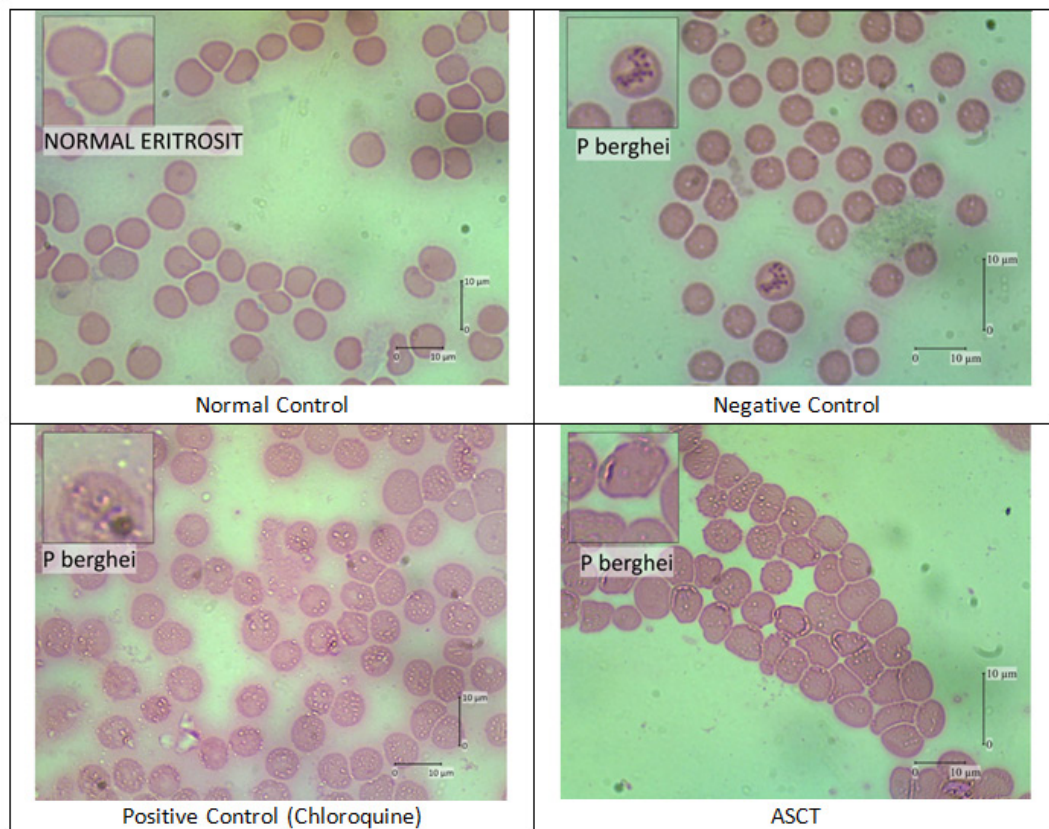


Fig. 2. Normal erythrocytes and erythrocytes infected with *P. berghei*

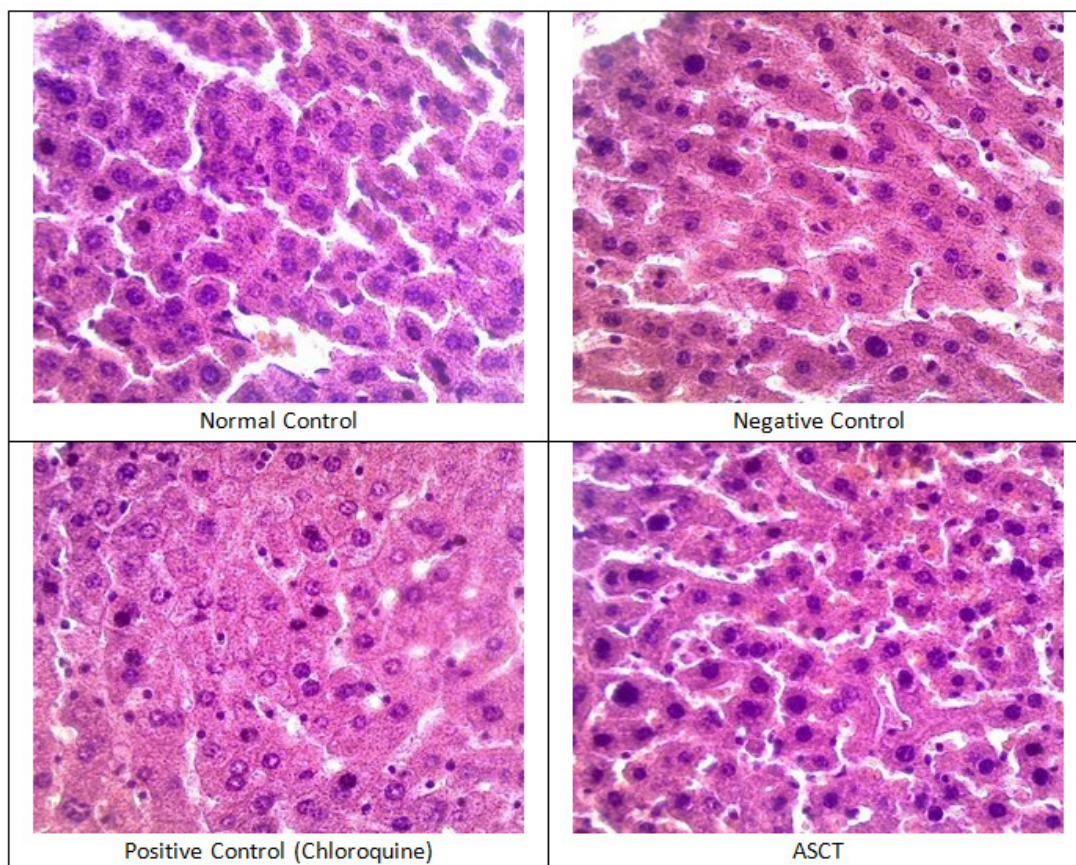


Fig. 3. Liver damage, necrosis, and degeneration of mice's livers in each group

antimalarial properties. In antimalarial potential, villalstonine has 1/15th potency compared to chloroquine as single compound in fighting malaria and strong antiplasmodial activity against the multidrug-resistant K1 strain of *P. falciparum* with an IC₅₀ value of 0.27 iM^{25, 26}. Vincadifformine and Pleiocarpamine are also compounds found in ASCE. Vincadifformine is recognized for its antimalarial biological activity against *P. falciparum*, exhibiting efficacy on the A FcM strain (a chloroquine-resistant strain with an IC₅₀ for chloroquine of approximately 230 ng/mL) and the Nigerian strain (a chloroquine-sensitive strain with a chloroquine IC₅₀ of about 41 ng/mL)²⁷. In contrast, the compound Pleiocarpamine demonstrates antimalarial activity as evidenced by test results utilizing an antiplasmodial assay with the induction of *P. falciparum* strain K1 and in vivo testing involving mice induced by *P. berghei*²⁸.

CONCLUSION

Based on these findings, it is possible to conclude that ASCT with ASCE as an active ingredient has an antimalarial effect by lowering the proportion of parasitemia in mice as well as the average liver damage score. In ASCE, several key chemicals, including villalstonine, vincadifformine, and pleiocarpamine, are predicted to be responsible for this effect.

ACKNOWLEDGMENT

The authors express their gratitude to PT. Agaricus Sido Makmur Sentosa for providing the ASCT in this study.

Conflict of Interest

The authors declare that there is no conflict of interest.

Funding Sources

This research was supported and funded by Pendanaan Program Bantuan Biaya Luaran Prototipe 2023 from Directorate General of Higher Education, Research and Technology Republic of Indonesia number 0921/E5.5/AL.04/2023.

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