Research Article

Cysteine Protease Inhibitors from the Methanol Extract of the Root Bark of Securidaca longepedunculata with Antimalarial Potentials in Chloroquine-Resistant P. berghei Parasite

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Abstract. Malaria parasite resistance against Artemisinin-based Combination Therapy (ACT) in some parts of the world necessitates the search for antimalarial compounds or plant extracts with novel mode of action and active against the chloroquine-resistant strain of the parasite to serve as alternative to ACT. Cysteine protease inhibitors’ fraction of the methanol extract of the root bark of Securidaca longepedunculata (CPI) was investigated for antiplasmodial activity against chloroquine-resistant P. berghei-infected mice and its inhibitory effect on papain, P. berghei cysteine proteases and heme biocystallization were also evaluated. The methanol extract of the root bark was obtained by soxhlet extraction with 1,000 mL of 70% (v/v) methanol for 48 hours and concentrated to dryness at 45°C. CPI was obtained using PBS (pH 7) extraction followed by cold acetone precipitation. Peter’s four-day suppressive and Rane’s four-day curative test was employed to assess the antimalarial potentials of CPI. Data was analysed with one way ANOVA followed by Dunnett’s post hoc test, differences were considered significant at p ≤ 0.05. The suppressive effect of CPI was significant (p ≤ 0.05) at 34 and 23 mg/kg doses. Doses of 34, 23 and 11 mg/kg produced significant (p ≤ 0.05) dose-dependent curative effect. CPI inhibited the proteolytic activity of papain enzyme and P. berghei cysteine proteases in vitro with IC_{50} values of 20.1 and 5.6 μg/mL respectively. The present study showed that cysteine protease inhibitors fraction of the methanol extract of the root bark of Securidaca longepedunculata is a potential source of novel antimalarial agents that could target malaria parasite cysteine proteases.

Keywords: Securidaca longepedunculata; Cysteine protease inhibitors; Plasmodium berghei; Chloroquine-resistance.

1. Introduction

Malaria continues to be a major public threat worldwide especially in Africa; it is a protozoan disease that is caused by parasite of the protozoan genus Plasmodium. According to the latest report from the World Health Organization (WHO), malaria was responsible for the death of 445,000 people around the world in the year 2016, most of which occurred in Africa and mostly affected children under the age of five [1]. Five species of Plasmodium have been identified as the causative agent of the disease in humans; Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae, Plasmodium knowlesi and Plasmodium ovale [2].

Recent reports of resistant isolate of human malaria parasites against the Artemisinin-based Combination Therapy [3, 4] which are the current first-line drugs for the management of the disease necessitate the search for novel antimalarial drugs. The two major classes of antimalarial drugs; artemisinins and quinines were discovered from plant sources [5, 6], hence the need to explore medicinal plant for novel antimalarial compounds. Inhibition of malaria parasite
cysteine proteases which are proteolytic enzymes necessary for the parasite survival in human host [7] is currently being studied as ideal target for developing new therapeutic agents. Malaria parasite cysteine protease inhibitors from plant sources are currently being evaluated for antimalarial potentials [8, 9].

The degradation of host hemoglobin by the parasite proteases released heme moieties which are toxic to the parasite and challenged its survival [10]. The parasite evolved several heme detoxification strategies one of which is the conversion of toxic heme to non-toxic hemozoin. Blocking the heme-hemozine conversion pathway is the major antimalarial mechanisms of the quinolones such as chloroquine and many antimalarial herbal remedies [10–12].

Securidaca longepedunculata is used in the treatment of a variety of ailments by traditional healers in many parts of the world; in Nigeria, the plant is called Uwar magunguna (mother of all medicines) in Hausa language because of its usefulness in treating various ailments. The methanol extract of the root bark of S. longepedunculata possessed potent activity against the chloroquine-sensitive strain of P. berghei parasite [13, 14]. Previous studies also showed that the plant is active against venereal diseases and syphilis [15], infections related to nervous and circulatory systems [16], skin cancer, skin infections, dysentery, typhoid and frequent stomach ache [17, 18], various parts of Securidaca longepedunculata are used against various diseases but roots are the most used part [19].

The aim of the present study is to evaluate the in vivo antimalarial potentials of cysteine protease inhibitors’ fraction from the methanol extract of the root bark of Securidaca longepedunculata in chloroquine-resistant P. berghei-infected Swiss Albino mice and its inhibitory effect on papain, P. berghei proteases and heme biocrystallization.

2. Materials and Methods

2.1. Chemicals and reagents. Hemin chloride, cysteine HCl and sodium acetate buffer were bought from Sigma Aldrich (USA). Chloroquine diphosphate and artemether were bought from Jiangsu Pengyao Pharmaceuticals Co., Ltd. (China). All other chemicals and reagents used were of analytical grade. These include, DMSO, PBS (pH 6 and 7.6), NaOH, EDTA, papain, gelatin, saponin, Triton-X100, giemsa, 70% (v/v) methanol, Dragendorff’s reagent, ferric chloride, chloroform, 10% (v/v) ammonia, glacial acetic acid, sulphuric acid, acetic anhydride, acetone, 10% (v/v) Molisch’s reagent, TCA and trisodium citrate.

2.2. Plant materials. The plant specimen comprising the leaves attached to the stalk and roots of Securidaca longepedunculata (Figure 1) was obtained behind Area BZ, Ahmadu Bello University (A.B.U) quarters, Samaru Sabon Gari local government area of Kaduna State, Nigeria on the 5th July, 2016. The plant sample was identified in the herbarium unit of the Department of Botany, A.B.U., by comparing with existing specimen (Voucher specimen number: 1402).

2.3. Experimental animals. Swiss Albino mice of both sexes with an average weight of 20.23 ± 2.92 g were obtained from the Department of Veterinary Parasitology, National Veterinary Research Institute (NVR), Vom, Plateau state, Nigeria. The animals were kept under standard laboratory conditions, fed with standard diet and water ad libitum in accordance with the Guide for the Care and Use of Laboratory Animals [20]. Ethical approval for the use of animals was granted by A.B.U committee on animal use and care with approval number: ABUCAUC/2018/018.

2.4. Parasite. Chloroquine-resistant Plasmodium berghei (ANKA) was obtained from Malaria Research Laboratory, Institute of Advanced Medical Research and Training (IMRAT) College of Medicine, University of Ibadan. The strain was maintained for the period of the study by serial blood passage from mouse to mouse.

2.5. Methanol extraction. The plant material was air-dried at room temperature for 2 weeks, pulverized in a mortar, and sieved to fine powder. About 208.60 g of the powdered material was used for the extraction. The methanol extract of the root bark of Securidaca longepedunculata was obtained by soxhlet extraction using 1,000 mL of 70% (v/v) methanol for 48 hours. The extract was concentrated to dryness at 45°C using rotary evaporator.

2.6. Isolation of cysteine protease inhibitors from the methanol extract. The method employed in the isolation of cysteine protease inhibitors from the methanol extract was closely similar to that of Bijina et al., [21]; 50 mL of 0.1 M phosphate buffer (pH 7) was added to 5 g of crude methanol extract, and the mixture was placed on a rotary shaker for 20 min at 30 rpm for complete dissolution. The resulting solution was centrifuged at 10,000 x g for 15 min at 4°C. The supernatant was pooled together and the pellet was discarded. Acetone at 0°C was added to the supernatant at ratio 4:1 with gentle stirring. Precipitate obtained after the cold acetone precipitation was air-dried and stored at 0°C as the CPI fraction.

2.7. Phytochemical screening. The phytochemical constituents of CPI were determined according to the method described by Evans [22].

2.8. Acute toxicity testing. Acute toxicity test of the extract was carried out according to the method describe by Lorkes [23]. In the first phase of the experiment, 9 mice were divided into three groups of 3 each. Groups 1, 2 and 3 mice were
administered with varying doses of 10, 100 and 1,000 mg/kg, respectively and the animals were observed for signs of toxicity and death within the first 4 hours and after 24 hours, respectively. In the second phase of the experiment, 4 mice were administered different doses based on the result of the first phase and were observed for any possible death after 24 hours. The LD$_{50}$ was calculated using the formula:

$$LD_{50} = \sqrt{\text{minimal lethal dose} \times \text{maximal survival dose}}.$$

2.9. Inhibition of $\beta$-hematin synthesis. $\beta$-hematin assay was carried out according to the method described by Basilico et al., [24]. A solution of hematin chloride consisting of 6.5 mM $\beta$-hematin in 40% DMSO was used. 50 $\mu$L of CPI and chloroquine of varying concentrations; 0.5, 1, 2, and 4 mg/mL in triplicates were incubated with 50 $\mu$L of hematin chloride solution for 20 min at 37°C. 100$\mu$L of sodium acetate and 25 $\mu$L of 17.4 M glacial acetic acid (pH 2.6) were added to initiate polymerization, and the reaction was allowed to continue for 48 hours at 37°C for complete polymerization. The resulting solution was then centrifuged at 10,000xg for 5 min and the supernatant was discarded. The pellet was washed three times with 200 $\mu$L DMSO until a clear supernatant was obtained. The pellet was dissolved with 200 $\mu$L of 0.1 M NaOH and the absorbance was recorded by a micro well plate reader at a wavelength of 630 nm [12]. The results were expressed as percentage inhibition of $\beta$-hematin synthesis and the IC$_{50}$ values of test extract was calculated from inhibition versus concentration graph.

Inhibition (%) = \frac{A_0 - A_i}{A_0} \times 100

$A_0$ = Absorbance without inhibitor

$A_i$ = Absorbance with inhibitor

2.10. Effect of cysteine protease inhibitors on papain. Papain (0.1 g) was dissolved in 100 mM PBS (pH 6.8). The enzyme was activated by the addition of 100 $\mu$L of 10 mM cysteine-HCl and 100 $\mu$L of 5 mM EDTA, and the final volume was made up to 10 mL with 100 mM PBS pH (6.8).

Enzyme assay was carried out according to the method described by Amlabu et al., [8]. About 100 $\mu$L of CPI with concentrations, 1, 5, 7, 10, 20 and 30 $\mu$g/mL in triplicates were incubated with 100 $\mu$L of enzyme (1% papain) for 30 min at 37°C. About 200 $\mu$L of 3% gelatin (Prepared in PBS pH 6.8) was then added to start proteolysis reaction. The mixture of enzyme and substrate was incubated for 90 min at 37°C. After incubation, the reaction was stopped by addition of 400 $\mu$L 20% (w/v) trichloroacetic acid and placed on a rotary shaker for 2 min at 30 rpm for complete precipitation of proteins, blank tube was prepared by adding 400 $\mu$L of 20% (w/v) trichloroacetic acid to the mixture of enzyme and CPI before the substrate was added. The precipitated protein was removed by centrifugation at 10,000xg and the absorbance of the supernatant was recorded at 280 nm. Result was expressed as percentage inhibition of enzyme activity and the IC$_{50}$ values of CPI were calculated from inhibition versus concentration graph;
2.11. Isolation of \textit{P. berghei} cysteine proteases. Isolation of \textit{P. berghei} cysteine proteases from parasitized red blood cells of \textit{P. berghei}-infected mice was carried out according to the method described by Amlabu et al., [8]. Eight mice infected with chloroquine-resistant \textit{P. berghei} (ANKA) with a rising parasitemia level of 10\% were euthanized and their blood collected in 3.8\% (w/v) sodium citrate in PBS (pH 7.2). The blood was centrifuged at 10,000xg for 10 min; plasma and buffy coat were removed by aspiration using a 200-2000 \mu L pipette. The red blood cells were washed four times with cold (0\(^\circ\)C) 0.1 M PBS (pH 7.2) until a clear supernatant was obtained, the supernatant was discarded and the pellet constitute infected red blood cells isolate.

The infected red blood cells were incubated with 0.1\% (w/v) saponin in PBS (pH 7.2) in the ratio of one part pellet to three part saponin for 60 min at 4\(^\circ\)C, the resulting solution which consist of 100\% lysed erythrocytes was centrifuged at 10,000xg for 15 min, the supernatant was discarded and the pellets were washed three times in 200 mM sodium acetate buffer (pH 4.0) until a clear supernatant was obtained, the pellets constitute \textit{P. berghei} parasite isolate.

The parasite isolate was incubated with 0.5\% (v/v) Triton X-100 in Tris-buffer saline for 90 min at 4\(^\circ\)C, the resulting solution after incubation was centrifuged at 10,000xg for 30 min at 4\(^\circ\)C, the pellet was re-extracted with Triton X-100 and the supernatant was pooled together as the crude enzyme isolate and was stored at about 4\(^\circ\)C for enzyme assay.

2.12. \textit{Plasmodium berghei} cysteine proteases enzyme inhibition assay. Enzyme assay was carried out according to the method described by Amlabu et al., [8]. 100 \mu L of CPI with concentrations, 1, 5, 7, 10, 20 and 30 \mu g/mL in triplicates were incubated with 100 \mu L of enzyme in 500 \mu L sodium acetate buffer (pH 4.0) for 30 min at 37\(^\circ\)C. About 200 \mu L of 3\% (w/v) gelatin prepared in PBS (pH 6.8) was then added to start proteolysis reaction, the mixture of enzyme and substrate was incubated for 90 min at 37\(^\circ\)C. After incubation the reaction was stopped by addition of 400 \mu L 20\% (w/v) TCA and placed on a rotary shaker for 2 min at 30 rpm for complete precipitation of protein, blank was prepared by adding 400 \mu L of 20\% (w/v) trichloroacetic acid to the mixture of enzyme and CPI before the addition of the substrate. The precipitated protein was removed by centrifugation at 10,000xg for 10 min and the absorbance of the supernatant was recorded at 280 nm. Result was expressed as percentage inhibition of enzyme activity and the IC\textsubscript{50} values of CPI were calculated from inhibition versus concentration graph;

\[ \text{Inhibition} (\%) = \frac{A_0 - A_i}{A_0} \times 100 \]

\[ A_0 = \text{Absorbance without inhibitor} \]
\[ A_i = \text{Absorbance with inhibitor} \]

2.13. Antimalarial studies. The doses of CPI used in the present study were 10, 20 and 30\% of the LD\textsubscript{50} equivalent of 34, 23 and 11 mg/kg.

2.13.1. Inoculation of mice. A donor mouse with a rising parasitemia of 20\% was euthanized and its blood collected in EDTA bottle and diluted with normal saline so that each 0.2 mL contained approximately \(1.0 \times 10^7\) infected red blood cells [25]. The experimental animals were infected with standard inoculum of \(1.0 \times 10^7\) parasitized erythrocytes in volumes of 0.2 mL intraperitoneally.

2.13.2. Suppressive test. Four-day suppressive test was carried out according to the method described by Peter and Anatoli [25]. Twenty five (25) mice of both sexes with an average weight of 20 g were infected with standard inoculum of the parasite and divided weight dependently into five groups of five mice each. Group’s 1-3 mice received, 34, 23 and 11 mg/kg equivalent to 10, 20 and 30\% of the LD\textsubscript{50} respectively. Group 4 mice received 2 mg/kg of standard antimalarial drug artemether and group 5 received 10 ml/kg normal saline. Treatment commenced 2 hours after parasite inoculation and continued for 3 consecutive days. On the 4th day, blood was collected for parasitemia determination.

2.13.3. Curative test. Four-day curative test was carried out according to the method described by Peter and Ryley [26]. Twenty five (25) mice of both sexes with an average weight of 20 g were infected with standard inoculum of the parasite and divided weight dependently into five groups of five mice each. Group’s 1-3 mice received, 34, 23 and 11 mg/kg equivalent to 10, 20 and 30\% of the LD\textsubscript{50} respectively. Group 4 mice received 2 mg/kg of standard antimalarial drug artemether and group 5 received 10 ml/kg normal saline. Treatment commenced 72 hours after parasite inoculation and continued for 3 consecutive days. On the 4th day, blood was collected for parasitemia determination.

2.13.4. Preparation of thin blood smear and parasitemia determination. Thin smears of blood films were collected from the tail end of each mouse on day four, the smears were placed on microscope slides and stained with 10\% Giemsa dye and examined under a light microscope at \(\times 1,000\) magnifications under oil emersion to assess the level of parasitemia:
Parasitemia (%) = \frac{\text{Average of parasitized RBC in five fields}}{\text{Average of RBC in five fields}} \times 100

\text{Suppression} (%) = \frac{P_o - P_t}{P_o} \times 100

\text{Curative} (%) = \frac{P_o - P_t}{P_o} \times 100

P_o = \text{Parasitemia of control (normal saline) group}
P_t = \text{Parasitemia of treated groups.}

2.14. Packed cell volume determination. Heparinized capillary tubes were filled with blood to about two-third of their lengths. The vacant end of each of the capillary tubes was sealed to protect the blood from spilling. The tubes were placed in hematocrit centrifuge with seal side towards the periphery and then centrifuged for 5-6 min. The percentage of packed cell volume was read from hematocrit reader [27].

2.15. Determination of Biochemical Parameters. The activities of Alanine Amino Transferase (ALT), Aspartate Amino Transferase (AST) and Alkaline Phosphate (ALP) were determined in serum using Randox kit (UK), Spectrum diagnostics kit (Egypt) and Agappe diagnostic kit (Switzerland), respectively, according to the manufacturer’s protocol.

Reduced glutathione (GSH) assay was carried out according to the method described by Rajagopalan et al., [28]. The principle of the assay is based on the reaction of 5, 5'-dithiobis-2-nitrobenzoic acid (DNTB) and reduced glutathione. About 1.5 mL of 10% TCA was added to 150 μL of serum (in PBS, pH 7.4) and centrifuged at 1,500 x g for 5 min. 1 mL of the supernatant was treated with 0.5 mL of Ellmans reagent and 3 mL of phosphate buffer (0. 2 M, pH 8.0). The absorbance was recorded at 412 nm. The quantity of GSH was obtained from the graph of the GSH standard curve.

Catalase activity (CAT) was measured according to the method described by Abel [29]. Catalase in serum catalyses the decomposition of hydrogen peroxide into water and oxygen molecules.

\[
\text{H}_2\text{O}_2(l) \overset{\text{Cat}}{\longrightarrow} \text{H}_2\text{O}(aq) + \text{O}_2(g)
\]

About 10 μL of serum was added to 5 mM potassium phosphate buffer (pH 7.0). About 0.1 mM of freshly prepared 30 mM hydrogen peroxide was added to start the reaction and the decomposition of hydrogen peroxide was recorded by UV spectrophotometer at 240 nm wavelength for 5 min. A molar extinction coefficient (E) of 0.041 mM\(^{-1}\text{cm}^{-1}\) was used to calculate catalase activity.

\[
\text{Cat} = \frac{\text{Absorbance}}{E}
\]

2.16. Statistical analysis. The data obtained were expressed as means ± SD and analyzed with SPSS version 20. One way analysis of variance (ANOVA) with subsequent Dunnett’s post hoc test was carried out. Differences were considered significant at \(p \leq 0.05\). Data was presented as tables or charts as appropriate.

3. Results

3.1. Extraction yield. The extraction of 208.6 g powdered material of the root bark of Securidaca longepedunculata with methanol gave 126.30 g of the extract which represents a yield of 60.50%. About 5 g of the methanol extract of the root bark gave 0.7 g of cysteine protease inhibitors fraction (CPI) which represent a yield of 14%.

3.2. Phytochemical analysis. Preliminary phytochemical screening revealed the presence of flavonoids, cardiac glycoside and saponins (Table 1). Alkaloids, tannins, anthraquinones, steroids and triterpenes were absent.

Table 1: Phytochemical constituents of cysteine protease inhibitors fraction.

| Constituent         | Inference |
|---------------------|-----------|
| Alkaloids           | -         |
| Tannins             | -         |
| Steroid and Triterpens | -     |
| Flavonoids          | +         |
| Cardiac glycosides  | +         |
| Saponins            | +         |
| Anthraquinones      | -         |
| + = present, - = absent. |

3.3. Acute toxicity testing. The intraperitoneal median lethal dose of CPI was estimated to be 113.13 mg/kg. Breathing abnormalities, rolling movement, hind limb extension and unconsciousness were observed within the first four hours of the experiment.

3.4. Inhibitory effect of cysteine protease inhibitors’ fraction on β-hematin polymerization. Cysteine protease inhibitors fraction failed to inhibit β-hematin polymerization with IC\(_{50}\) value of 23.9 mg/mL (Figure 2).

The standard drug chloroquine diphosphate inhibited β-Hematin polymerization with IC\(_{50}\) value of 2.8 mg/mL (Figure 3).

3.5. Inhibitory effect of cysteine protease inhibitors on papain enzyme and P. berghei cysteine proteases. Cysteine protease inhibitors fraction from the methanol extract of the root bark of S. longepedunculata demonstrated potent in vitro
Figure 2: Inhibitory activity of protease inhibitors fraction of *S. longepedunculata* on β-hematin polymerization. Values are means ± SD (n=3), CPI = Cysteine Protease Inhibitors, IC<sub>50</sub> = Concentration of CPI that produced 50% inhibition of β-hematin polymerization.

Figure 3: Inhibitory activity of chloroquine diphosphate on β-hematin polymerization. Values are means ± SD (n=3), CQ = Chloroquine diphosphate, IC<sub>50</sub> = Concentration of CQ that produced 50% inhibition of β-hematin polymerization.

Figure 4: Inhibitory activity of cysteine protease inhibitors of *Securidaca longepedunculata* on papain enzyme. Values are means ± SD (n=3), CPI = Cysteine Protease Inhibitors, IC<sub>50</sub> = Concentration of CPI that produced 50% inhibition of Papain enzyme activity.
inhibition of papain enzyme activity with IC$_{50}$ value of 20.1 μg/mL (Figure 4).

The fraction also showed potent inhibition of *P. berghei* cysteine proteases in vitro with IC$_{50}$ value of 5.6 μg/mL (Figure 5).

3.6. Suppressive and curative effect of cysteine protease inhibitors fraction of *Securidaca longepedunculata* in chloroquine-resistant *P. berghei*-infected mice. CPI produced non-dose dependent suppression of parasitaemia with the highest (34 mg/kg) and median (23 mg/kg) doses producing significant (p≤0.05) suppression. The standard drug, artemether (2 mg/kg), gave the highest significant (p≤0.05) parasitaemia suppression when compared with normal saline control group (Table 2).

**Table 2:** Suppressive effect of cysteine protease inhibitors’ fraction of *S. longepedunculata* in chloroquine-resistant *P. berghei*-infected Mice.

| Treatment (/kg) | Parasitemia (%) | Chemosuppression (%) |
|----------------|-----------------|----------------------|
| CPI (34 mg)    | 2.93 ± 1.08$^*$ | 44.43                |
| CPI (23 mg)    | 2.61 ± 1.36$^*$ | 50.43                |
| CPI (11 mg)    | 4.72 ± 1.49     | 10.38                |
| Artemether (2 mg) | 1.38 ± 0.49$^*$ | 73.77                |
| N/S (10 mL)    | 5.27 ± 1.21     | –                    |

Data presented are means ± SD and analysed using one way ANOVA followed by Dunnett’s post hoc test. Values with the superscript $^*$ differs significantly (p≤0.05) from the normal saline group within the same column; CPI = Cysteine protease inhibitors, N/S = Normal Saline.
Figure 7: Effect of cysteine protease inhibitors on reduced glutathione levels in *P. berghei*-infected Mice. CPI$_\alpha$, CPI$_\epsilon$ and CPI$_\mu$ = Cysteine Protease Inhibitors at 34, 23 and 11 mg/kg respectively, ART = Artemether; 2 mg/kg, N/S = Normal Saline; 10 ml/kg, GSH = Reduced Glutathione, Data presented are means ± SD (n=4) and analysed using one way ANOVA followed by Dunnett’s post hoc test * = Significant at (p≤0.05).

CPI showed dose dependent significant (p≤0.05) curative effect with the highest dose (34 mg/kg) producing the highest curative effect of 59.77% (Table 3). The standard drug artemether (2 mg/kg) showed 81.89% curative effect.

Figure 8: Effect of cysteine protease inhibitors on catalase levels in *P. berghei*-infected Mice. CPI$_\alpha$, CPI$_\epsilon$ and CPI$_\mu$ = Cysteine Protease Inhibitors at 34, 23 and 11 mg/kg respectively, ART = Artemether; 2 mg/kg, N/S = Normal Saline; 10 ml/kg, Data presented are means ± SD (n=4) and analysed using one way ANOVA followed by Dunnett’s post hoc test * = Significant at (p≤0.05).

3.7. Changes in body weight of Mice infected with *P. berghei* and treated with cysteine protease inhibitors of *S. longepedunculata*. Mice infected with chloroquine-resistant *P. berghei* parasite and treated with CPI showed no significant (p≤0.05) change in body weight on day 4 compared to the normal saline group (Table 4). However the percentage decrease in body weight of all treated groups on day 4 was slightly greater than that of the normal saline group (8.24%). CPI group (23 mg/kg) produced the highest percentage decrease in body weight of 12.65. While artemether group showed the lowest decrease in weight (7.65%).

3.8. Changes in pack cell volume (PCV) of Mice infected with *P. berghei* and treated with cysteine protease inhibitors of *S. longepedunculata*. The control group showed a 10.7% decrease in pack cell volume (PCV) on day 4 (Table 5). CPI at 34, 23 and 11 mg/kg has demonstrated significant (p≤0.05) protection ability against loss in PCV. Treatment with 34 mg/kg and 11 mg/kg produced 2.11 and 8.02% increase in PCV respectively.

3.9. Effect of cysteine protease inhibitors on some biochemical and antioxidant parameters in *P. berghei*-infected mice.
Table 3: Curative effect of cysteine protease inhibitors’ fraction of S. longepedunculata in chloroquine-resistant P. berghei-infected Mice.

| Treatment (kg) | Parasitemia (%) | Curative (%) |
|---------------|-----------------|--------------|
| CPI (34 mg)   | 3.07 ± 0.83*    | 59.77        |
| CPI (23 mg)   | 3.22 ± 1.00*    | 57.73        |
| CPI (11 mg)   | 3.49 ± 0.85*    | 54.18        |
| Artemether (2 mg) | 1.38 ± 0.49* | 81.89        |
| N/S (10 mL)   | 7.62 ± 2.50     |              |

Data presented are means ± SD and analysed using one way ANOVA followed by Dunnett’s post hoc test. Values with the superscript * differs significantly (p≤0.05) from the normal saline group within the same column; CPI = Cysteine protease inhibitors, N/S = Normal Saline.

Table 4: Changes in body weight of Mice infected with P. berghei and treated with cysteine protease inhibitors of S. longepedunculata.

| Treatment (kg) | Weight (g) | Decrease (%) |
|---------------|------------|--------------|
| D0            | D4         |              |
| CPI (34 mg)   | 20.75 ± 4.92 | 18.25 ± 4.50 | 12.04 |
| CPI (23 mg)   | 19.75 ± 2.99 | 17.25 ± 2.06 | 12.65 |
| CPI (11 mg)   | 19.25 ± 1.26 | 17.50 ± 1.29 | 9.09  |
| Artemether (2 mg) | 20.25 ± 2.06 | 18.70 ± 2.31 | 7.65  |
| N/S (10 mL)   | 19.4 ± 2.61  | 17.8 ± 2.68  | 8.24  |

Data presented are means ± SD and analysed using one way ANOVA. Values with the superscript * differs significantly (p≤0.05) from the normal saline group within the same column; CPI = Cysteine protease inhibitors, N/S = Normal Saline, D0 = First-day of drug administration, D4 = Fourth-day of drug administration.

Table 5: Changes in pack cell volume of Mice infected with P. berghei and treated with cysteine protease inhibitors of S. longepedunculata.

| Treatment (kg) | PCV (%) | Change (%) |
|---------------|---------|------------|
| D0            | D4      |            |
| CPI (34 mg)   | 45.5 ± 3.87 | 47.50 ± 3.42* | +2.11 |
| CPI (23 mg)   | 44.25 ± 2.63 | 45.25 ± 2.50* | +2.20 |
| CPI (11 mg)   | 43.00 ± 2.58 | 46.75 ± 2.99* | +8.02 |
| Normal Saline (10 mL) | 41.4 ± 3.44 | 37.4 ± 2.41 | -10.7 |

Data presented are means ± SD and analysed using one way ANOVA followed by Dunnett’s post hoc test. Values with the superscript * differs significantly (p≤0.05) from the normal saline group within the same column; CPI = Cysteine protease inhibitors, D0 = First-day of drug administration, D4 = Fourth-day of drug administration.

There was no significant difference (p≥0.05) in serum levels of ALT in treated mice compared to the normal saline group. However, AST and ALP serum levels were significantly (p≤0.05) reduced in 34, 23 and 11 mg/kg CPI treated groups (Figure 6). Artemether (2 mg/kg) treated group also produced significant (p≤0.05) reduction in the levels of AST and ALP. Only 0.28 mg/kg CPI-treated group showed significant (p≤0.05) increase in the levels of GSH compared to the normal saline group (Figure 7).

Mice infected with chloroquine-resistant P. berghei and treated with CPI showed no significant change (p≤0.05) in the serum levels of catalase compared to the normal saline group on day 4 (Figure 8).

4. Discussion

It was earlier reported that about 80% of the world’s population relied on medicinal plants for their primary health care [30]. With the discovery of artemisinins from Artemisia annua and quinines from Cinchona tree bark [5, 6] nature has proven to be a reliable source of novel antimalarial compounds. These necessitate the search for more novel antimalarial drugs from plant sources. Malaria parasites species that cause human disease are unable to infect non primate animal models [31, 32]. The use of P. berghei-infected mice as in vivo model for testing putative antimalarial compounds or extracts is supported by the identification of several conventional antimalarial drugs like chloroquine, halofantrine, mefloquine and artemisinins [31, 32], the use of in vivo models in drug testing takes into account pro-drug effect and any possible involvement of the immune system.

The 4-day suppressive test is commonly used for antimalarial screening and the determination of percent inhibition of parasitaemia is the most reliable parameter [25]. Rodent models to test for antimalarial potential of compounds against chloroquine-resistant parasite have been developed to facilitate search for novel compounds or extracts that are active against the resistant strain [33, 34]. The methanol extract of the root bark of Securidaca longepedunculata was chosen because of the potent antimalarial activity demonstrated against the chloroquine-sensitive parasite in earlier studies [13, 14].

Based on the classification of Matsumura [35] and Corbett [36], CPI is moderately toxic. The doses of CPI administered in both the suppressive and curative tests were less than one-third of its LD50 value and were well tolerated. This is in line with the findings of Dikasso et al., [37], that the therapeutic dose of a test substance should be less than one-third of its LD50 value. CPI demonstrated significant curative effect in chloroquine-resistant P. berghei-infected Swiss Albino mice. Antiplasmodial activity has been related to a range of several classes of secondary plant metabolites including alkaloids, flavonoids, tannins, steroids and triterpenes [38]. The presence of flavonoids could be responsible for the antiplasmodial activity observed. Parasite cysteine proteases are involved in the process of erythrocyte invasion [39], therefore, extract rich in cysteine protease inhibitors are good candidates for suppression of parasitaemia as demonstrated by CPI fraction in the present study. This is in line with a previous study where cysteine protease inhibitors from...
Calotropis procera demonstrated good suppressive effect in P. berghei-infected mice [9]. The standard drug artemether showed very good suppressive and curative effects which is consistent with previous studies [13, 14].

CPI did not significantly protect against loss in body weight of P. berghei-infected mice in the present study. According to Vasantha et al., [40] the presence of phytochemicals like flavonoids is a characteristic feature of any plant extract with appetite suppressant ability. It could be that CPI fraction contains large quantities of flavonoids. The slight weight reduction observed with the infected and untreated mice on day 4 of the study is in line with earlier reports that reduction in body weight is one of the signs of malaria infection in mice [41].

In the present study, the 10.7% reduction in PCV on day 4 observed in the control group is another sign of malaria infection as stated by Langhorne et al., [41]. Animals suffer from anaemia because of red blood cells destruction, either by parasite multiplication or by spleen reticuloendothelial cell action [42]. Oxidative stress has been reported to be another reason for the development of anaemia [43]. The significant increase in the levels of PCV in mice treated with CPI is in line with the findings of Ovuakporaye [44], which stated that plants with antimalarial property always increase the levels of the blood elements. The presence of flavonoids in CPI may have contributed to the observed increase in PCV due to their established antioxidant properties. According to Emeka and Obioa [45], extract that increases or protects against loss in PCV may contain constituents that trigger the production of more blood cells.

There is a liver stage in the life cycle of the parasite in the course of malaria disease progression [46]. The elevated levels of ALP and AST in the infected untreated group may be attributed to damage to the liver via oxidative stress upon infection or release of merozoites. The significant reduction in the levels of AST and ALP in CPI-treated groups is in line with the report of Rabia et al., [47] that plants with flavonoids possess hepatoprotective and antioxidant ability. The significant elevation in the levels of GSH in CPI suggests that CPI possessed antioxidative potentials.

According to Basilico et al., [24], a compound could be considered to have heme polymerization inhibitory activity if it has heme polymerization inhibitory IC₅₀ value smaller than the limit of chloroquine diphosphate, i.e., 12 mg/mL. Therefore, CPI fraction with IC₅₀ value >12 mg/mL did not demonstrate in vitro β-hematin inhibitory activity. It was earlier reported that alkaloids are the major class of phytochemicals with β-hematin inhibitory activity [10]. The absence of alkaloids could be responsible for the inability of the CPI fraction to inhibit β-hematin polymerization. Although CPI fraction did not possess heme polymerization inhibitory activity yet it has demonstrated very good curative and suppressive activities against the parasite. These findings suggest that inhibition of heme polymerization is most likely not the primary mechanism of action of CPI against P. berghei parasite. Extracts or compounds with IC₅₀ values less than 23 μg/mL were described as possessing excellent activity against the standard protease papain [48]. Based on this, it can be deduced that CPI fraction possessed potent activity against papain and P. berghei cysteine proteases.

5. Conclusion

Cysteine protease inhibitors fraction from the methanol extract of the root bark of Securidaca longepedunculata possessed potent suppressive and curative effect against chloroquine-resistant P. berghei parasite. Inhibition of heme biocrystallization is most likely not its primary mechanism of action. The fraction also demonstrated potent inhibition of P. berghei cysteine proteases in vitro which suggest that it may be acting via inhibition of parasite cysteine proteases. It could be a source of novel antimalarial compounds that will target malaria parasite cysteine proteases. Further studies are needed to identify and characterized bioactive compounds responsible for these activities.

Competing Interests

The authors declare no competing interests.

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