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ANTIPLASMODIAL ACTIVITY OF *Cleome viscosa* (L.) AGAINST CHLOROQUINE SENSITIVE *Plasmodium falciparum* (3D7 STRAIN) AND CHLOROQUINE SENSITIVE *Plasmodium berghei* (ANKA)

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AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

Background: Malaria is a deadly parasitic disease, caused by parasites that are transmitted to people through the bites of infected female Anopheles mosquitoes. An estimated 228 million cases of malaria occurred worldwide. Most malaria cases in 2018 were in African Region (93%), followed by South-East Asia Region with 3.4% of the cases and the Eastern Mediterranean Region with 2.1%. The spreading and development of resistance is a great problem to malaria treatment and control. So the plant based formulations are an alternative source for development of new drugs for the treatment of malaria. Hence, the present study was aimed to evaluate the antiplasmodial activity of the plant *Cleome viscosa.*

Methods: The collected plant parts were washed with tap water and dried in shade under laboratory condition. The crude extracts were extracted through soxhlet apparatus, the residues were concentrated in rotary vacuum evaporator. The parasites were cultured in RPMI medium supplemented with O+ serum in 96-well plate. The THP-1 cell lines were also cultured in RPMI medium in 96-well plate. *In vivo* studies were conducted in BALB/c albino mice.

Results: Of all the extracts, the ethyl acetate extract of leaves of *C. viscosa* had excellent antimalarial activity with IC50 value of 7 µg/ml. During cytotoxicity evaluation of crude extracts, none of the extracts had shown cytotoxicity on THP-1 cell line. The in vivo study has revealed that the ethyl acetate leaf extract of *C. viscosa* displayed a very good activity against *Plasmodium berghei.*

Conclusion: The ethyl acetate leaf extract of *C. viscosa* was found to be a good source for the development of new medicine or drug for the treatment of drug resistant malaria. Thus, further research is needed to characterize the bioactive molecules of the extracts of *C. viscosa* that are responsible for inhibition of malaria parasite.

Keywords: Antimalarial activity; *Cleome viscosa*; cytotoxicity; *Plasmodium berghei*; *Plasmodium falciparum*; THP-1 cell line.

1. INTRODUCTION

The year 2015 was an extraordinary year for malaria control due to fall of malaria infections worldwide particularly Sub-Saharan Africa [1]. Between 2000 and 2015, the mortality rate among children under five fell by 65% worldwide and by 71% in Africa [2].

Due to the emergence of resistance to Chloroquine, most malaria endemic countries have stopped using

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Chloroquine as the first line treatment for malaria to artemisinin combination therapy [3]. Unfortunately Artemisinin-resistant strain has been reported from Thai-Cambodia since 2009 and is spreading to other parts of the world. Thus has hasten the need for new antimalarials [4]. The World Health Organization (WHO) has recommended artemisinin-based combination therapy with lumefantrine, amodiaquine, mefloquine, sulphadoxine- pyrimethamine (SP) as the first-line treatment of malaria and artemisinin [5] was derived from plant source.

This fact has encouraged the continuing search for new natural product-derived antimalarial drugs. Several plants are used in traditional medicine for the treatment of malaria and fever in malaria endemic areas [6]. Thus, the present study was undertaken to investigate the *in vitro* antimalarial activity of the plant *C. viscosa* crude extracts against *P. falciparum* along with cytotoxicity against THP-1 cell line. Also the study was aimed to assess the *in vivo* antimalarial activity of the plant extract against *Plasmodium berghei* in experimental BALB/c mice.

Cleome viscosa L.belongsto Capparaceae familyis a widely distributed herb with yellow flowers and long slender pods containing seeds. *C. viscosa* a sticky herb with strong penetrating odour, is an annual weed growing in the tropics. The plant found usefulness in Ayurvedic medicine for the treatment of liver diseases, bronchitis, diarrhea, inflammations and fever. The fresh juice of the crush seed is reported for the treatment of infantile convulsions and in mental disorder. Juice from the plant, diluted in water and given in small quantities is useful for treating fever and leaves for ulcer and wound. The analgesic, antipyretic and anti-diarrheal activities of the extract have been established [7].

2. MATERIALS AND METHODS

2.1 Collection of Plant Materials

Freshsamples of leaves stem and flowers of *C. viscosa* were collected from Acharya Nagarjuna University Campus, Guntur district, Andhra Pradesh, India. The confirmation of the plant species was done by Prof. S.M. Khasim, Department of Botany, Acharya Nagarjuna University, Guntur, Andhra Pradesh, India.

2.2 Extract Preparation

Shade-dried plant samples were subjected to extraction in 90% different organic solvents methanol, ethyl acetate and chloroform at 50--60°C in a soxhlet apparatus (Borosil). The filtrates were concentrated by rotary vacuum evaporation (>45°C) and then freeze dried (20°C) to get solid residue. The extraction percentage was calculated by using the following formula:

$$\text{Percentage of extraction} = \frac{\text{Weight of the extract (g)}}{\text{Weight of the plant material (g)}} \times 100$$

These plant extracts were dissolved in dimethyl sulphoxide (DMSO) and monitored for the presence of phytochemical constituents by following the method of Sofowora [8] and Kepam [9].

2.3 Parasite Cultivation

The plant extracts were screened for antiplasmodial activity against chloroquine sensitive *P. falciparum* 3D7 strain. *P. falciparum* strain was obtained from ongoing cultures in the laboratory. These were cultured according to the method of Trager and Jenson [10] in candle jar desiccator.

2.4 *In vitro* Antimalarial Screening

The *P. falciparum* malaria parasite culture suspension of 3D7 (synchronized with 5% sorbitol to ring stage) was seeded (200 µl/well) in 96-well tissue culture plates. Plant extracts were added to the wells in two fold dilutions, at different concentrations, i.e. 100, 50, 25, 12.5 and 6.25 µg/ml. Chloroquine treated parasites were kept as positive control and DMSO treated parasites were kept as negative control groups. The parasites were cultured for 30 h in candle jar desiccators. The cultures were incubated at 37°C for 48 h in an atmosphere of 2% O_2, 5% CO_2 and 93% N_2. At 18 h before termination of the assay, [^{3}H] Hypoxanthine (0.5 µCi/well) was added to each well of 96-well plate. The effect of extracts in the cultures was evaluated by the measurement of [^{3}H] Hypoxanthine incorporation into the parasite nucleic acids [11]. Each treatment had four replicates; at end of the experiment, one set of the parasite infected red blood cells was collected from the wells and blood smears were prepared. These smears were fixed in methanol and air dried. The smears were stained with Acridine Orange (AO) and Giemsa stain. Stained smears were observed under UV illumination microscope (Carl Zeiss) for confirmation of [^{3}H] Hypoxanthine assay.

The parasites were harvested on glass filter papers using NUNC Cell Harvester and CPM (count per minute) was recorded in gamma scintillation counter. Control readings were considered to be as 100% parasite growth and the parasite inhibition was calculated for plant extract treated samples [12]. The parasite inhibition was calculated as follows:

$$\% \text{ Inhibition} = \frac{\text{Average CPM of Control} - \text{Average CPM of plant extracts}}{\text{Average CPM of Control}} \times 100$$

2.5 Cytotoxicity of Extracts on THP-1 Monocyte Cells

Cytotoxic properties of active plant extracts were assessed by functional assay using THP-1 cell line. The cells were cultured in RPMI-1640 medium. Briefly, cells (0.2×10^6 cells/200 µl/well) were seeded into 96-well flat-bottom tissue-culture plates in complete medium. Plant extracts with different concentrations (100, 50, 25 and 12.5 and 6.25 µg/ml) were added after 24 h of seeding and incubated for 48 h in a humidified atmosphere at 37°C and 5% CO_2. DMSO as negative inhibitor and ellipticine as positive inhibitor were kept as negative control and positive control. At the end of the experiment 10 µl of MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) stock solution (5 µg/ml in 1 × Phosphate Buffered Saline) was added to each well, gently mixed and incubated for another 4 h. After spinning the plate was centrifuged at 1500 rpm for 5 min, the supernatant was discarded, subsequently 100 µl of DMSO (stopping agent) was added. After formation of formazan, it was read on a micro titer plate reader (Versa max tunable multiwell plate reader) at 570 nm and the percentage of cell viability was calculated using the following formula. The selectivity index of *in vitro* toxicity was calculated for each extract as follows:

$$\text{SI} = \frac{\text{IC50 of THP}-1 \text{ cells}}{\text{IC50 of } P.\ falciparum}$$

$$\% \text{ Cell Viability} = \frac{\text{Mean absorbance in test wells}}{\text{Mean absorbance in control wells}} \times 100$$

2.6 Chemical Injury to Erythrocytes

For this, 200 µl of erythrocytes were incubated with 100 µg/ml of the extract, a dose equal to the highest used in the antiplasmodial assay. After 48 h of incubation, the assay was terminated and thin blood smears were prepared and fixed in methanol and stained with Giemsa stain and observed for morphological variations of erythrocytes [12].

2.7 *In vivo* Studies

A working solution of 50 mg/ml was prepared for *in vivo* (mice) studies. The potent plant extract with concentrations from 200 to 1000 mg/kg body weight were prepared in PBS (Phosphate Buffered Saline) for *in vivo* antimalarial activity against *P. berghei* in BALB/c mice. For negative control, PBS and for positive control chloroquine (5 mg/kg body weight/day) were used.

Healthy BALB/c female mice aged 6--8 weeks (25--30 g) were used for the present study. Animal experiments were designed and conducted in accordance with the guidelines and permission obtained from Institutional Animal Ethics Committee of University College of Pharmacy, Acharya Nagarjuna University, Guntur, Andhra Pradesh, India.

The chloroquine sensitive *P. berghei* ANKA strain was maintained *in vivo* in BALB/c mice by weekly inoculation of 1×10^7 infected red blood cells in naïve mice. Then the parasitaemia was counted with haemocytometer and the parasites adjusted to 0.5×10^6 with PBS sterile solution. Each animal was injected intraperitoneally with 200 µl (0.2 ml) with 0.5×10^6 parasites inoculated on first day, i.e. Day 0.

2.8 The 4-day Suppressive Test

This test was used to evaluate the schizontocidal activity of the methanolic leaf extract of *C. viscosa* against *P. berghei* infected mice according to the method described by Peter et al. [13]. The treatment was started three hours after mice had been inoculated with the parasites on Day 0 and then continued daily for four days from Day 0 to Day 3. After completion of treatment, the blood smear was observed on Day 4 to determine parasitaemia and percentage of inhibition.

2.9 Parasitaemia Measurement

Thin smears of blood were made from the tail of each mouse at the end of each test. Five different fields on each slide were examined to calculate the average parasitaemia as shown below.

$$\text{Parasitaemia (\%)} = \frac{\text{Number of parasitized RBC}}{\text{Total number of RBC}} \times 100$$

Finally, percentage of parasitaemia suppression of the extracts was calculated using the following formula.

$$\text{Suppression (\%)} = \frac{\text{Mean parasitaemia of control negetive group}}{\text{Mean parasitaemia of treated group}} \times 100$$

2.10 Monitoring of Body Weight

During 4-day suppressive test, body weight of each mouse was measured before infection (Day 0) and on Day 4 using a sensitive digital weighing balance.

2.11 Packed Cell Volume Measurement

Packed cell volume (PCV) was measured to predict the effectiveness of the test extract in preventing haemolysis resulting from increasing parasitaemia associated with malaria. The PCV was determined using a standard Micro-Haematocrit Reader. The measurenent was taken before inoculating the parasite and after treatment using the following relationship:

$$\text{PCV} \quad (\%) = \frac{\text{Volume of erythrocytes in a given volume of blood}}{\text{Total blood volume}} \times 100$$

2.12 Statistical Analysis

The mean and standard deviations of the treated and control groups were calculated at 95% confidence intervals for inhibition, mortality, parasitaemia, body weight and PCV. The IC_{50} values were determined with dose-response curves; the results were analyzed statistically by two-tailed student's *t*-test to identify the differences between the treated group and control group using Minitab 11.12.32. Bit software. The data were considered significant at $p < 0.05$.

3. RESULTS

The weight of leaves, stems and pods of *C. viscosa* (Plate 1) extracted in methanol, ethyl acetate and chloroform were 1.88, 1.05, 2.10 g; 1.22, 5.97, 2.19 g and 3.80, 1.49, 2.17 g respectively. The percentage yields of extracts ranged from 2.10 to 11.94%. It was found that ethyl acetate extract of stem (11.94%) showed maximum yield followed by methanol extract of roots (7.6%). The phytochemical studies revealed that the methanol, ethyl acetate and chloroform extracts of leaves, stem, and pod of *C. viscosa* had avariety of phytochemical constituents namely alkaloids, triterpenes, flavonoids, tannins, coumarins, carbohydrates, phenols, saponins, phlobatannins and steroids (Table 1).

Table 1. Phytochemical constituents in different extracts of leaves, stem and pods of *Cleome viscosa*

Tested compound	Leaves			Stem			Pods		
	ME	EA	CH	ME	EA	CH	ME	EA	CH
Alkaloids	+	-	+	+	+	-	-	+	+
Coumarins	+	-	+	-	+	-	-	-	-
Carbohydrates	-	-	+	-	-	+	-	-	-
Phenols	+	-	-	-	+	-	-	+	-
Saponins	-	-	+	-	-	-	+	+	-
Tannins	-	+	-	-	-	-	+	-	-
Flavonoids	+	-	+	+	-	+	-	-	+
Terpenoids	-	+	-	+	-	-	+	+	-
Phlobatannins	-	-	-	+	+	+	+	-	-
Steroids	-	+	-	+	-	-	-	+	-

+ Present, - Absent, ME- Methanol, EA- Ethyl acetate, CH- Chloroform

Whole Plant

Leaf

Stem

Pod

Plate 1. *Cleome viscosa* L. plant and its parts

Table 2. Antiplasmodial activity against *P. falciparum* 3D7 strain of different crude extracts of *Cleome viscosa*

Plant part	Extract	Percentage of inhibition (M±SD, *p* - value)					IC_{50} (µg/ml)
		6.25 µg/ml	12.5 µg/ml	25 µg/ml	50 µg/ml	100 µg/ml	95% *CI* (LCL-UCL)
Leaves	ME	6.44±1.03, 0.0039	15.36±3.21, 0.018	36.76±3.96, 0.0044	65.38±1.68, 0.0002	93.78±2.57, 0.0003	37.10±2.48 (30.95-43.25)
	EA	49.09±2.31, 0.0008	72.18±4.70, 0.0015	89.57±2.15, 0.0002	96.30±0.84, 0.0000	98.78±0.50, 0.0000	7.00±0.85 (4.88-9.122)
	CH	19.77±2.96, 0.0083	45.25±3.39, 0.0020	71.10±3.09, 0.0007	96.15±0.90, 0.0000	99.03±0.40, 0.0000	14.33±1.53 (10.54-18.13)
Stem	ME	1.58±0.35, 0.14^{NS}	4.92±1.09, 0.040	10.43±0.78, 0.0033	21.01±1.88, 0.0037	37.69±1.87, 0.0010	>100
	EA	12.14±1.74, 0.0087	21.67±3.67, 0.011	51.47±2.81, 0.0011	73.68±5.09, 0.0017	97.44±1.42, 0.0001	24.33±2.08 (19.16-29.50)
	CH	0.00±0.00, NS	1.18±0.16, 0.20^{NS}	6.72±1.97, 0.065^{NS}	16.10±1.41, 0.0039	28.73±2.53, 0.0035	>100
Pods	ME	RBCs lysed					
	EA	11.02±2.36, 0.018	20.43±3.32, 0.010	34.82±4.02, 0.0051	63.88±4.56, 0.0019	94.52±1.56, 0.0001	38.83±3.33 (30.56-47.10)
	CH	0.63±0.12, 0.81^{NS}	3.05±0.18, 0.033	8.46±1.15, 0.012	16.39±2.83, 0.015	30.05±2.55, 0.0032	>100
DMSO (Negative control)	-	0.74±0.65	1.65±0.41	2.47±0.16	3.14±0.02	4.04±0.07	-
CQ (Positive control)	-	-	-	-	-	-	3.74±0.75

Values are represented as mean of 3 replicates ± standard deviation at 95% confidence intervals with lower and upper limits and p-value is significant at <0.05, NS-not significant, ME-methanol, EA-ethyl acetate, CH-chloroform, DMSO-Dimethyl sulphoxide, CQ-chloroquine

Table 3. Cytotoxicity of different crude extracts of *Cleome viscosa* against THP-1 cell line

Plant part	Extract	Percentage of inhibition (M±SD, *p* - value)					IC_{50} (µg/mL) 95%*CI* (LCL-UCL)	SI
		6.25 µg/ml	12.5 µg/ml	25 µg/ml	50 µg/ml	100 µg/ml		
Leaves	ME	3.36±0.52, 0.0079	7.78±0.46, 0.0014	15.62±2.25, 0.0075	30.27±2.71, 0.0029	46.65±3.25, 0.0017	>100	>2.69
	EA	4.19±0.91, 0.0094	9.26±1.91, 0.015	17.35±2.82, 0.0093	34.67±4.02, 0.0048	55.92±3.63, 0.0015	86.33±7.57 (77.08-92.18)	12.33
	CH	5.84±0.23, 0.0050	10.43±1.69, 0.0019	22.04±1.69, 0.0021	40.03±1.25, 0.0004	65.97±2.66, 0.0006	69.00±3.61 (60.04-77.96)	4.81
Stem	ME	0.00±0.00, NS	0.00±0.00, NS	0.00±0.00 NS	5.15±1.09, 0.028	9.46±0.71, 0.0028	>100	>1.00
	EA	0.00±0.00, NS	0.00±0.00, NS	0.00±0.00 NS	0.00±0.00, NS	0.00±0.00 NS	ND	ND
	CH	1.03±0.19, 0.22^{NS}	6.39±1.29, 0.015	8.87±1.54, 0.012	15.00±2.44, 0.011	27.74±1.99, 0.0019	>100	>1.00
Pods	ME	2.38±0.55, 0.025	7.26±1.90, 0.025	12.26±2.05, 0.010	22.68±3.24, 0.0076	40.20±2.93, 0.093^{NS}	>100	ND
	EA	0.00±0.00, NS	0.00±0.00 NS	0.00±0.00 NS	3.19±1.48, 0.17	3.43±0.93, 0.072 ^{NS}	>100	>2.57
	CH	1.03±0.38, 0.22^{NS}	4.16±1.30, 0.038	10.29±2.20, 0.017	16.75±2.13, 0.0064	33.21±3.08, 0.0031	>100	>1.00
DMSO (Negative control)	-	0.00±0.00	0.40±0.10	0.64±0.12	1.32±0.29	1.51±0.16	-	
Ellipticine (Positive control)	-	-	-	-	-	-	0.59±0.25	

Values are represented as mean of 3 replicates ± standard deviation at 95% confidence interval with upper and lower limits and p-value is significant at <0.05, NS- not significant, ME- methanol, EA- ethyl acetate, CH-chloroform, SI-selective index (SI = IC_{50} THP-1 cell line/IC_{50} P. f 3D7 strain), ND-Not determinate, DMSO-Dimethyl sulphoxide

Table 4. Parasitaemia, inhibition and survival time during 4-day suppressive test after treatment with ethyl acetate leaf extract of *Cleome viscosa* against *Plasmodium berghei* infected BALB/c experimental mice

Test substance	Dose (mg/kg/day)	% Parasitaemia (M±SD, *p*-value)	% Inhibition	Mean survival time (Days ± SD, *p*-value)
Ethyl acetate Leaf extract	200	33.20±1.42, 0.18^{NS}	5.84	6.3±31.52, 0.40^{NS}
	400	25.24±1.48, 0.0035	28.13	6.00±1.00, 0.39^{NS}
	600	18.78±1.97, 0.0013	46.73	8.67±1.53, 0.072^{NS}
	800	15.57±1.41, 0.0005	55.84	10.33±0.58, 0.0004
	1000	12.56±1.89, 0.0005	64.37	10.66±1.53, 0.030
Vehicle (-)	1 ml	35.26±1.44,	-	5.33±0.58
Chloroquine (+)	5	0.86±0.32	97.56	15.0±00.00

The values are represented as mean of 3 values ± standard deviation and significant at p <0.05 (compared with negative control), NS- Not significant, (-) Negative control, (+) Positive control

Table 5. Body weight during 4-day suppressive test after treatment with ethyl acetate leaf extract of *Cleome viscosa* against *Plasmodium berghei* infected BALB/c experimental mice

Test substance	Dose (mg/kg/day)	Weight on Day 0 (g) (M±SD)	Weight on Day 4 (g) (M±SD, *p*-value)	% Change
Ethyl acetate Leaf extract	200	25.76±0.67	25.00±0.47, 0.65^{NS}	2.95
	400	26.13±1.43	25.93±0.48, 0.19^{NS}	0.76
	600	26.15±1.72	26.42±1.80, 0.24^{NS}	-1.03
	800	26.37±1.00	26.63±1.54, 0.16^{NS}	-0.98
	1000	26.18±0.71	27.13±1.12, 0.066^{NS}	-3.63
Vehicle (-)	1 ml	26.10±1.53	24.65±1.03	5.55
Chloroquine (+)	5	25.81±0.91	26.73±0.69	-3.56

The values are represented as mean of 3 values ± standard deviation and significant at p <0.05 (compared with negative control), NS- not significant, (-) Negative control, (+) Positive control

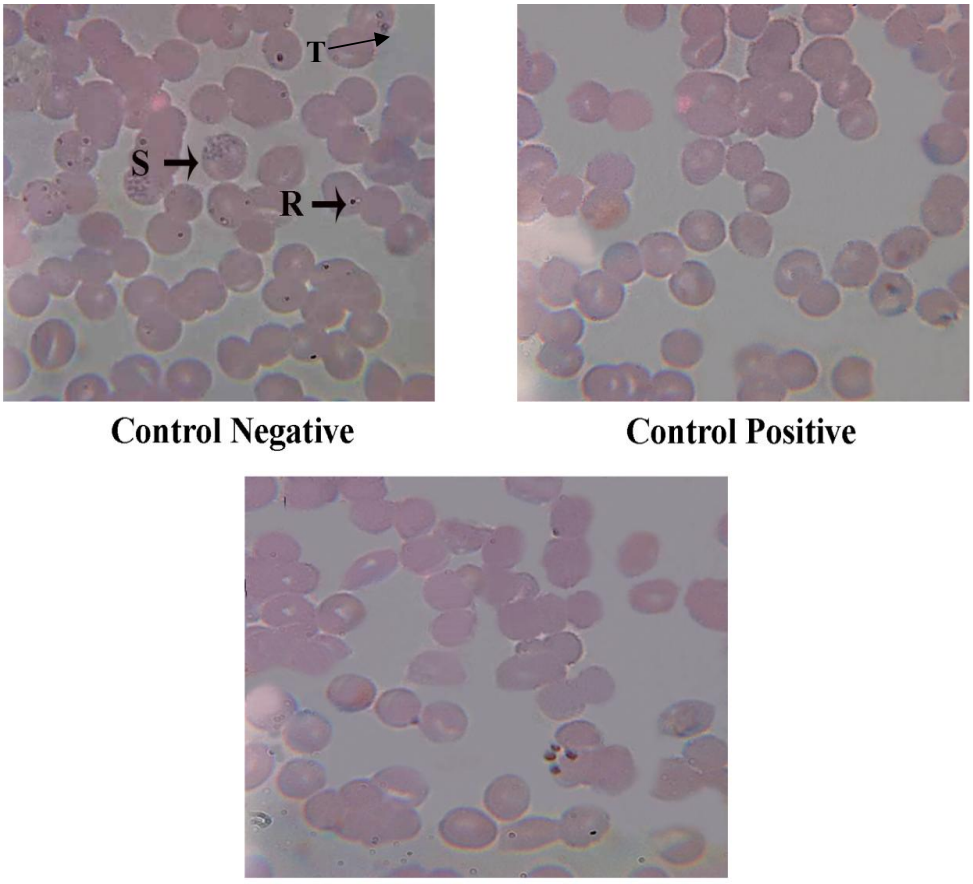


Plate 2. Microscopic observations after treatment with *C. viscosa* ethyl acetate leaf (CV EAL) extract against *P. falciparum* 3D7 strain at highest concentration (100 µg/ml) with Giemsa stain at 1000x magnification (R- ring stage, T- trophozoite, S- schizont)

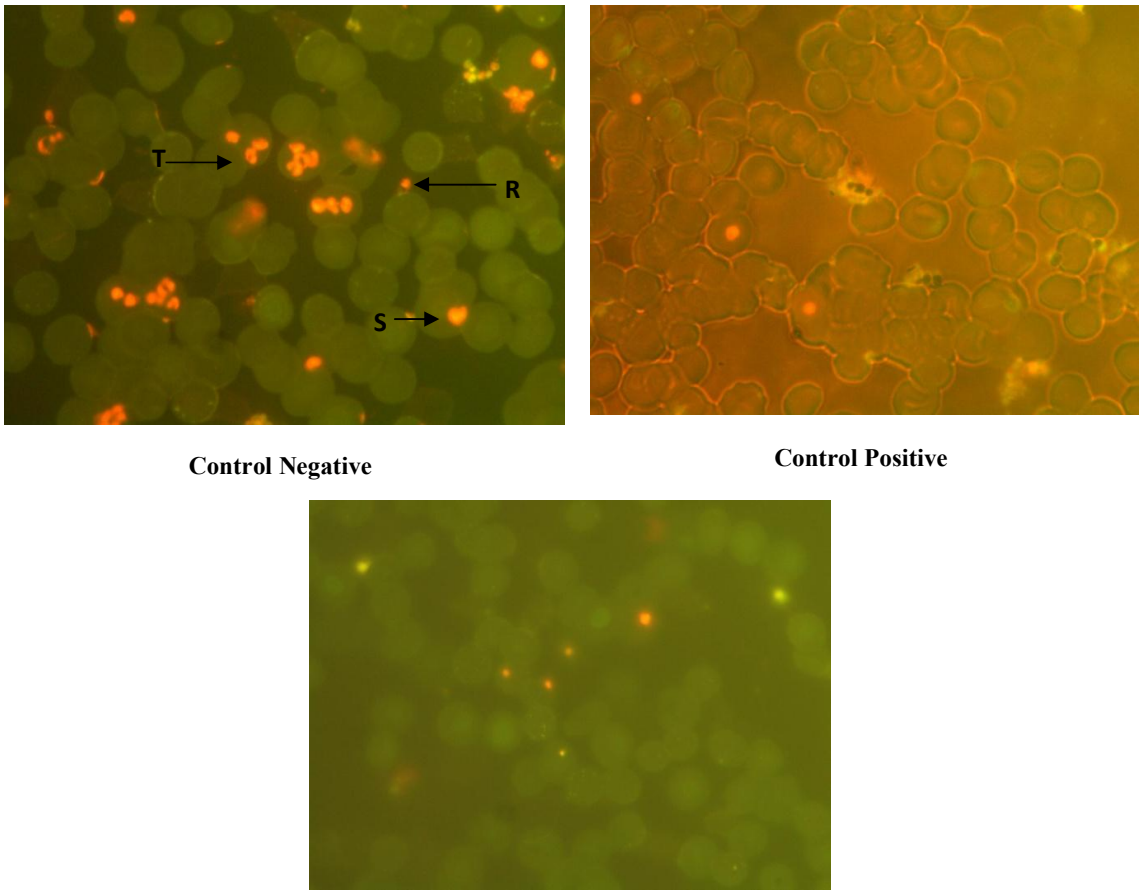


Plate 3. Microscopic observations after treatment with *C. viscosa* ethyl acetate leaf (CV EAL) extract against *P. falciparum* 3D7 strain at highest concentration (100 µg/ml) with Acridine Orange at 1000x magnification (R- ring stage, T- trophozoite, S- schizont)

Table 6. Packed cell volume during 4-day suppressive test after treatment with ethyl acetate leaf extract of *Cleome viscosa* against *Plasmodium berghei* infected BALB/c experimental mice

Test substance	Dose (mg/kg/day)	% PCV on Day 0 (M±SD)	% PCV on Day 4 (M±SD, *p*-value)	% Reduction
Ethyl acetate extract	200	44.72±2.62	42.62±3.84, 1.00^{NS}	4.69
	400	45.27±1.07	45.73±1.04, 0.29^{NS}	-1.01
	600	43.00±1.60	43.57±1.41, 0.71^{NS}	-1.33
	800	42.55±2.09	43.63±1.86, 0.70^{NS}	-2.54
	1000	44.38±3.85	45.12±1.72, 0.39^{NS}	-1.87
Vehicle (-)	1 ml	44.38±3.85	42.60±3.61	4.01
Chloroquine (+)	5	43.14±2.15	44.67±2.33	-3.55

The values are represented as mean of 3 values ± standard deviation and significant at $p < 0.05$ (compared with negative control), NS- not significant, (-) Negative control, (+) Positive control

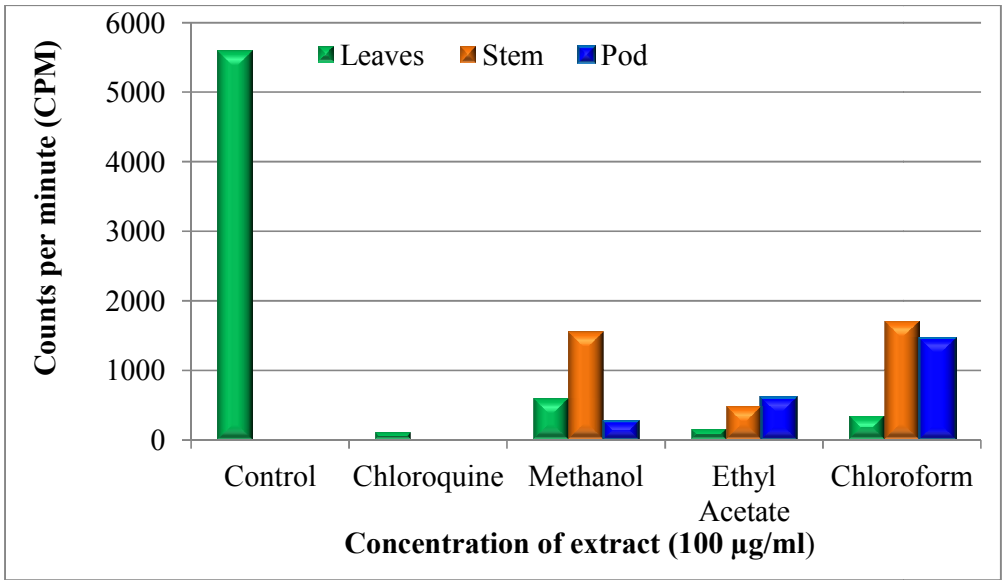
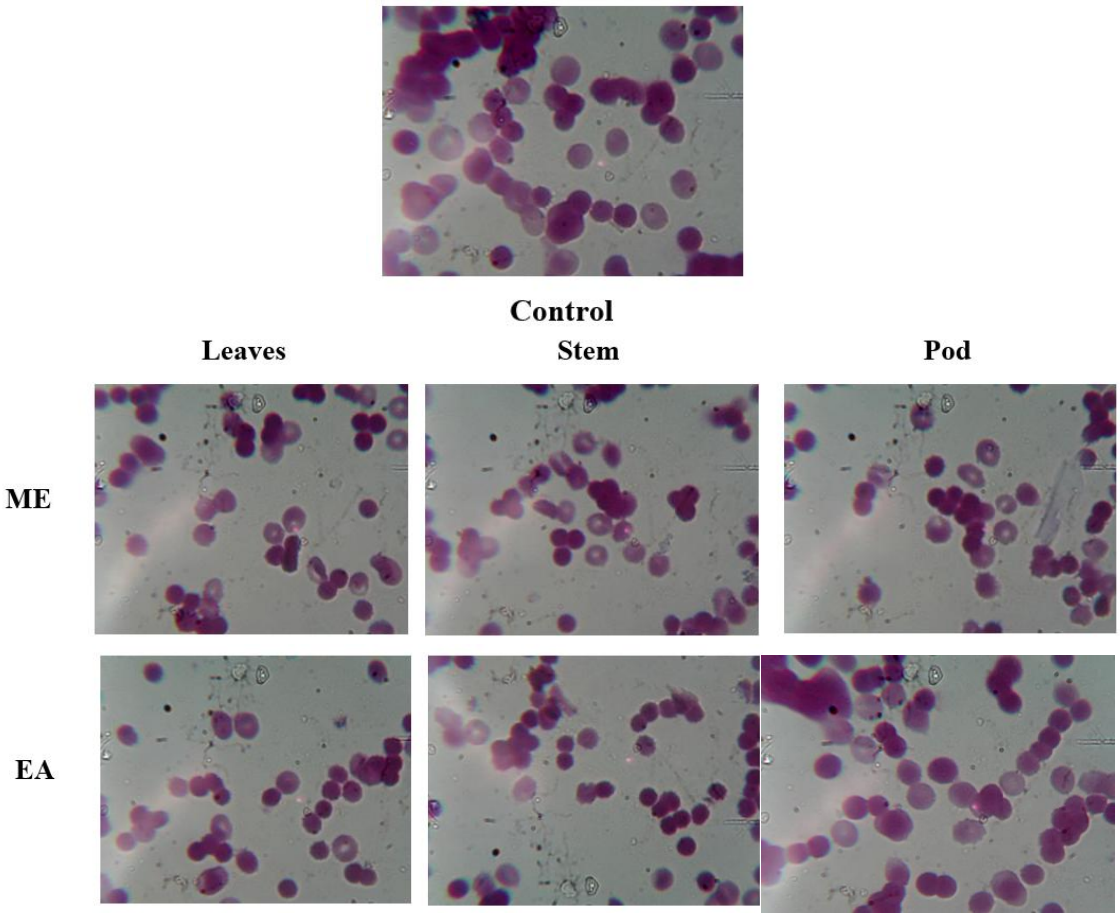


Fig. 1. Comparison of CPM among control (untreated), chloroquine treated and plant extract treated against *P. falciparum* 3D7 strain (Ethyl acetate leaf extract of *C. viscosa* shown low CPM similar to chloroquine CPM)



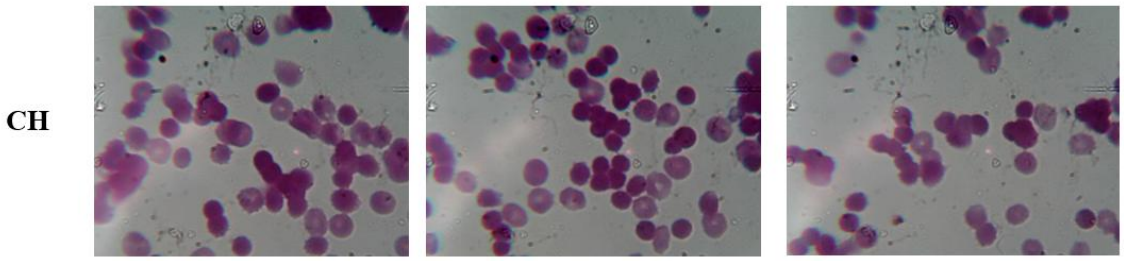


Plate 4. Screening for chemical injury to erythrocytes after treatment with different crude extracts of *Cleome viscosa* at higher concentration of 100 µg/ml (ME- methanol, EA- ethyl acetate CH- chloroform)

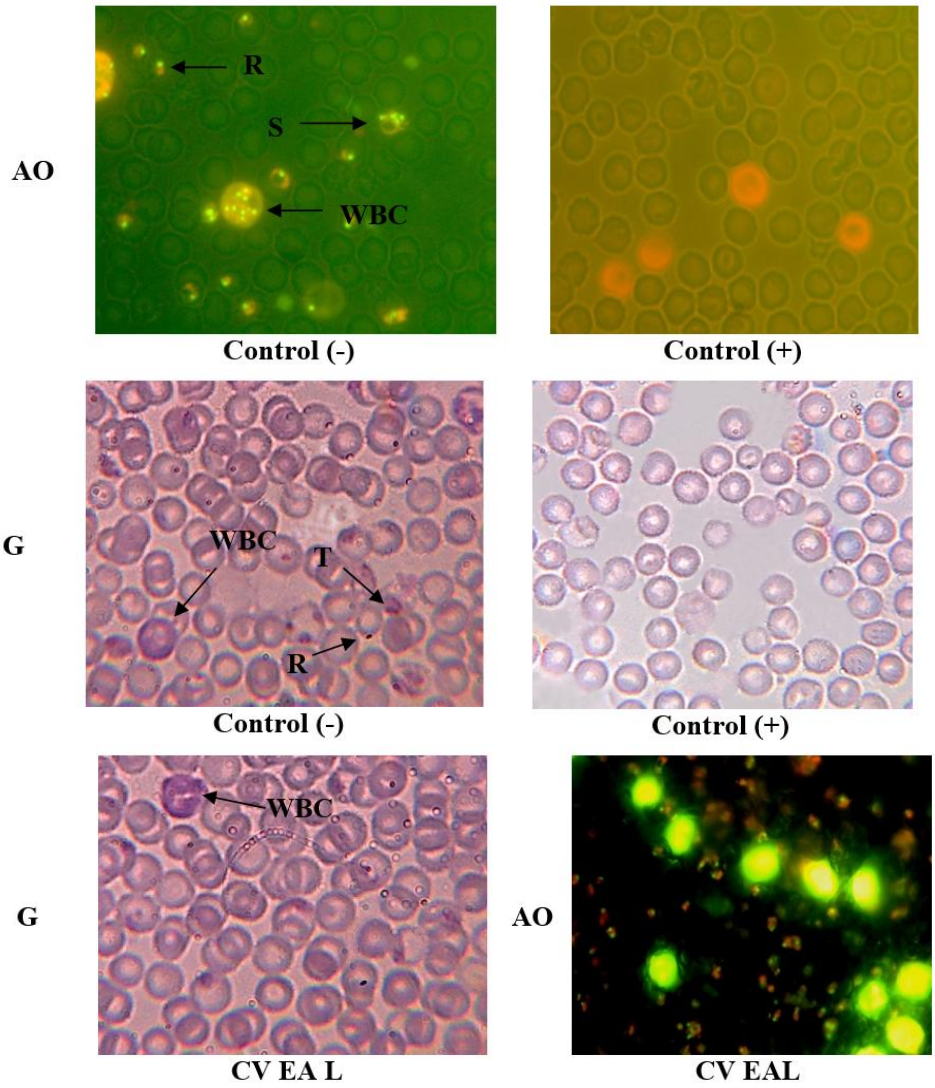


Plate 5. Microscopic observations after treatment with *Cleome viscosa* ethyl acetate leaf (CV EA L) extracts against *Plasmodium berghei* strain at highest concentration (1000 mg/kg b.wt./day) with Acridine Orange (AO) and Giemsa (G) stain at 1000x magnification (R- ring stage, T- trophozoite, S- schizont, WBC- white blood cell)

In the present study, methanol, ethyl acetate and chloroform crude extracts of leaves, stem and pod of *C. viscosa* were evaluated for their antimalarial potencies. The IC_{50} values of the tested plant extracts against *P. falciparum* are listed in Table 2. The *in vitro* antiplasmodial activity of biological active substances were categorized into four groups based on IC_{50} value, i.e. <5 µg/ml --- very active, 5--50 µg/ml --- active, 50--100 µg/ml --- weakly active, >100 µg/ml – inactive [14].

Based on the above categorization, the IC_{50} values of the methanol, ethyl acetate, and chloroform extracts of leaves, stem and pods of *C. viscosa* varied from 7 – 38.83 µg/ml against CQ-sensitive *P. falciparum* strain. The methanol extracts of leaves (37.10 µg/ml); ethyl acetate extract of leaves (7 µg/ml), stem (24.33 µg/ml) and pod (38.83 µg/ml); chloroform extract of leaves (14.33 µg/ml) showed good antimalarial activity and were significant at $p < 0.05$. Among these extracts, the ethyl acetate extract of leaves showed an excellent antimalarial activity (IC_{50} = 7 µg/ml). The methanol extracts of stem, chloroform extracts of stem and pod showed less antimalarial activity with IC_{50} values >100 µg/ml. Moreover methanol extract of pod was not determined because of its negative effect on RBC.

The microscopic observation on inhibition of *P. falciparum* by ethyl acetate extract of leaves (100 µg/ml) of *C. viscosa* is shown in Plate 2 and Plate 3.The counts per minute (CPM) during the treatment of all the extracts of *C. viscosa* at the highest concentration (100 µg/ml) are represented in Fig. 1.

The *in vitro* cytotoxicity studies against THP-1 cell line were conducted for all the extracts. All the 9 extracts of *C. viscosa* showed IC_{50} values >20 µg/ml. An extract was classified as non-toxic when the IC_{50} value was >20 µg/ml. Based on the above, all the plant extracts were not harmful for *in vivo* studies. The selectivity indices indicated the low toxicity of tested extracts and safe for therapies (Table 3).

The microscopic observation of uninfected erythrocytes incubated with the extracts of *C. viscosa* and uninfected erythrocytes from the blank column of the 96-well plate almost all showed no morphological differences after 48 h of incubation (Plate 4). However, the methanol extract of pod ruptured RBC cells. Hence, this is the first report of *invitro* antiplasmodial activity of *C. viscosa* against CQ-sensitive *P. falciparum* 3D7 strain.

The results of the present study indicated that ethyl acetate extract of leaf of *C. viscosa* displayed very good activity against *P. berghei in vivo* in BALB/c experimental mice. During the study period, ethyl acetate extract of leaf caused a moderately low ($p < 0.05$) and dose-dependent decrease in parasite counts, unlike the chloroquine treated group while the control group showed a daily increase in parasitaemia.

During the early infection, oral administration of 200, 400, 600, 800 and 1000 mg/kg body weight/day concentration of extract caused chemo suppression of 5.84, 28.13, 46.73, 55.84 and 64.37 % respectively which was statistically significant at $p < 0.05$ as compared to negative control. The standard drug chloroquine (5 mg/kg b.wt./day) caused 97.56 % chemo suppression which was more significant when compared to the extract treated groups (Table 4). The highest concentration of extract used (1000 mg/kg b.wt./day) showed 64.37 % chemo suppression which was slightly similar to that of the standard drug chloroquine (5 mg/kg b.wt./day).

The comparative analysis indicated that ethyl acetate extract of leaf of *C. viscosa* showed statistically significant difference on 4-day parasitaemia at 200 mg/kg b.wt./day (33.2%), at 400 mg/kg b.wt./day (25.24%), at 600 mg/kg/day (18.78%) and at 800 mg/kg b.wt./day (15.57%) compared to the negative control. Low level parasitaemia was observed at highest dose (1000 mg/kg b.wt./day) of ethyl acetate extract of *C. viscosa* with 12.56% (Table 4) and statistically significant at $p < 0.05$.

The mean survival time (MST) of the chloroquine treated mice (positive control) was 15 days. The MST of infected mice (negative control) was 5.33 days. The MST of ethyl acetate leaf extract treated mice at 200, 400, 600, 800 and 1000 mg/kg b.wt./day was 6.33, 6.00, 8.67, 10.33 and 10.66 days respectively. This was significantly longer ($p < 0.05$) than the value of the negative control mice which survived only for 5.33 days but the effect was slightly lower than standard drug chloroquine treated mice (Table 4).

In 4-day suppressive test, all the doses of the extract showed a preventive effect in weight reduction and normalized the weight in infected mice at all dose levels compared to the negative control mice. The increase in body weight was not dose-dependent (Table 5).

The ethyl acetate leaf extract of *C. viscosa* exhibited protective activity against the reduction in packed cell volume (PCV) levels compared to the negative control but was not dose-dependent (Table 6). The Packed Cell Volume (PCV) in all the experimental groups dropped significantly following malaria infection for the 72 h and prior to treatment. However, both chloroquine and plant extract treated groups

subsequently recorded an increase in PCV after treatment. There was a significant (p <0.05) increase in the PCV in mice treated with 200-1000 mg/ kg b.wt/day compared to the control.

4. DISCUSSION

The present investigation was undertaken to evaluate the *in vitro* antiplasmodial activity of plant *C. viscosa* in different extracts such as methanol, ethyl acetate and chloroform from leaves, stem and pods along with cytotoxicity. Among the tested extracts, the ethyl acetate extract of leaf of *C. viscosa* (IC_{50} = 7 µg/ml) has shown better *in vitro* antiplasmodial activity against chloroquine (CQ)-sensitive *P. falciparum* (3D7) strain. Similarly Rasoanaivo *et al.* screened the extracts of Madagascan plants in search of antiplasmodial compounds [14].

Previously, Elufioye et al. [15] investigated *in vivo* antimalarial effect of methanolic extract of *C. viscosa* whole plant and fractions (n-hexane and ethyl acetate) against early, established andresidual malaria infections. The *in vivo* antimalarial effect against early infection, curative effectagainst established infection and prophylactic effect against residual infection were studied inchloroquine-sensitive *Plasmodium berghei* NK-65 strain infected mice. The results showed that themethanol extract of *C. viscosa* whole plant and partitioned fractions demonstrated significant(p <0.05) dose-dependent antimalarial activity in all the three antimalarial evaluation models thusjustifying the inclusion of this plant in recipes for treating malaria. Also *in vitro* and *in vivo* antimalarial activities of methanolic extract of stembark of *Pongamia pinnata* [16] and methanolic leaf extract of *Calotropis gigantea* [17] were studied and corroborates with the present findings.

It is interesting to note that the majority of the chloroform extracts did not show any activity. However, the ethyl acetate extracts showed better antiplasmodial activity out of the total extracts followed by methanol. In this study, some of the selected extracts did not display *in vitro* antiplasmodial activity in an applicable range. A possible clarification could be of factors such as chemo types, environmental parameters, harvesting and storage conditions that could collectively influence the plant metabolites prior to and following harvestings, which in turn would be reflected in the bioactivity [18].

The cytotoxic effect against THP-1 cell lines revealed that all the 9 extracts showed IC_{50}>100 g/ml. The cytotoxicity >20 g/ml is considered as non-toxic to animals which is safe for further studies. Based on the above, all the plant extracts were not harmful and are safe for therapeutic studies. The selectivity index (SI) of most of the extracts indicates the safety for therapy. Those that showed high SI (>10) should offer the potential for safer therapy [19,20].

In addition, none of the test extracts of experimental plant have shown any of the chemical injury to the erythrocytic membrane throughout the experimentation except the methanolic extract of pod. Commonly, the erythrocytic membrane is a fragile structure that can be significantly altered by drug interactions. The mechanical stability of the erythrocytic membrane is an excellent indicator of *in vitro* studies for cytotoxicity screening because of its structural dynamics favoring interactions with drugs and this signifies the possible use of these extracts as antiplasmodial drugs in future. The mechanism of action might be due to the inhibition of haemozoin biocrystallization by the alkaloids and inhibition of protein synthesis by triterpenoids [21].

As ethyl acetate extract of leaves from *C. viscosa* has shown good *in vitro* antimalarial activity, it has been screened for *in vivo* antimalarial activity against *P. berghei* in experimental mice by following 4-day suppressive test. So *in vivo* antiplasmodial activity can be classified as moderate, good and very good if an extract demonstrated percentage of parasitaemia suppression equal to or greater than 50% at a dose of 500, 250 and 100 mg/kg body weight/day respectively [22]. Based on this classification, the crude extract of the studied plant has shown good antiplasmodial activity.

The study revealed that, the ethyl acetate extract of leaf of *C. viscosa* has shown a significant (p <0.05) and dose-dependent chemo suppression in the test, low parasitaemia levels and more mean survival time (MST). Drugs lead to decreased parasitaemia and subsequent recovery of symptomatic malaria. They also reduce parasitaemia through different ways like reducing parasite nutrient intake, interfering with parasite metabolic pathways like haeme metabolic pathway which is involved in the metabolism of iron [23]. Drugs also negatively influence the parasite reproduction and growth [24].

The plant extract reduced the level of parasitaemia and increased the mice survival time. Chloroquine had a good chemo suppression of 100% as determined on post-infection and a 100% survival rate post-infection. This observation confirms the earlier work by Musila [25] who reported antiplasmodial activity of the *digitata*, *Canthium glaucum,Launaea cornuta* and aqueous and organic extracts of *Adansonia Zanthoxylum chalybeum.* Among these, he concluded

that, the aqueous extracts of *Adansonia digitata* were able to reduce the number of *Plasmodium* parasites in mice.

Our present observation also supports earlier work by Chandel [26], who reported that the ethanolic extract of the leaves of *Ajuga bracteosa* were able to reduce the number of *Plasmodium* parasites in mice. Also, Ogbuehi et al. [27] reported the suppressive, repository and curative activity of the methanolic root extracts of *Anthocleista noblis, Nauclea latifolia* and *Napoleona imperialis* from south-east medicinal plants from Nigeria. All the plant extracts promisingly reduced the parasitaemia. Anosa et al. [28] studied *in vivo* antimalarial activity of ethanolic stem bark extracts of *Enantia polycarpa* in mice infected with *P. berghei*. The extracts exhibited promising activity against both the early and established infection and achieved 75.8% and 72% chemo suppression and increased the MST after administration which is in correlation with the present study.

Anemia, body weight loss and body temperature reduction are the common symptoms of malaria infected mice [22]. Thus, an ideal antimalarial agents obtained from plants are expected to prevent body weight loss in infected mice due to the rise in parasitaemia. Despite the fact that the increase in weight was not consistent with increase in dose, the tested crude extract significantly prevented weight loss associated with the decrease in parasitaemia level in suppressive test. The preventive effect of extract might be due the presence of saponins, flavonoids, glycosides and phenolic compounds found in the crude extract [29].

The PCV was measured to evaluate the efficiency of the ethyl acetate extract of leaves of *C. viscosa* in preventing haemolysis due to escalating parasitaemia level. Constant decrease in the packed cell volume observed in the untreated group of mice could be as a result of increased level of parasitaemia resulting in decreased red blood cells and hemoglobin. The fundamental cause of anaemia incorporates the following mechanisms: the clearance and/or destruction of infected RBCs, the clearance of uninfected RBCs, and erythropoietic suppression and dyserythropoiesis. Each of these mechanisms has been associated with anaemia in both human and mouse malaria [30].

5. CONCLUSION

The present work reveals that, out of the nine extracts of the studied plant *C. viscosa*, the ethyl acetate leaf extract found to have potent antimalarial activity against *P. falciparumin vitro* and against *P. berghei in vivo*. Moreover, these plant extracts exhibited no toxicity to THP-1 cell line. Thus, the present work provides the scope of using these compounds for further therapeutic studies for new antimalarial drug formulations. More research is needed to identify and characterize the potent molecules that suppress the malaria parasite for development of new drug therapies in view of growing resistance to malaria.

ETHICAL APPROVAL

Animal experiments were designated and approved with Ref. No. ANUCPS/IAEC/AH/P18/2016 by Institutional Animal Ethics Committee (IAEC) of ANU College of Pharmacy, Acharya Nagarjuna University, Guntur, Andhra Pradesh, India.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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