Antimalaria Activity of Cassia spectabilis DC Leaf and Its Inhibition Effect in Heme Detoxification

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Research

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Abstract

Background In previous studies, Cassia spectabilis DC leaf has shown a good antimalarial activity. Therefore, this study is a follow-up study of leaf activity and mechanism of C. spectabilis DC as an antimalarial. Methods In vitro antimalarial activity testing using P. falciparum which was done with bioassay guide isolation in order to obtain the active compound. In vivo testing towards infected P. berghei mice was conducted to determine the effects of antimalarial prophylaxis and antimalarial activity in combination with artesunate. Whereas, heme detoxification inhibition testing as one of the antimalarial mechanisms was carried out using the Basilico method. Results The results showed that active antimalarial isolate obtained from C. spectabilis DC leaf had a structural pattern that was identical to (-)-7-hydroxyspectaline. Prophylactic test on infected P. berghei mice obtained the highest dose of inhibition percentage of 90% ethanol extract of C. spectabilis DC leaf was 68.61% while positive (doxycycline) control at 100 mg kg-1 was 73.54%. In antimalarial testing in combination with artesunate, it was found that administering 150 mg kg-1 (three times a day) of C. spectabilis DC (D0 – D2) + artesunate (D2) was better than the standard combination of amodiaquine + artesunate with 99.18% and 92.88% inhibition percentage. For the inhibitory activity of heme detoxification from ethanol extract 90%, C. spectabilis DC leaf had IC50 value of 0.375 mg mL-1 which was better than chloroquine diphosphate. Conclusion These results showed that C. spectabilis DC leaves possesses potent antimalarial activity and may offer a potential agent for effective and affordable antimalarial phytomedicine.

Background

Research to obtain new antimalarial drugs, both synthetic drugs and those derived from natural materials, especially from plants, is still ongoing.

One of the plants in Indonesia that has been traditionally recognized to treat malaria is *Cassia spectabilis* DC from the Caesalpiniaceae family. Previous study related to this plant with *in vivo* shows that this plant is quite potential to be continued(1). New antimalarial active compounds have also been obtained from plant in the same genus, namely Cassiarin A alkaloid compound from *C. siamea* plant (2, 3). For this reason, an *in vitro* research for antimalarial active compound from *C. spectabilis* DC plant with bioassay guide isolation is conducted.

Prevention of malaria can be done in various ways, one of which is chemoprophylaxis. Chemoprophylaxis is one way that can be done to reduce the risk of malaria infection and alleviate clinical symptoms if getting infected with malaria, chemoprophylaxis is aimed at many people who travel to malaria endemic areas for not too long time to avoid malaria infection. In Indonesia, therapeutic choice used for malaria prophylaxis is doxycycline and tetracycline (4). Both can be used as chemoprophylaxis for malaria but there are still many undesirable effects from this drug (5). Therefore, this research needs to be done to find and develop drugs that can be used as an effective antimalarial prophylaxis, safe, have few side effects, cheap, and easy to obtain, especially those from plants, namely *C. spectabilis* DC leaf.

Furthermore, antimalarial drug development and discovery is expected to provide new drugs with potential and safe drug mechanisms for humans. Research related to the biochemical process of malaria parasites plays an important role in the development of new antimalarial drugs. It will also test the effect of *C. spectabilis* DC leaf ethanol extract on biochemical activity in the malaria parasite food vacuole, including determining the potential for extracts in the detoxification of malaria parasite heme and antimalarial activity of the parasite in each life cycle.

In addition, parasitic resistance to some of the existing antimalarial drugs is the biggest problem in overcoming this disease, especially for malaria endemic areas (6). The combination therapy with artemisinin derivatives or commonly referred to artemisinin-based combination therapy (ACT) is highly recommended by WHO as the preferred therapy that is able to control the spread of resistance from *P. falciparum* (7, 8). Previous studies have found effective dose of 90% ethanol extract of *C. spectabilis* DC leaf is amounting to 150 mg kg^{-1} by giving three times a day orally (1).

Based on the foregoing, a combination of ethanol extract of *C. spectabilis* DC leaf extract will also be tested with artesunate which is one of the artemisinin derivatives to determine the inhibition of growth in *P. berghei* mice with *in vivo*. The combination therapy will be carried out in various models and from the therapeutic model an overview of the resulting antimalarial activity will be obtained. It is hoped that after this study, an appropriate combination model between artesunate and 90% leaf ethanol extract will obtain *C. spectabilis* DC that produces the greatest therapeutic effect.

Methods

Plant Material

C. spectabilis DC leaf was obtained and determined at Purwodadi Botanical Garden-Indonesian Institute of Sciences [Lembaga Ilmu Pengetahuan Indonesia, LIPI], Pasuruan, East Java, Indonesia. Specimen was deposited as the herbarium in Department of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, Universitas Airlangga (No : 02/W/XI/2016)

Experimental Animals

In this study, experimental animals used were BALB/c strain male mice obtained from Faculty of Veterinary Medicine of Universitas Airlangga with a weight of about 20-30 grams. Before being tested, the experimental animals were acclimatized for two weeks. Testing using experimental animals in this study had received permission from the Ethic Commission of Faculty of Veterinary Medicine of Universitas Airlangga (No: 2.KE.181.10.2018)

Extraction

The extract was made by macerating dried *C. spectabilis* DC leaf powder using 90% ethanol as many as three times. The macerate obtained was then evaporated using a rotavapor.

Isolation

1000 grams of dried powder of *C. spectabilis* DC leaf was macerated with n-hexane, then the pulp powder was extracted again with methanol. The extract was practiced in liquid-liquid with ethyl acetate and 3% tartaric acid. The aqueous layer was adjusted at pH 9 with saturated $NaHCO_3$ and extracted with chloroform. The chloroform fraction was then separated using column chromatography (chloroform-ethyl acetate-methanol) produced nine fractions. The antimalarial active fraction was continued to do column chromatography using silica gel 60 F_{254} (chloroform/methanol, 1; 00: 1). This isolation produced four sub-fractions. Antimalarial active sub-fraction was purified on TLC preparative with chloroform/methanol (3:2) to obtain active isolate. Identification of active isolate was carried out using the TLC-densitometry, UV-Vis spectrophotometry, FTIR spectroscopy, NMR, and HRFAB-MS.

In vitro antimalarial activity test

Stock sample solutions were prepared in DMSO and diluted to the required concentration with complete media (RPMI 1640 plus 10% human plasma, 25 mM of HEPES, and 25 mM of $NaHCO_3$) until the final concentrations of the sample on the well culture plate were 10, 1, 0.1, 0.01, and 0.001 µg mL^{-1}. The test was carried out in duplicate. The plates were incubated in CO_2 condition at 37ºC in a wax tube for 48 hours (9).[9] For test stage-specific antimalarial activity *in vitro*, incubation is performed at 6, 12, 24, and 48 hours. After the incubation, the contents of the well were harvested and the red cells were transferred to clean microscopic slides to form a series of thick films. The film was stained for 10 minutes in a 10% Giemsa solution (pH 7.3). Each 50% of inhibitory concentration (IC_{50}) was calculated based on the inhibition percentage towards *P. falciparum* using probit analysis.

In vivo antimalarial prophylactic activity test

In vivo test for antimalarial prophylactic activity of *C. spectabilis* DC leaf 90% ethanol extract used the modified Peters method (10).[10] Adult mice were randomly divided into six groups, each with seven animals per group. Group one as a negative control group was given a 0.5% Na CMC suspension solution. Groups 2, 3, 4, and 5 were given extract suspension solutions with doses of 100, 200, 400, and 800 mg kg^{-1}, respectively. Group six as a positive control group was given a doxycycline of 13 mg kg^{-1} suspension solution. Each solution was given orally once a day for four days, and on the fourth day the mice were infected with *P. berghei*. Thin blood smears from each mouse were made 72 hours after infection, then calculated the percentage of their parasitemia and inhibition.

Suppressive effect of ethanolic extract of *C. spectabilis* DC leaf combined with artesunate

The suppressive effect of ethanolic extract of *C. spectabilis* DC leaf in combination with artesunate, respectively against *P. berghei* infection in mice was determined using Peters' 4-day suppression test procedure(10).[10] A donor mouse densely infected with parasites was anaesthetized with chloroform and the blood was collected through cardiac puncture. The presence of parasitaemia was established by

microscopic examination of a thin blood film. The blood was diluted with PBS (Sigma) so that each 0.2 mL of blood contained 1 x 10^7 *P. berghei* infected with erythrocytes. A total of 0.2 mL of diluted blood was injected intraperitoneally into 36 healthy mice. The infected animals were randomly divided into six groups namely A, B, C, D, E, and F. The animals were treated shortly after inoculation on day zero (D_0). Group A was given artesunate at 36.4 mg kg^{-1}, on D_0 – D_2. Group B was given ethanolic extract of *C. spectabilis* DC leaf at 150 mg kg^{-1} (three times a day) concurrently with artesunate at 36.4 mg kg^{-1} (once a day), on D_0 – D_2. Group C was given ethanolic extract of *C. spectabilis* DC leaf at 150 mg kg^{-1} (three times a day) on D_0 – D_2 and artesunate at 36.4 mg kg^{-1} (once a day), on D_0. Group D was given ethanolic extract of *C. spectabilis* DC leaf at 150 mg kg^{-1} (three times a day) on D_0 – D_2 and artesunate at 36.4 mg kg^{-1} (once a day), on D_2. Group E was given amodiaquine at 72.8 mg kg^{-1} concurrently with artesunate at 36.4 mg kg^{-1}, once a day, on D_0 – D_2. Group F received 0.2 mL of 0.5% Na CMC solution as control group. Oral route was used for all administration. On day three (D_3), thin film was made from each mouse-tail blood on a microscopic slide. The slide was fixed with methanol, stained with Giemsa, and examined under the microscope. Parasitaemia level was determined by counting the average number of parasites at least 1000 erythrocytes. Percentage of parasitaemia inhibition was then calculated.

Heme polymerization inhibition test

Testing of inhibition of heme polymerization using the Basilico method(11)[11] has been modified. The test concentration was made at 2, 1, 0.5, 0.25, 0.1, and 0.01 mg mL^{-1}. Making a standard hematin curve started from making a 1 mM hematin solution in 0.2 M NaOH.

Testing of inhibition of heme polymerization was by taking 100 µL of 1 mM hematin solution in 0.2 M NaOH then adding 50 µL of the test material. Detoxification reaction of heme started with adding 50 µL of glacial acetic acid solution (pH 2.6) to the microtube which already contained a hematin solution and test material. Detoxification was carried out at the temperature of 37^oC for 24 hours. Next, the microtube was centrifuged at 8000 rpm for 10 minutes, then the filtrate was separated and the precipitate was washed with 200 µL of DMSO three times. The β-hematin crystalline precipitate obtained was dissolved in 200 µL of NaOH 0.1 M to form alkaline hematin which could be measured for absorbance. A total of 100 µL of the alkaline hematin solution was transferred to 96-well microplates and the absorbance was read using ELISA reader at a wavelength of 405 nm. The effects of each test substance on β-hematin production were calculated and compared with negative controls.

Results

Extraction and Isolation

Spectrum identification of ^{1}H-NMR and ^{13}C-NMR stated that isolate 8.3.1 had an identical structural pattern specifically with (-)-7-hydroxyspectaline (Fig. 1), a natural homologue that was previously isolated from *C. spectabilis* DC flower and fruit (12).[12] In spectra ^{1}H-NMR, it was possible to observe the broad

singlet on δ 3.60 ppm (1H, s); and δ 3.69 ppm (1H, s) which is hydroxyl signal, two singlets at δ 1.91 ppm (2H, s, C-4) and δ 1.68 ppm (2H, s, H-5) are ethyl benzene compound signals, and some cassettes at δ 1.38 ppm (2H, s, H-1); δ 1.22 ppm (2H, s, H-2-8 '); δ 1.51 ppm (2H, s, H-9), and one triplet at 35 2.35 ppm (2H, t, H-10 '). Spectrum^{13} C-NMR showed a signal of one ketone at δ 179.0 ppm, five carbon benzene compounds at δ 56.96 ppm; δ 67.13 ppm; δ 29.23 ppm; δ 25.77 ppm; and δ 48.29 ppm, six aliphatic carbons at δ 34.32 ppm; δ 25.87 ppm; δ 29.23 ppm; δ 29.33 ppm; δ 22.89 ppm; and δ 38.87 ppm, one methyl at δ 30.23 ppm, and one hydroxyl carbon at δ 65.27 ppm.

Fig. 1. Molecular structure of (-) - 7-hydroxyspectaline

In vitro antimalarial activity test

In vitro antimalarial activity from *C. spectabilis* DC leaf extracts and fractions (Table 1) were based on the classification according to Gessler et al., where extract with IC_{50} less than 10 µg mL^{-1} was considered very good, 10 to 50 µg mL^{-1} was considered moderate, and more than 50 µg mL^{-1} was considered to have low activity(13).[13]

Table 1. The percentage inhibition of extracts and fractions of *C. spectabillis* DC leaf against *P. falciparum* 3D7 strain

Extract/faction	Weight (g)	Average inhibition (%) of *P. falciparum* at concentration (µg mL^{-1})					IC_{50} (µg mL^{-1})
		100	10	1	0.1	0.01	
Hexane extract	118.34	0	0	0	0	0	0
Methanol extract	141.68	100	68.67 ± 5.19	14.36 ± 2.26	0	0	1-10
Ethyl acetate fraction	100.21	100	96.56 ± 4.86	75.47 ± 3.38	16.41 ± 3.78	0	0.41
Chloroform fraction	4.06	100	79.80 ± 1.87	56.36 ± 12.12	31.38 ± 12.58	0	0.55

Table 2. The percentage inhibition of chloroform fractions of *C. spectabillis* DC leaf against *P.falciparum* 3D7strain

Fraction	Weight	Average inhibition (%) of *P. falciparum* at concentration (µg mL^{-1})					IC_{50}
	(mg)	10	1	0.1	0.01	0.001	(µg mL^{-1})
C.1	20.00	27.97 ± 5.46	0	0	0	0	0
C.2	4.10	0	0	0	0	0	0
C.3	13.90	31.82 ± 3.51	6.39 ± 3.95	0	0	0	>10
C.4	17.90	93.07 ± 6.36	59.02 ± 1.38	28.22 ± 8.39	10.54 ± 9.24	0	0.384
C.5	7.70	100	62.74 ± 6.03	12.44 ± 17.59	0	0	>0.1
C.6	104.40	97.40 ± 3.67	93.50 ± 6.81	65.50 ± 18.95	14.92 ± 7.44	0	0.060
C.7	94.20	91.90 ± 1.23	84.84 ± 2.91	68.22 ± 4.91	48.83 ± 11.03	0	0.011
C.8	366.10	100	100	84.39 ± 7.54	66.26 ± 3.58	42.90 ± 4.50	0.002
C.9	36.50	100	87.36 ± 6.41	65.51 ± 3.78	58.52 ± 1.33	37.96 ± 15.12	0.005

Table 3. The percentage inhibition sub-fractions of the C.8 fraction of *C. spectabillis* DC leaf against *P.falciparum* strain 3D7 *in vitro*

Sub-faction	Weight	Average inhibition (%) of *P. falciparum* at concentrations (µg mL^{-1})					IC_{50}
	(mg)	10	1	0.1	0.01	0.001	(µg mL^{-1})
SFC.8.1	8.65	99.56 ± 0.59	86.42 ± 3.19	70.95 ± 0.17	62.71 ± 3.82	53.30 ± 4.67	0.001
SFC.8.2	11.25	0	0	0	0	0	0
SFC.8.3	32.50	100	72.84 ± 6.81	62.58 ± 6.01	46.59 ± 0.47	31.08 ± 7.40	0.016
SFC.8.4	20.50	76.74 ± 16.36	0	0	0	0	>1

Table 4. The percentage inhibition of isolate 8.3.1 and isolate 8.3.2 resulted from sub-fraction C.8.3 of *C. spectabillis* DC leaf against *P. falciparum* strain 3D7 *in vitro*

Isolate	Weight	Average inhibition (%) of *P. falciparum* at concentration (µg mL^{-1})					IC_{50}
	(mg)	10	1	0.1	0.01	0.001	(µg mL^{-1})
Isolate 8.3.1	4.30	97.23 ± 1.49	80.11 ± 0.69	67.61 ± 1.93	46.77 ± 0.91	27.71 ± 1.35	016
Isolate 8.3.2	2.60	100	77.21 ± 2.81	50.56 ± 10.48	31.96 ± 13.61	0	0.070

Isolation from chloroform extract obtained nine fractions. *In vitro* antimalarial activity test of these fractions showed that the C.8 fraction was the most active, with IC_{50} at 0.02 µg mL^{-1}, as shown in Table 2. Purification of the C.8 fraction gave four sub-fractions and their antimalarial activity was shown in Table 3. Sub-fraction SFC.8.1 and SFC.8.3 showed antimalarial activity towards *P. falciparum* 3D7 with IC_{50} respectively 0.012 and 0.015 µg mL^{-1}. Purification of sub-fractions SFC.8.3 provided two isolates and their antimalarial activity was shown in Table 4. Isolate 8.3.1 and isolate 8.3.2 showed antimalarial activity towards *P. falciparum* 3D7 with IC_{50} respectively 0.016 and 0.070 µg mL^{-1}.

Effect of *C. spectabilis* on parasitic stage development

In vitro antimalarial activity test for the effect of 90% ethanol extract of *C. spectabilis* DC leaf towards *P. falciparum* 3D7 was performed at different incubation periods (0, 6, 12, 24, and 48 hours). This was done to determine the activity of 90% ethanol extract of *C. spectabilis* DC leaf on the growth of parasites in different cycles. The extract concentration used was 100 µg mL^{-1}. The results of this experiment are shown in Table 5.

Table 5

Percentage of parasitemia, growth, and inhibition of *P. falciparum* 3D7 at 6, 12, 24, and 48 hours of incubation period

Sample	Incubation time (hours)	Parasitic stage			% parasitemia	% growthinhibition	%
		Ring	Trophozoite	Sporozoite			
DMSO	0	41	5	1	0.76	-	-
	6	38	11	2	0.84	0.08	-
	12	36	15	2	0.86	0.10	-
	24	45	19	7	1.14	0.38	-
	48	95	30	4	2.06	1.30	-
90% ethanol extract of *C. spectabilis* DC leaf	0	41	5	1	0.76	-	-
	6	38	10	1	0.81	0.05	37.5
	12	34	11	5	0.79	0.03	70
	24	22	13	2	0.59	0	100
	48	17	0	0	0.28	0	100

The growth of parasitic stages in the incubation period of 0-12 hours had almost no sharp difference. However, after the incubation period 12, it could be seen that parasitic growth showed the opposite direction. For negative controls, it moved upward and the parasite given the test solution went to 0. After 24 hours, the incubation period of the parasite was inhibited 100% compared to the control (Fig. 2).

Fig. 2. Percentage of 90% ethanol extract parasitemia of *C. spectabilis* DC leaf and control at each incubation time. DMSO = dimethyl sulfoxide, EECS = 90% ethanol extract of *C. spectabilis* DC leaf.

Fig. 3. Effects on stages development of *P. falciparum* 3D7 *in vitro* at difference incubation time. Parasitic morphology and the development of specific stages were assessed at the beginning of incubation (0 hour) and at 6, 12, 24, and 48 hours. The parasites were classified in three groups: ring (R), trophozoite (T), and sporozoite (S). The parasitic differential count was reported as a percentage of the total red blood cells infected. Five bars in each group according to different incubation times (left to right): 0, 6, 12, 24, and 48 hours. DMSO = dimethyl sulfoxide, EECS = 90% ethanol extract of *C. spectabilis* DC leaf.

In vivo antimalarial prophylactic activity test

In vivo antimalarial prophylactic activity test resulted 90% ethanol extract of *C. spectabilis* DC leaf was shown in Table 6.

Table 6. Prophylactic effects of 90% ethanol extract of *C. spectabilis* DC leaf and doxycycline towards *P. berghei* infection in mice

Sample	Dose (mg kg^{-1})	% parasitemia	% inhibition
90% ethanol extract of *C. spectabilis* DC leaf	100	5.95 ± 1.21	40.14
	200	4.06 ± 1.00	59.16
	400	3.97 ± 0.89	60.06
	800	3.12 ± 0.44	68.61
Doxycycline 100 mg*	13	2.63 ± 0.88	73.54
Na CMC	-	9.94 ± 1.81	-

*adult human dose

Extracts with a dose of 800 mg kg^{-1} provided the greatest inhibitory effect (68.61%) compared to other doses. With probit analysis calculation, ED_{50} value obtained was 161.20 mg kg^{-1}.

Suppressive effect of ethanolic extract of *C. spectabilis* DC leaf combined with artesunate.

Table 7 showed the results of suppressive tests by artesunate and *C. spectabilis* DC extract-drug combinations. Suppressive effects produced by the three extract-artesunate combinations were higher than artesunate alone. Moreover, the suppressive effects of group D (ethanolic extract of *C. spectabilis* DC leaf at 150 mg kg^{-1} (three times a day) on D_0 – D_2 and artesunate at 36.4 mg kg^{-1} on D_2) (99.18%) were higher than those produced by artesunate alone (82.60%) and artesunate-amodiaquine combination (92.88%).

Table 7 Suppressive effect of ethanolic extract of *C. spectabilis* DC leaf combined with artesunate against *P. berghei* infection in mice

Treatment	Parasitaemia count		Suppression (%)
	D_0	D_3	
Na CMC	1.84 ± 0.59	9.14 ± 2.38	-
C. spectabilis DC + Artesunate ($D_0 - D_2$)	2.01 ± 0.64	3.11 ± 1.30	84.93
C. spectabilis DC ($D_0 - D_2$) + Artesunate (D_0)	2.92 ± 0.73	3.40 ± 1.21	90.14
C. spectabilis DC ($D_0 - D_2$) + Artesunate (D_2)	2.22 ± 1.14	2.08 ± 1.28	99.18
Artesunate	2.27 ± 0.76	3.40 ± 1.24	82.60
Amodiaquine + Artesunate	1.62 ± 0.74	1.97 ± 0.68	92.88

Heme polymerization inhibition test

IC_{50} value from 90% ethanol extract of *C. spectabilis* DC leaf was shown in Table 8.

Table 8. Effect of 90% ethanol extract of *C. spectabilis* DC leaf towards heme polymerization inhibitory activity compared with chloroquine diphosphate

Ingredients	Concentration (mg/mL)	Level of hematin (mm)	Inhibition (%)	IC_{50} (mg/mL)
90% ethanol extract of *C. spectabilis* DC leaf	2	67.32 ± 4.49	76.49 ± 1.57	0.375
	1	85.34 ± 1.42	70.20 ± 0.50	
	0.5	118.49 ± 2.92	58.62 ± 1.02	
	0.25	186.37 ± 6.55	34.91 ± 2.29	
	0.1	223.85 ± 4.35	21.82 ± 1.52	
	0.01	252.00 ± 1.85	11.99 ± 0.64	
Chloroquine diphosphate	2	99.80 ± 2.50	65.15 ± 0.97	0.682
	1	125.65 ± 4.03	56.12 ± 1.41	
	0.5	158.00 ± 4.63	44.82 ± 1.62	
	0.25	186.78 ± 3.95	34.76 ± 1.38	
	0.1	207.05 ± 1.96	27.69 ± 0.68	
	0.01	229.53 ± 9.26	19.84 ± 3.24	
DMSO	-	286.33 ± 2.92	-	-

Discussion

In this research, the examination of active alkaloid compounds as antimalarials is in accordance with the bioassay guide isolation conducted with several stages of antimalarial activity testing, namely at the extract, fraction, and isolate stages of the *C. spectabilis* DC leaf *in vitro* and equipped with identification of groups of compounds from active isolates using UV-Vis spectrophotometer, TLC-densitometry, FTIR spectroscopy, and NMR.

Based on bioassay guide isolation, CS-1 active isolates are found which is continued to be identified by TLC-densitometry, FTIR spectroscopy, and NMR. Identification using TLC-densitometry shows that CS-1 isolate is in the range l 200-300 nm, with a value of Rf = 0.65. Identification using FTIR spectroscopy has an absorption peak at 472.53 cm^{-1}; 657.68 cm^{-1}; 786.9 cm^{-1}; 864.05 cm^{-1}; 1101.28 cm^{-1}; 1382.87 cm^{-1}; 1411.8 cm^{-1}; 1562.23 cm^{-1}; 1639.38 cm^{-1}; 2854.45 cm^{-1}; 2927.74 cm^{-1}; 3434.98 cm^{-1}. Identification using NMR spectroscopy shows that CS-1 isolate has proton and carbon spectra results that are identical to the compound (-)-7-hydroxycassine which is a piperidine alkaloid compound that has been successfully isolated from *C. spectabilis* DC flower and fruit

Then, 90% ethanol extract of *C. spectabilis* DC leaf is carried out as an *in vivo* prophylactic activity test on *P. berghei*. Dosage selection is based on research conducted by Munoz *et al.* where researchers use doses of up to 1000 mg kg^{-1} of bodyweight of mice(14).[14] Because of that, in this antimalarial test, the mice are selected in the range of 100-800 mg kg^{-1}. The test dose of 800 mg kg^{-1} gives the greatest inhibitory effect (68.61%) compared to the other test dose groups while doxycycline as a standard drug gives an inhibition of 73.54%.

The effect of ethanol extract of *C. spectabilis* DC leaf to the malaria parasite heme detoxification process is assessed according to the results of the *in vitro* β-hematin inhibition test. The results obtained from the ethanol extract of *C. spectabilis* DC leaf is the IC_{50} value of 0.375 mg mL^{-1} while chloroquine as an antimalarial standard compound is 0.682 mg mL^{-1}. The results of this study indicate that the ethanol extract of the *C. spectabilis* DC leaf has a greater inhibitory activity than chloroquine. Frölich *et al.* state that compound that has inhibition of β-hematin formation greater than 60% is said to have high potential as β-hematin inhibitor (good inhibitor) and conversely the percentage inhibition of less than 40%, is a low inhibitor of the formation of β-hematin(15).[15] Based on this argument, it can be stated that the ethanol extract of the *C. spectabilis* DC leaf has a high potential as an inhibitor of formation b-hematin compared to chloroquine as a standard drug.

The potential for *C. spectabilis* DC leaf ethanol extract towards the inhibition of hemozoin formation causes morphological disturbances and growth of malaria parasites. This is in accordance with the study of several libraries showing that heme detoxification barriers cause growth disturbance and death of malaria parasites due to membrane lysis and disruption of the activity of several enzymes(16-19).[16-19] This is evidenced by the inhibitory activity of parasitic growth during the 12-hour incubation period which increases compared to controls. This indicates that the malaria parasite has carried out the process of hemoglobin degradation to become globin and heme. Heme free (Fe(II)PPIX) which is toxic, then detoxifies the parasite into a non-toxic form, starting with free heme oxidation (ferrous state) to become hemin (ferric state).

Growth disturbance from parasites seen 12 hours after the incubation period showed that parasitic growth has been inhibited by 70% compared to parasites that are not given test material. Furthermore, after 24 hours the parasite incubation period, it is 100% inhibited compared to the control.

The combination of standard antimalarials with natural extracts is still rarely done even though natural materials are a source of antimalarial natural materials. This combination therapy has the advantage of being able to increase the effectiveness of extract as an antimalarial. It also has the potential to slow down the occurrence of parasitic resistance to standard antimalarials(20). The purpose of using combination antimalarial drugs is to increase the effectiveness of therapy and prevent or slow the onset of resistance to a single drug. Selection of *C. spectabilis* DC leaf ethanol extract combination with artesunate referring to the basis of malaria treatment which is a standard antimalarial drug recommendation from WHO which is ACT with the combination of amodiaquine and artesunate selected in this study(21).

In this research, *C. spectabilis* DC leaf ethanol extract used is a dose of 150 mg kg^{-1} given three times in accordance with the results of previous study (1).[1] The use of which is combined with artesunate which is an artemisinin derivative that has blood schizonticidal activity with fast onset of action and gametocide which can reduce malaria transmission in endemic areas(22).[22] On the other hand, artesunate used is at a dose of 36.4 mg kg^{-1} which is the result of conversion based on body weight of mice. The provision of artesunate with different length of time is motivated by the case of resistance to artesunate starting to emerge so that through this study, it is expected to be able to see the inhibition of artesunate if given just one or two days compared to the administration of artesunate for three days in accordance with WHO recommendation. In addition, this study also uses a negative control that is 0.5% Na CMC and a positive control used amodiaquine with dose of 72.8 mg kg^{-1} and artesunate dose of 36.4 mg kg^{-1} each once a day for three days which refers to ACT where the administration of antimalarial drugs for three days has been able to effectively inhibit the growth of parasites. In addition, according to WHO recommendation, the desired antimalarial drug is effective, safe, and used in a short time.

A combination of *C. spectabilis* DC leaf ethanol extract with artesunate or amodiaquine is given sequentially and not mixed into one test suspension because drug preparations will become unstable(23).[23] Giving concurrently and not in one formula have the disadvantage of reducing patient compliance to take the drug, but the combination therapy can shorten the duration of treatment therapy(21).[21]

The surprising thing also turns out to be a combination of *C. spectabilis* DC leaf ethanol extract given three times a day at dose of 150 mg kg^{-1} for three days with an artesunate dose of 36.4 mg kg^{-1} given on the last day (third day), which shows a higher inhibitory activity (99.18%) compared to a standard drug that is a combination artesunate with amodiaquine for three days (92.88%). The existence of these results is very supportive for the purpose of this study which seeks to reduce the use of artesunate in an effort to combat resistance case because artesunate is only given at the end of the day of therapy which means it can shorten the treatment period. Thus, it is expected that the combination of artesunate with *C. spectabilis* DC leaf ethanol extracts as a new antimalarial combination drug can replace the artesunate combination drug with amodiaquine used so far.

Conclusion

The results show 90% ethanol extract of *C. spectabilis* DC leaf has a very good antimalarial activity which is shown from the *in vitro* and *in vivo* activities. Antimalarial active isolate obtained from *C. spectabilis* DC leaf has a structural pattern that is identical to (-)-7-hydroxyspectaline. Combination of 150 mg kg^{-1} (three times a day) of *C. spectabilis* DC (D_0 – D_2) with artesunate obtains better results compared with the standard combination of 72.8 mg kg^{-1} amodiaquine + 36.4 mg kg^{-1} artesunate with 99.18% and 92.88% inhibitions. The 90% ethanol extract of *C. spectabilis* DC leaf also found to be active in inhibiting the heme detoxification process. Therefore, this plant can be potentially used as a new source for the development of new plant-based antimalarial agent.

List Of Abbreviations

ACT	artemisinin-based combination therapy
CO_2	carbon dioxide
DMSO	dimethyl sulfoxide
ED_{50}	median effective dose
ELISA	enzyme-linked immunosorbent assay
Fe(II)PPIX	ferrous-protoporphyrin IX
FTIR	fourier-transform infrared
HEPES	N-2-hydroxyethylpiperazine-N-ethanesulfonic acid
HRFAB-MS	high-resolution fast atom bombardment mass spectrometry
IC_{50}	the half maximal inhibitory concentration
Na CMC	sodium carboxymethylcellulose
$NaHCO_3$	sodium bicarbonate
NaOH	sodium hydroxide
NMR	nuclear magnetic resonance
PBS	phosphate buffered saline
RPMI	Roswell Park Memorial Institute
TLC	thin-layer chromatography
UV-Vis	ultraviolet-visible

Declarations

Ethics approval and consent of participate

Not applicable

Consent of Publication

Not applicable

Availability of data and materials

Data supporting of this article is included within the article

Conflicting Interest

The authors declare that they have no conflict of interest.

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Author Contribution

Conceived and designed the experiment: WE, DRB, HA and TSW. Analysed the data: WE and HA. Contributed reagents/materials/analysis tools: WE. Wrote the paper: WE, DRB and TSW. All authors read and approved the final manuscript

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Figures

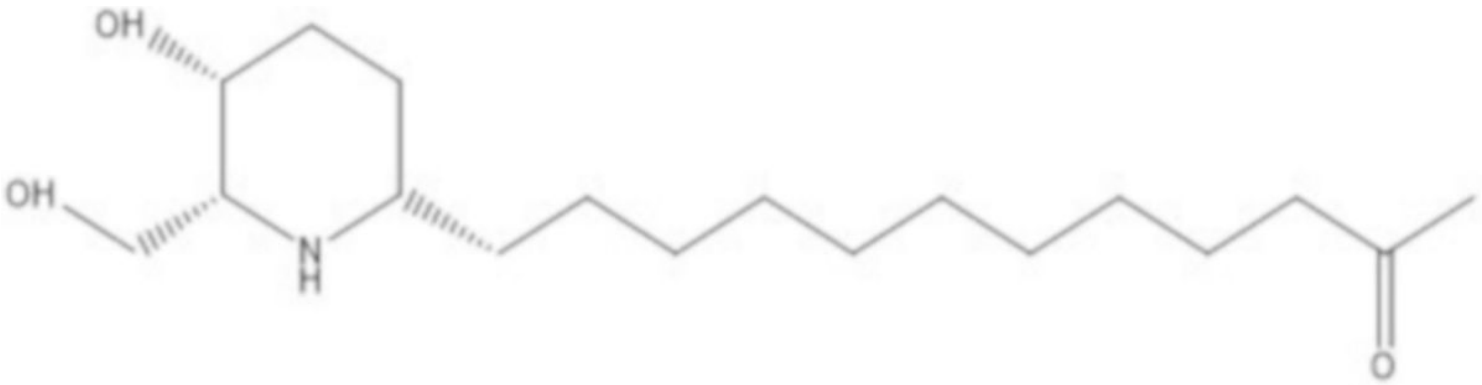

Figure 1

. Molecular structure of (-) - 7-hydroxyspectaline

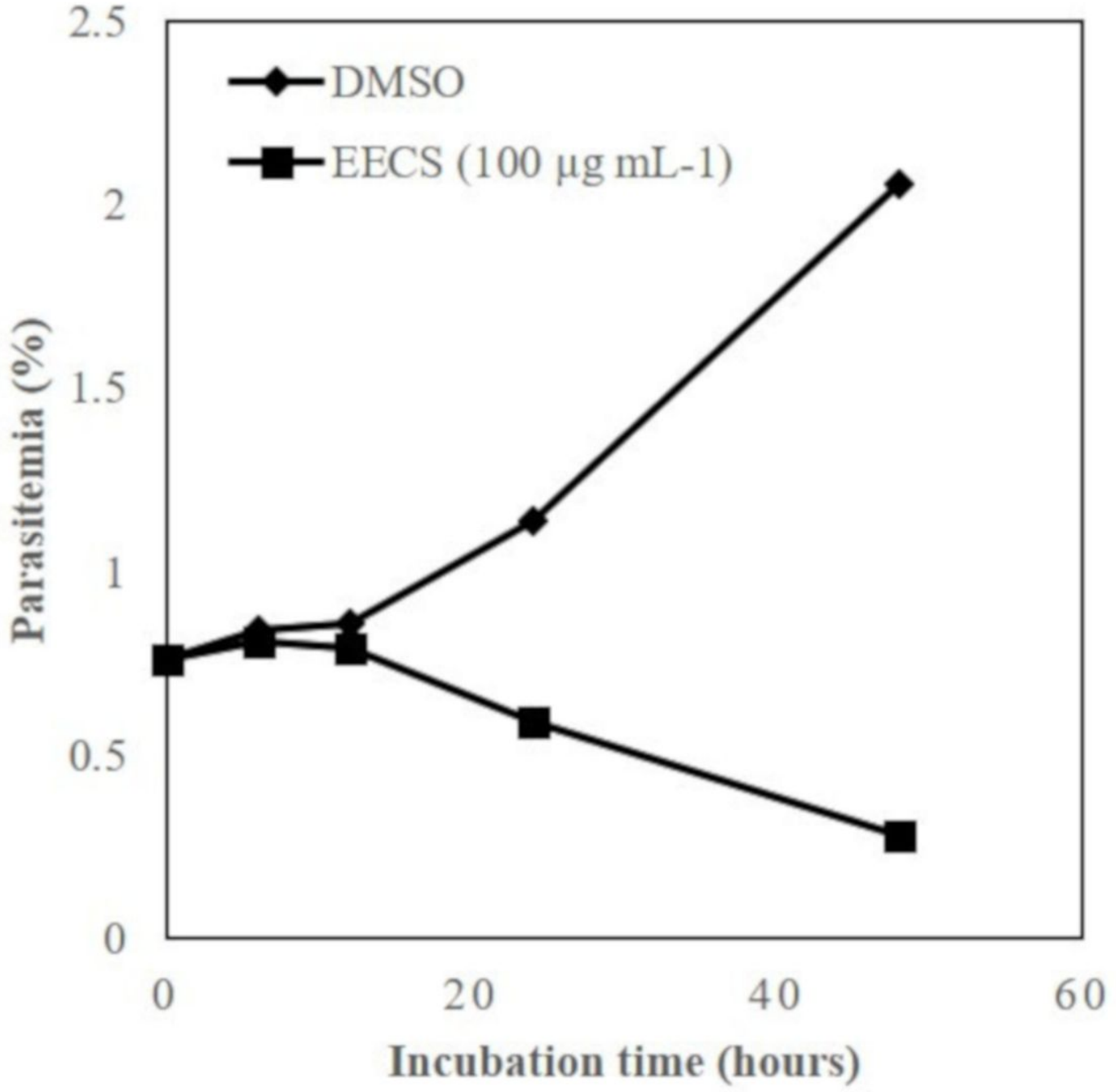

Figure 2

The percentage parasitemia of 90% ethanol extract of C. spectabilis leaf and control at each incubation time. DMSO = dimethyl sulfoxide, EECS = 90% ethanol extract of C. spectabilis leaf.

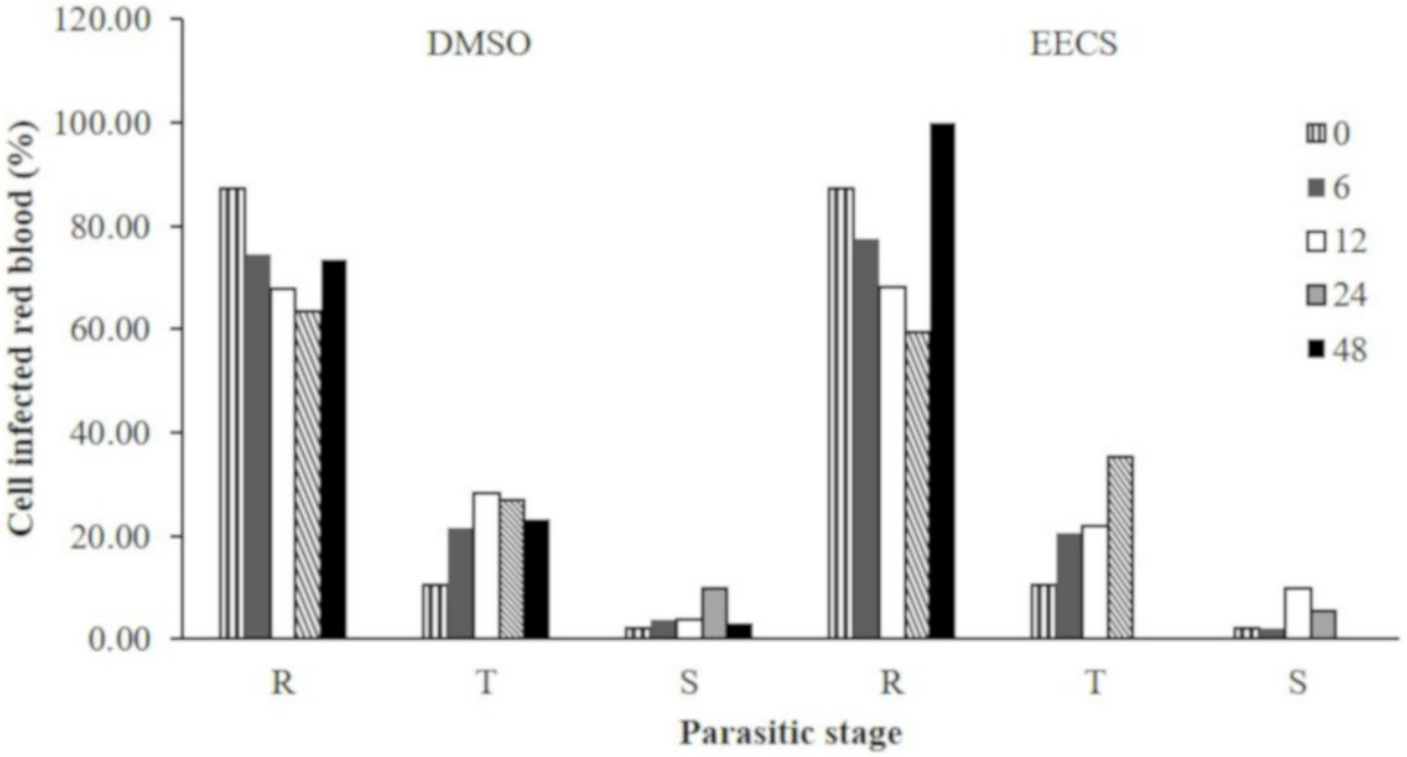

Figure 3

Effects on stages development of P. falciparum 3D7 in vitro at difference incubation time. Parasitic morphology and the development of specific stages were assessed at the beginning of incubation (0 hour) and at 6, 12, 24, and 48 hours. The parasites were classified in three groups: ring (R), trophozoite (T), and sporozoite (S). The parasitic differential count was reported as a percentage of the total red blood cells infected. Five bars in each group according to different incubation times (left to right): 0, 6, 12, 24, and 48 hours. DMSO = dimethyl sulfoxide, EECS = 90% ethanol extract of C. spectabilis DC leaf.