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Cysteine Protease Inhibitors from *Calotropis procera* with Antiplasmodial Potential in Mice

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Authors' contributions

This work was carried out in collaboration between all authors. Author AA conceived, performed the experiment and completed the data analysis, interpretations and the final completion of the manuscript. Authors IAU, SI, EO and AYK assisted in the design of the experiment and preparation of the manuscript.

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ABSTRACT

Inhibitory activity of crude extract of cysteine protease inhibitors from *Calotropis procera* against *Plasmodium berghei* infected mice was investigated. Various extracts obtained from the leaves, roots, flowers, latex and stem bark of *Calotropis procera* using different extraction media that included Sodium Chloride, Sodium Hydroxide, Hydrochloric acid, Sodium phosphate buffer and distilled water were used to evaluate for Cysteine Protease inhibitory activity against Papain enzyme. Sodium phosphate buffer extract of the latex of *Calotropis procera* with the highest Protease inhibitor activity was concentrated by cold acetone precipitation, freeze-dried and used for further studies. Subchronic toxicity study of the extract was carried out in a mouse model. Inhibitory effect of the extract on *Plasmodium berghei* Cysteine protease and *in vivo* studies on infected mice was investigated using incremental doses of 20, 30, and 50 mg/kg body weight/day. The crude extract exhibited inhibitory activity against Cysteine protease with an IC_{50} value of 25.50 µg/ml. The

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highest percentage parasite suppression of 48% was obtained for the group treated with 50 mg/kg bodyweight/day of the extract. Subchronic toxicity study using the effective dose of the extract at 50 mg/kg body weight/day showed a significant elevation in body weight of the animals and Alanine transaminase activity. A dose-dependent relationship was observed in the elevation of total protein and glucose concentrations in treated animals, but there were no significant changes in percentage PCV, triacylglycerol concentration, Aspartate transaminase and Alkaline Phosphatase activities. The result of this study shows that crude extract obtained from Latex of *Calotropis procera* is active against plasmodium Cysteine Protease in rodent malaria model and could be considered as a potential source for antimalarial drug development.

Keywords: Cysteine protease; inhibition; Plasmodium berghei; Calotropis procera.

1. INTRODUCTION

Malaria is a major cause of morbidity and mortality and it is estimated that more than half of the World population were at risk of malaria with an estimated 0.7-1 million deaths per year and over 97 countries are malaria-endemic [1,2]. Evolution of resistance to most affordable drugs such as chloroquine and gradual decline in the efficacy of artemisinin based combination therapies(ACTs) in *Plasmodium* species [3], with no effective vaccine in sight and resistance of vector to insecticides, necessitate the need for novel entities, ideally directed against new targets such as malarial cysteine proteases [4,5]. The life cycle of malaria parasite exhibits two stages: Exo-erythrocytic and erythrocytic life cycles. The erythrocytes life cycle was responsible for all clinical manifestations and it begins when parasites (merozoites) invade erythrocytes and develop to larger, more metabolically active form (trophozoites) followed by multinucleated schizont stages which bust out of red blood cells and reinvade the erythrocytes [4,6]. Plasmodium Cysteine protease is required for the invasion, rupture of erythrocytes and subsequent degradation of haemoglobin from the host [5]. The amino acid released from haemoglobin degradation is used for the synthesis of parasite protein and hence survival of this parasite inside the host organism [7,8,9]. Inhibition of haemoglobin degradation offers a valid target for development of novel chemotherapeutic agents.

Cysteine protease inhibitor (CPI) in plants functions as storage proteins, regulators of endogenous proteolysis, defence against insect and pathogens attack [10]. Protease inhibitors from plants have been reported to inhibit the growth of a variety of pathogenic bacterial and fungal strains [11,12,13]. Cysteine protease inhibitor have been identified and studied in many plant sources such as rice [14], maize [15] soybean, cowpea potato, Chinese cabbage and carrot [16]. Efficacy of synthetic peptides CPI and non-protein CPI from plants in treatment of diseases caused by *Trypanosoma cruzi*, *Leishmania major,* viruses and cancer treatment has been established [9,17-19].

Calotropis procera, a plant of *Asclepiadaceae* family are widely distributed in West Africa, has received special attention because of its use in folk medicine [20]. Previous reports have confirmed that this plant possesses various pharmacological properties that may be used for the treatment of inflammatory disorders such as arthritis, cancer, sepsis [6,21-23] and also antiplasmodial activity [24]. Despite numerous reports on the protein content and presence of proteases [25-28] and chemotherapeutic proteins [15], to the best of our knowledge information concerning the identification of protease inhibitor in this plant is lacking. Thus, the plant was chosen based on its high cysteine proteases content and that it may contain inhibitor protein(s) regulating the activity of endogenous proteolysis. Understanding of the inhibitory mechanism they employed may offer prospects for treatment of the various disorders resulting from defective control of proteolytic processes. This research was designed to evaluate the toxicity and antiplasmodial properties of the cysteine protease inhibitor crude extract of *Calotropis procera,* in order to come up with a safe efficacious, reliable and sustainable alternative to be used in malaria chemotherapy.

2. MATERIALS AND METHODS

Chemicals: Azocasein, Benzyl-arginine para nitroanilide (BApNA) and Papain were from Sigma. All other reagents used were of analytical grade.

Plant Material: The plant was collected and identified in the Department of Biological

Sciences, Ahmadu Bello University, Zaria, Kaduna State; with a voucher number of 900219. The leaves, latex, flowers, roots, and stem bark were used for the study.

Animals: Swiss albino mice of either sex, aged 4 - 6 weeks old and weighing 20 - 25 g each were used as experimental animals. The mice was conveniently housed, free access to commercial food pellets and water and natural 12 h daylight/night cycles. Experiments were conducted in strict compliance with internationally accepted principles for laboratory animal use and care as contained in the [29].

Parasite: *Plasmodium berghei* NK65 chloroquine sensitive strain was obtained from National Institute of Pharmaceutical Research and Development (NIPRD) Abuja, Nigeria and maintained in the laboratory by serial passage in mice.

2.1 Extraction of Protease Inhibitor

Twenty-five (25 g) of fresh leaves, latex, root, flowers and stem bark of *Calotropis procera* were blended with 100 ml each of sodium chloride 15% (w/v), sodium hydroxide 0.2% (w/v), 0.05 M hydrochloric acid, 0.1 M phosphate buffer (pH 7) and distilled water as described by [30]. The clear supernatant obtained after homogenization and centrifugation (at 10,000×g, 15 min, and 4°C) was assayed for protease inhibitory activity and protein content.

2.2 Acetone Precipitation

Four (4) volumes of cold acetone were added with stirring to the crude extract. After 20 minutes of stirring, the precipitate was collected by suction filtration and washed with cold acetone. The precipitate was air dried and stored at 4°C. Acetone-derived powder was suspended in 50mM phosphate buffer, pH 7.6. This was designated as the crude acetone extract [31].

2.3 Protease Inhibitor Assay

Protease inhibitor activity was assayed according to the method described in [32]. An aliquot of 500 µl of the extract was pre-incubated for 10 min at 37°C with 500 µl papain prepared in 100 mM phosphate buffer, pH 6.8, with 0.3 mM EDTA and 2 mM cysteine - HCl. The assay was initiated by the addition of the 1 ml substrate solution (0.5% (w/v) azocasein), incubated for 20 min at 37°C, and 2 ml of trichloroacetic acid (TCA, 20% w/v) was added at the end of the experiment to terminate the reaction. After 20 min at room temperature, the mixture was centrifuged at 10,000×g for 10 min and the absorbance of the supernatant was measured at 410 nm. BApNA assays was performed by adding 290 µL of 50 mM Tris–HCl (pH 7.6) and 200 µL of 1.25 mM BApNA solution to the previously pre-incubated cysteine protease enzyme (papain) and crude inhibitor extract for 10 minutes at 25°C. After 30 min at 37°C, the reaction was stopped by adding 150 µL of acetic acid (30%). The absorbance of the resulting color was measured at 405 nm. Percentage inhibitory activity was then calculated.

$$\% \text{ inhibitory activity} = A_C - A_I / A_C \times 100.$$

A_C=Absorbance without inhibitor

A_I= Absorbance with inhibitor

2.4 Protein Estimation

Protein concentration was determined according to the method of Bradford, using bovine serum albumin (BSA) as a standard [33].

2.5 LD_{50} Determination and Subchronic Toxicity Test

LD_{50} was obtained using the method described by [34]. Subchronic toxicity was determined according to the method [34]. Mice (20) were divided into four groups of five mice each, the first group served as control, given only 20 ml/kg/bw phosphate buffer saline (PBS), while the remaining 3 groups received 20 mg/kg/bw, 30 mg/kg/bw and 50 mg/kg/bw respectively of crude extracts intraperitoneally (i.p) for 21 days. Weights of mice and packed cell volume (PCV) of each group were compared before and after a 21 days period. Serum glucose was assayed with Randox glucose diagnostic kit based on glucose oxidase method. Total proteins were evaluated with method of the, [33]. Serum triglycerides measured by method ([35]. Aspartate aminotransferase (AST) and Alanine aminotransferase (ALT) were valuated using the method of Reitman and Frankel as described by [36]. Alkaline phosphatase (ALP) was assayed based on the method of Kind and King as described by [37].

2.6 Inoculation of Mice and Parasite Count

A donor mouse with a parasitemia level of 20% was sacrificed and blood was drawn into a

Heparinised syringe and diluted with phosphate buffered saline. Infection was initiated by injecting 0.2 ml of the parasite preparation from a donor mouse to healthy mice via the intraperitoneal route. Parasitemia was monitored by microscopic Giemsa-stained thin blood smears. The number of parasitized erythrocytes in about 10-50 fields were counted twice and the average was computed to give the parasitemia of each mouse.

Percentage parasitemia was calculated as:

% Parasitiemia =
(Number of parasitized RBC/ Total number of RBC) x 100

2.7 Preparation of Parasite Cysteine Protease Extracts

Blood obtained from infected mice at the peak of parasitemia was incubated with 0.01% saponin in 100 mM phosphate buffer saline (PBS) pH 6.0 at 37°C for 10 min to lyse red blood cell (RBC) and was washed four times in ice-cold PBS (10,000 x g,5min). The released parasite was lysed by addition of 0.5% Triton X-100 in Tris-buffer saline followed by incubation at 4°C for 90 min as described by [38]. The supernatant was collected after an initial centrifugation at 10,000 x g for 30 min at 4°C and the pellets from preparation was re-extracted twice. The extracts were then pooled together as crude enzyme sample.

2.8 *In vivo* Antiplasmodial Suppressive Test of Crude Extract

The extract from the latex of the plant which exhibited the highest specific papain inhibition was used for *in vivo* 4-day antiplasmodial suppressive test. Mice were maintained and bred at the animal facility of the University. Thirty- five mice were grouped into seven groups (1to7). They were infected with diluted parasitized erythrocytes from a donor mouse with 0.2 ml (10^8 parasitized erythrocytes/ml) each intraperitoneally (i.p). Groups 1 and 2 were not infected but treated with phosphate buffer saline (PBS) and crude extract respectively. Groups 3 and 4 were infected and treated with (PBS) and 5 mg/kg/b.w Artesunate serving as Negative and positive control respectively. Groups 5, 6 and 7 were infected and treated with 20 mg/kg/b.w,30 mg/kg/b.w and 50 mg/kg/b.w. of the extract. Treatment of mice commenced 2 hours after infection and repeated daily for four days consecutively (i.e 2, 24, 48, and 72 h after inoculation). Mean Body weight, packed cell volume, percentage parasitemia and percentage survival were determined for each experimental groups.

%Parasite inhibition of the extract=
(Parasitemia in negative control - Parasitemia intreatment group) x100/ Parasitemia in negative control group.

2.9 Statistical Analysis

Data obtained were analyzed using One-way Analysis of Variance (ANOVA). Variant 152 means were separated using Duncans Multiple range Pos hoc Test. Results were 153 presented as Mean ± Standard deviation. Differences in survival curves between groups were analysed using the Log-rank (Mantel-Cox) test.

3. RESULTS

Crude cysteine protease inhibitor (CPI) from the different part of *Calotropis procera* was extracted using various aqueous solvents. Among all the solvents used, phosphate buffer extract had the highest specific inhibitory activity for crude CPI from the latex of *Calotropis procera* (Fig. 1) and this was precipitated using cold acetone, followed by its evaluation toxicity test and *in vivo* antimalarial activity.

The crude CPI extract from latex showed inhibitory activity against Cysteine protease of *Plasmodium berghei* with the IC_{50} value of 25.50 µg/ml (Fig. 2). Median lethal dose (LD_{50}) of the crude CPI was 490 mg/kg body weigh T (Table 1).

Subchronic toxicity effect of crude CPI extract on mean body weight and packed cell volume (PCV) showed no significant difference ($p<0.05$) in both parameters in respect of treatment groups compared to control. However, mice treated with 50 mg/kg crude CPI showed a significance different ($p<0.05$) in percentage changes in mean body weight and packed cell volume, before and posttreatment respectively compare to placebo control (Figs. 3 and 4).

Some biochemical parameters in mice administered the crude CPI extract showed Alanine transaminase activity, Total protein and glucose concentrations to be elevated as

dosage concentration increased (Table 2), but no significant changes ($p<0.05$) were observed in PCV (Fig. 4), triacylglycerol concentration, Aspartate transaminase, and Alkaline Phosphatase activities compared to control (Table 2).

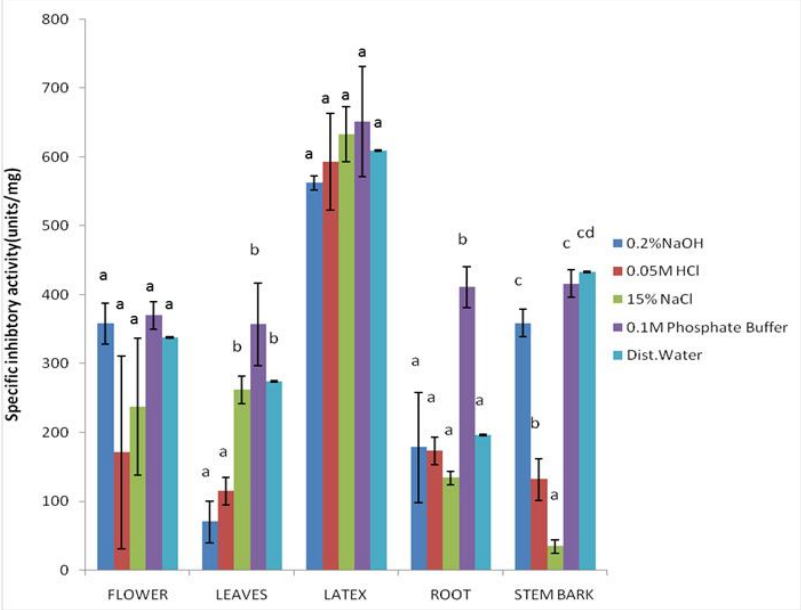

Fig. 1. Specific protease inhibitory activity *Calotropis procera* parts with different solvents
Papain inhibitory activity was quantified by incubating papain and plant extract with 0.5% Azocasein. Values are Mean ± SD (n=3)

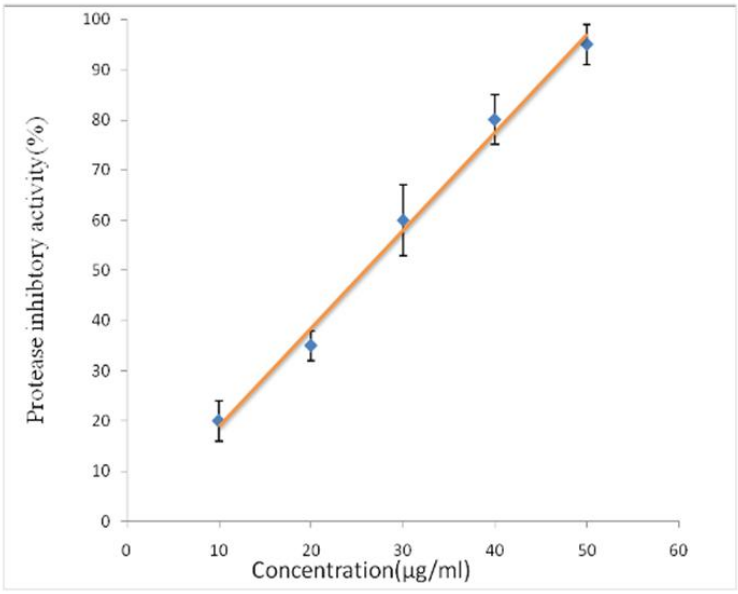

Fig. 2. *Plasmodium berghei* protease inhibtory activity of the acetone precipitated latex of *Calotropis procera*
Inhibitory activity was quantified by preincubating crude parasite lysate extract with plant acetone extracts and 1.25 mM BAPNA use as substrate. IC_{50} was calculated to be 25.5 µg/ml; Values are Mean ± SD (n=3)

Table 1. Determination of median lethal dose (LD_{50})

Plant sample	Experiment	Dose (mg/kg)	Death	
			After 24 hrs	LD_{50}(mg/kg/b.w)
	Phase1	10	0/3	
		100	0/3	
Calotropis procera		1000	3/3	490.00
(Latex)	Phase2	200	0/3	
		400	0/3	
		600	2/3	

The LD50 value was determined by calculating the geometric mean of the animal survived.
LD50 = √ (Highest non lethal dose) × (Lowest lethal dose)

Table 2. Effect of biochemical parameters on mice treated with crude CPI extract from *calotropis procera* latex

	Glucose (mg/dl)	Protein (mg/dl)	Triacylglycerol (mg/dl)	AST(U/L)	ALT(U/L)	ALP(U/L)
PBS (control)	95.73±0.42^{a}	4.86±0.09^{a}	150.88±2.34^{a}	36.56±0.47^{a}	39.24±0.39^{a}	129.88±0.63^{a}
20 mg/ kg/b.w	93.28±0.42^{a}	5.44±0.22^{b}	145.54±1.25^{ab}	36.40±0.80^{a}	40.64±0.71^{ab}	128.94±0.74^{a}
30 mg/ kg/b.w	103.02±2.00^{b}	4.74±0.28^{a}	140.66±0.67^{b}	37.32±.94^{a}	38.50±0.31^{a}	128.04±0.34^{ab}
50 mg/ kg/b.w	108.04±1.07^{c}	5.52±0.08^{b}	148.12±2.69^{a}	37.64±1.44^{a}	42.82±1.43^{b}	126.70±0.73^{b}

Values are mean ± SD (n=5)
Values with different superscript letters within the same vertical column are significantly different (P < 0.05) tested by Duncan Multiple Range Test

Crude CPI extract produced a dose-dependent 4-day chemo suppressive effect (Fig. 5) at the various doses used in the study corresponding to suppression of 39.42%, 47.67%, 48.00% by 20 mg/kg, 30 mg/kg and 50 mg/kg crude CPI respectively (Table 3). All infected mice treated with 20, 30 and 50 mg/kg extract survived much longer than untreated group (Fig. 6), infected but untreated group animals were all dead by day 11 of treatment. Only mice treated with 50mg/kg survived for 25 days of treatment period with parasites persisting in circulation; the group treated with the standard drug had parasites cleared completely on the 5th day of treatment and the group survived more than 25 days. This indicates a direct relationship between dose of extract and survival time. The log-rank (Mantle-Cox) analysis showed that the survival curves are significantly different. Table 4 shows a significant increase in mean body weight and packed cell volume of *plasmodium berghei* infected treated groups compared to placebo control group.

4. DISCUSSION

Several parasites responsible for mammalian diseases are dependent on cysteine protease for various life-cycle functions. Inhibition of these proteases by natural peptide that will not have off target binding could be useful in the treatment of these parasitic diseases especially in *Plasmodium* infections [4,5].

Table 3. Four days suppressive test of crude CPIextracts

Calotropis procera (latex)		
	%Parasitaemia	%Suppression
Infected+PBS (negative control)	14.56±0.80	
Infected+artesunate 10 mg/ml (positive control)	1.82±0.34	87.50±0.24^{c}
Infected+20 mg/kg/b.wCPI	8.82±.86	39.42±0.42^{a}
Infected+30 mg/kg/b.w CPI	7.62±0.62	47.67±0.52^{b}
Infected+50 mg/kg/b.w CPI	7.57±0.35	48.00±.32^{b}

Values are Mean ± SD; Values with different superscript letters within the same column are significantly different (P < 0.05) tested by Duncan Multiple Range Test

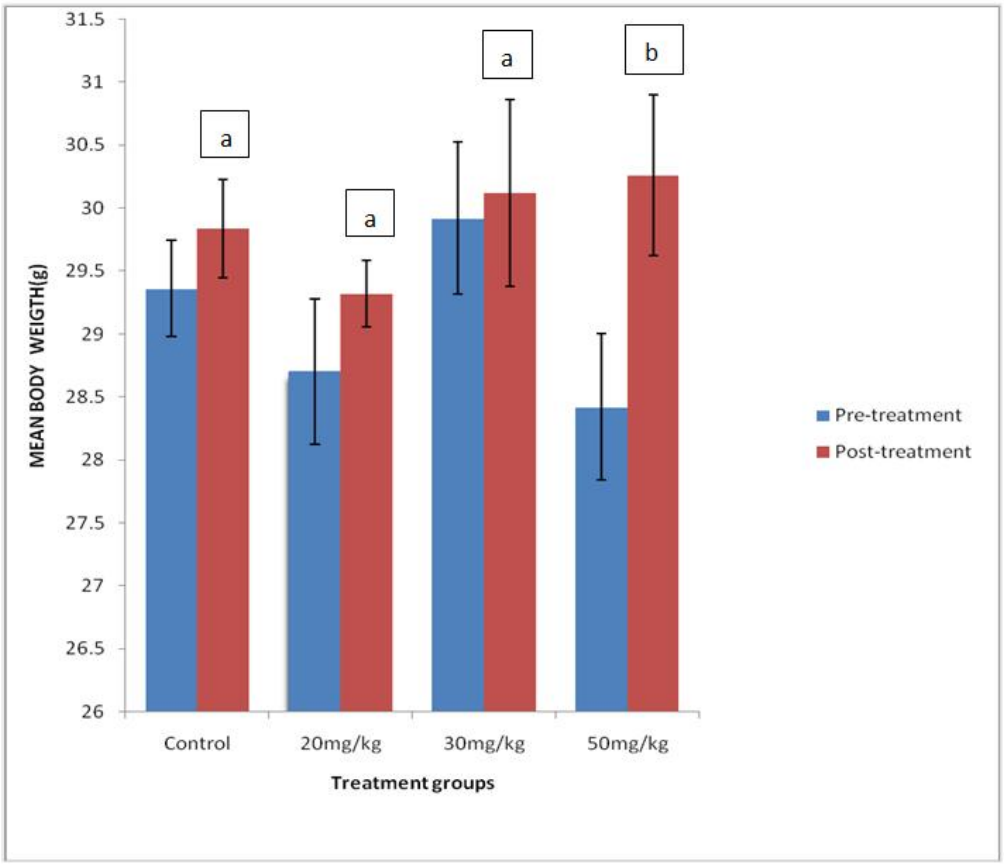

Fig. 3. Mean body weight of experimental mice treated with CPI from latex of *Calotropis procera* for 21 days

Values are Mean ± SD (n=5)

Different letters within the same parts of plant indicate significant different (<0.05) tested by Duncan Multiple Range Test

Table 4. Effect of crude CPI extract on the body weights of *P. berghe*i infected mice

	Body weight(g)		PVC (%)	
	Pre treatment	**Post treatment**	**Pre treatment**	**Post treatment**
Normal Control (PBS)	26.20±2.48	35.00±1.87^{a}	50.60±2.07	52.40±3.84^{b}
Inhibitor control (IC)	27.80±1.30	37.60±1.14^{a}	47.60±2.07	48.60±7.07^{ab}
Infected+PBS (negative control)	28.00±1.58	19.50±2.12^{b}	43.80±7.85	33.00±6.69^{a}
Infected+artesunate	24.20±1.92	33.40±2.88^{a}	49.20±1.92	50.00±2.19^{b}
Infected+20 mg/kg	27.00±1.58	32.40±4.77^{a}	46.00±3.16	46.40±4.63^{ab}
Infected+30 mg/kg	28.60±3.64	31.80±4.91^{a}	47.60±2.07	45.00±2.23^{b}
Infected+50 mg/kg	29.00±1.58	33.60±6.22^{a}	50.60±2.07	52.40±3.84^{b}

Values are Mean ± SD

Values with different superscript letters are significantly different (P < 0.05) tested by Duncan Multiple Range Test

Malaria is responsible for substantial morbidity and mortality in several nations, principally in tropical and subtropical regions [39]. Also, the drugs available for the cure of this desease are unsatisfactory because of their limited efficacy and increasing drug resistance. Thus, new, safer, and more efficacious drugs are urgently needed [3].

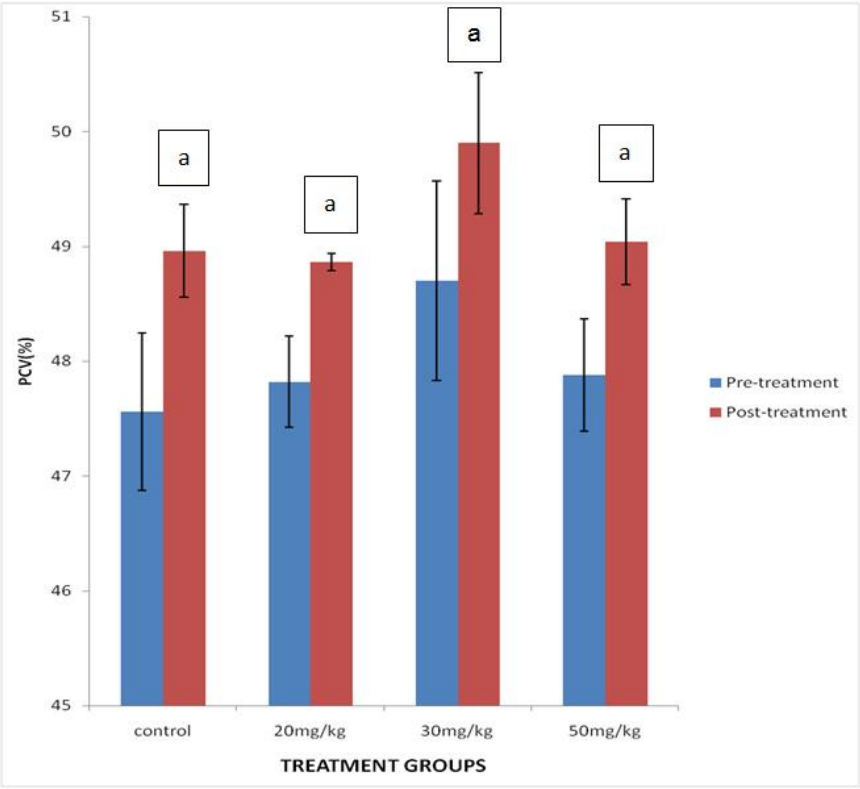

Fig. 4. Mean packed cell volume (PCV) of experimental mice treated with CPI from *Calotropis procera latex* for 21 days

Values are Mean ± SD;

Same letters indicate non significant different (<0.05) tested by Duncan Multiple Range Test

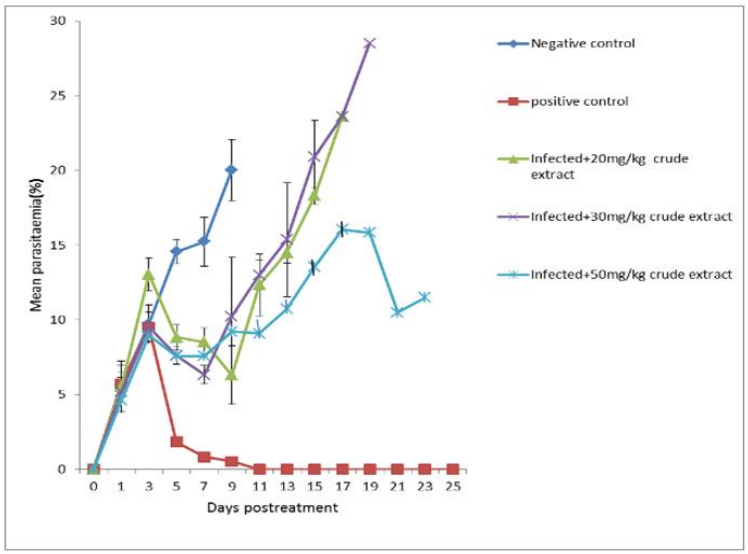

Fig. 5. Parasiteamia level of the experimental mice infected with *P. berghei* and treated with crude CPI from *Calotropis procera latex*

Values are Mean ± SD; CPI-Cysteine protease inhibitor

In this studies, higher specific cysteine protease inhibition was exhibited by phosphate buffer extract from the latex of *Calotropis procera* compared to other solvents, thus, phosphate buffer was selected as the potent extraction medium for maximal extraction of protease inhibitor from the latex without any loss in activity. This is based on the fact that reports on solubilization of therapeutic proteins using various solvent system showed that phosphate buffer extraction conferred stability to these agents(8). Saline extract from the latex of *C. procera* had anticancer activities [23]. Other researchers have used different extraction media such as 0.01 N NaOH [40], distilled water [22,41] and 0.1 M phosphate buffer, pH 7.5 [30].

The crude acetone extract from latex of *C. procera* inhibited plasmodium cysteine protease with the IC_{50} value of 25.50 µg/ml indicating that the crude extract bind to parasite protease at low concentration. The median lethal dosage (LD_{50}) of 490 mg/kg for this extract is classified as moderately toxic according to [42].

In the subchronic toxicity experiment, a gain in mean body weight of the mice in all the groups. This may be attributed to the normal growth of the mice with age and also, may be due to improved feed consumption and to the protein nature of the extract. Crude extracts did not produce any significant changes in packed cell volume (PCV) of the treated animals in all the doses used when compared to the control group. This suggests that the extract may have no toxicological effects on the hemopoietic system.

The Liver is the major organ involved in drug biotransformation. Levels of serum liver biomarker enzymes are assayed to evaluate any toxic effects on the liver [43]. Increases in AST levels are considered to be indicative of

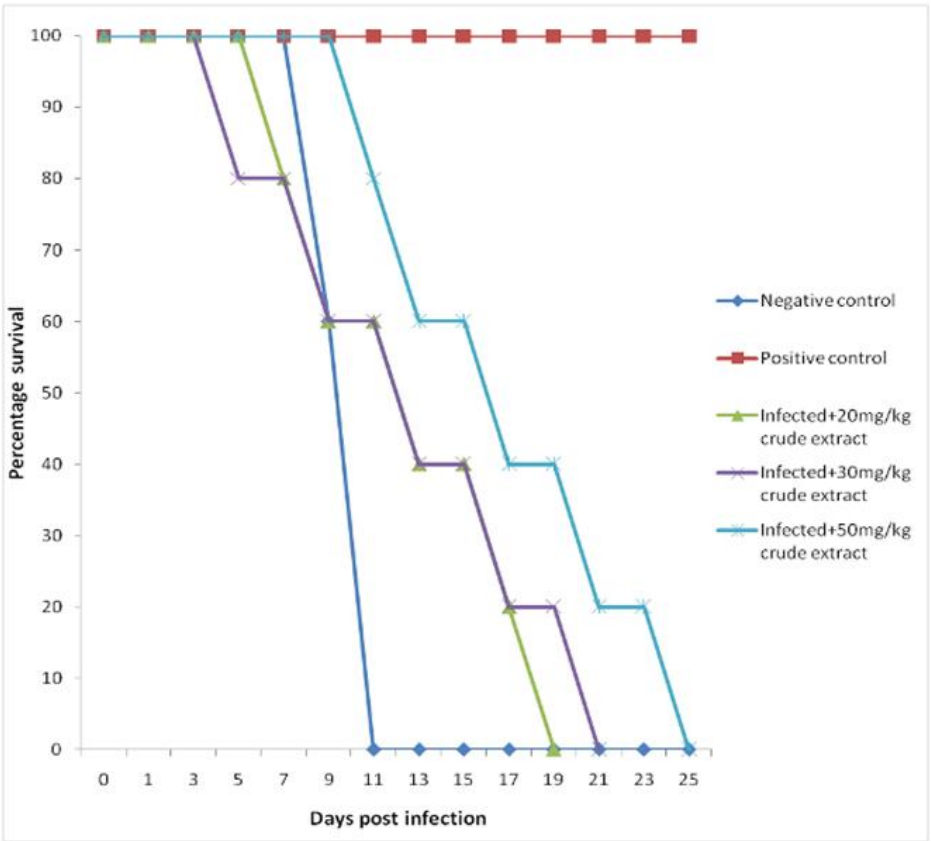

Fig. 6. Percentage survival of the experimental mice infected with *P. berghei* and treated with crude CPI from *Calotropis procera latex*

p<0.05, Log-rank (Mantel Cox) test (n = 5)

extensive tissue necrosis and hepatic cell damage. Increased ALT levels in the serum reflect hypertrophy and other conditions of the liver [44]. ALP is an enzyme in the cells lining the biliary ducts of the liver that has been used to diagnose biliary obstruction [45]. In this study, ALT levels increased only in the mice treated with 50 mg/kg, and the ALP and AST were not significantly different from the control group. However, the increase in ALT levels was modest and did not go above normal ranges in mice. A significant increase in the mean total protein level of the treated mice at the dose of 50 mg/kg may be attributed to protein nature of the extract.

The untreated mice had parasite count increased and a marked decrease in PCV decreased markedly until the death of the animal, which may be as a result of the destruction of red blood cells by the parasites leading to erythro-phagocytosis. Interestingly, our data also show that at death, the extract treated mice with 20 and 30 mg/kg had much higher parasitemias than control mice which died between 9 to 10 days after infection. Previous studies have shown that this strain of *P. berghei* causes a rodent form of cerebral malaria and leads to death at relatively low parasitemia [39]. In contrast, the extract-treated mice survived 7 to 14 days longer. A dose of 50 mg/kg/day crude CPI clearly delayed progression of parasitemia, as mice lived through the lethal infection, for almost 14 days more than the infected and untreated mice, and their parasitemias reached very high levels, signifying that extract treatment supported them to escape the early death from *P. berghei* infection. One possibility is that slower growth of the parasite in the presence of the extract inhibitor, is that it may be modulating the immune response of the host and thereby alters the outcome of the infection by preventing the cascade of events that leads to early death seen with *P. berghei* infection [39]. However, it is also possible that initial suppression of the parasite by extract and posttreatment rise may be due to the fact that crude extract is inhibiting Cysteine protease secreted by the mature parasite (trophozoite, schizont and merozoite), but had no effect on the ring stages of the parasite. The ring stage is known to produce little or none Cysteine protease enzyme and might be escaping the effect of inhibitor. The findings in this study indicate the preventive potential of the extract, since cysteine protease activity is involved in the invasion of RBC and degradation of immunoglobulins of the host. Several *in vitro* and *in vivo* studies using inhibitors such as peptidyl fluoromethyl ketones, vinyl sulfones, aldehydes and non-peptidic inhibitors impedes cysteine proteases activity of *Plasmodium* parasites and show the curative effect in mice infected with *Plasmodium berghei* parasite [5,19]. Similarly, Karthik *et al*. identified a serine protease inhibitor isolated from Marine Actinobacteria that cured *Plasmodium berghei* infected mice [4].

The survival curves for plant extract dosage and standard drug (artesunate) treated mice differ significantly from the control mice. In addition, the survival curves for plant extract at higher dosage and artesunate-treated mice were also significantly different from each other indicating a lower efficacy and safety of extract compare to standard drug.

Crude cysteine protease inhibitor from Latex of *C.procera* was active against *plasmodium berghei* Cysteine Protease, with considerable *in vivo* antiplasmodial activity in a mouse malaria model. The extract also exhibited low toxicity in the experimental animals. We are currently involved in the purification of this inhibitor from the plant using Hydrophobic chromatography.

5. CONCLUSION

Crude CPI extract of *C.procera* latex inhibited cysteine protease of *plasmodium berghei* invitro. CPI treatment has antiplasmodial potential which is effective in delaying parasitemia rise in mice and improved survivability caused by *P. berghei* NK65 infection. Toxicity studies of the extract indicate Mean lethal dose (LD_{50}) that is moderately safe to the experimental animals. The results obtained suggest that the CPI possess antiplasmodial activity and could be considered as a potential source for antimalarial drug development.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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