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Effects of *Clerodendrum violaceum* Leaf Extract on Some Liver Function Indices in *P. berghei*-Infected Mice

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Abstract: The effects of daily oral administration of ethanolic extract of *Clerodendrum violaceum* leaves (13 mg kg⁻¹ body weight) for 5 days on some liver function indices of *Plasmodium berghei*-infected mice were investigated. The evaluations were done on days 3, 8 and 14 post-infection. The indices studied include serum albumin, globulin, total protein, total bilirubin and conjugated bilirubin concentrations with the specific activities of Glutamate Pyruvate Transaminase (GPT), Glutamate Oxaloacetate Transaminase (GOT), Alkaline Phosphatase (ALP) and γ -Glutamyl Transferase (GGT) in the liver and serum. Treatment of infected mice with extract was able to significantly ($p < 0.05$) ameliorate the alterations in all the parameters observed in infected untreated mice, comparing favourably with chloroquine treatment. Administration of extract to uninfected mice showed no significant effect ($p > 0.05$) on the parameters studied both in the serum and liver compared to the uninfected untreated control, except that it significantly increased ($p < 0.05$) liver GPT activity. The results of this study suggest that the ethanolic extract of *Clerodendrum violaceum* leaves is able to considerably reverse the alterations caused by malaria infection in all parameters studied and is relatively non-toxic. Further studies on *Clerodendrum violaceum* as a source of antimalarial remedy are indicated on the basis of these results.

Key words: Plant, ethanolic extract, enzymes, biomolecules

INTRODUCTION

Traditional medicines have been used to treat malaria for thousands of years. In many developing countries, a large proportion of the population relies heavily on traditional practitioners and medicinal plants to meet primary health care needs (Bodeker and Willcox, 2000). Although modern medicine may be available in these countries, herbal medicines have often maintained popularity for historical and cultural reasons, one of which is the extract of *Clerodendrum violaceum* leaves used for the treatment of malaria in south-western region of Nigeria.

Clerodendrum violaceum Gürke (Verbenaceae) belongs to the genus *Clerodendrum* L. It is commonly called Clerodendrum in English and Ewe isedun in Yoruba (Nigeria). It is a shrub with greenish flowers of up to one inch across. The genus *Clerodendrum* is widely distributed in the tropics and subtropics, with a few species extending into the temperate regions. It is found in countries like Ghana, Zimbabwe, Congo, Cameroon and Nigeria in Africa. In Southwestern Nigeria, it is found in Lagos, Oyo and Kishi. The antimalarial activities of species belonging to this genus have been reported by Simonsen *et al.* (2001) and Muregi *et al.* (2004, 2007). Also of recent, we demonstrated in our laboratory the antimalarial activity of the ethanolic extract of the leaves of *Clerodendrum violaceum* (unpublished data) which is the species found in Kishi, Oyo and environs, thus authenticating the claims of people in such areas who use it for the treatment of malaria.

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However, the use of this plant demands information on its toxicity risk in the management of malaria. It is in the light of this that the present study was set out to provide information on the toxic effects, if any, of *Clerodendrum violaceum* leaves on the liver using animal model.

MATERIALS AND METHODS

Chemicals and Assay Kits

Absolute ethanol (Riedel-de Haën) was obtained from Sigma-Aldrich Laborchemikalien GmbH, Germany. Chloroquine diphosphate salt was obtained from Sigma Chemical Company, St. Louis, Mo, USA. Methanol was obtained from Eagle Scientific Limited, Nottingham. Giemsa stain was obtained from Anosantec Laboratories, UK. Immersion oil was obtained from Panzonar Laboratory Supplies, Button road, Canada. Assay kits for alkaline phosphatase, glutamate pyruvate transaminase, glutamate oxaloacetate transaminase were obtained from Randox laboratories Ltd. (Co. Antrim, UK). The assay kit for γ -glutamyl transferase was obtained from Biolabo reagents, France. All other reagents used were of analytical grade and were prepared in all glass-distilled water.

Animals

Sixty adult Swiss albino mice with an average weight of 20 g were obtained from the animal breeding unit of the Department of Biochemistry, University of Ibadan, Oyo state. The mice were housed in plastic cages and maintained under standard laboratory conditions with free access to rat pellets and tap water *ad-libitum*. The research adhered to the Principles of Laboratory Animal Care (NIH publication number 85-23, revised in 1985).

Parasites

A chloroquine-sensitive strain of *Plasmodium berghei* (NK-65) was obtained from the Institute for Advanced Medical Research and Training (IAMRAT), College of Medicine, University of Ibadan, Oyo state.

Plant Materials

Fresh leaves of *Clerodendrum violaceum* were harvested in Oyo town of Oyo state, Nigeria, in August, 2006. The plant was botanically authenticated at the Department of Plant Biology, University of Ilorin, Ilorin, Nigeria, by Mr. F.O. Egbedo and at Forestry Research Institute of Nigeria (FRIN), Ibadan, Oyo state, Nigeria.

Plant Extract Preparation and Phytochemical Screening

Fresh leaves of the plant were dried in the shade at room temperature and pulverized to powder using an electric blender. About 200 g of the powder was percolated in 1600 mL of absolute ethanol and kept in the shade for 48 h after which it was filtered. The filtrate was collected in a beaker, exposed to air and allowed to evaporate at room temperature to yield the extract concentrate (Adebayo *et al.*, 2003), giving a percentage yield of 8.3%. Preliminary qualitative and quantitative phytochemical screening of the plant extract was carried out employing standard procedures (Harborne, 1983).

Animal Grouping and Extract Administration

The animals were randomly divided into 5 groups: A, B, C, D and E of twelve mice each. Animals in groups C, D and E were inoculated from the same donor mouse. The percentage parasitaemia and the red blood cell count of the donor mouse was first determined using a haemocytometer and appropriate dilutions of the infected blood with isotonic saline was done. Each mouse in the infected groups was inoculated on day 0 intraperitoneally with 0.2 mL of infected blood containing about 1×10^7

P. berghei parasitized red blood cells. Treatment was withheld for 72 h to allow for establishment of infection and was commenced when parasitaemia was established by screening for malaria parasites in tail blood of infected animals after fixing in methanol and staining with Giemsa (Ryley and Peters, 1970).

Aqueous preparations of the extract (corresponding to 13 mg kg⁻¹ body weight, a value arrived at from ethno-botanical survey) and chloroquine (corresponding to 4 mg kg⁻¹ body weight) were made before administering orally to the mice. The administration of the extract and chloroquine which lasted for 5 days is as follows:

- Group A (uninfected mice): Received an appropriate volume of sterile distilled water
- Group B (uninfected mice): Received the aqueous preparation of the extract (13 mg kg⁻¹ body weight daily)
- Group C (infected mice): Received an appropriate volume of sterile distilled water
- Group D (infected mice): Received the aqueous preparation of the extract (13 mg kg⁻¹ body weight daily)
- Group E (infected mice): Received the aqueous solution of chloroquine (4 mg kg⁻¹ body weight daily)

Sample Collection and Preparation

Four mice were sacrificed from each of the five groups on days 3, 8 and 14 post-infection by slight ether anesthesia and were then dissected. Blood was collected by cardiac puncture into clean, dry sample tubes containing no anticoagulants. The clotted blood was then centrifuged at 1000 rpm for fifteen minutes and the clear serum supernatant was carefully collected using a Pasteur pipette. The serum samples were stored frozen until needed for analysis. Also, the liver of each animal was quickly removed, cleansed of superficial connective tissue and blood. The liver was then divided into two parts, a part homogenized in ice-cold 0.25 M sucrose solution (1:5 w/v) and the other fixed in 10% formalin for histopathological studies. The homogenates were stored frozen overnight to ensure maximum release of enzymes from cells (Ngaha *et al.*, 1989).

Biochemical Assay and Histopathological Studies

Alkaline phosphatase (ALP) activity was determined by the method of Wright *et al.* (1972) while the activities of the transaminases (GOT and GPT) were assayed by the method of Reitman and Frankel (1957). Serum albumin concentration was determined by the method of Doumas *et al.* (1971), serum globulin concentration was determined by the method of Tietz (1995) while total and conjugated bilirubin concentrations were determined by the method of Malloy and Evelyn (1937). Protein concentration was determined using the Biuret method described by Gornall *et al.* (1949). Histopathological studies were carried out with the procedure of Krause (2001).

Statistical Analysis

Values are expressed as Mean±SEM. The data were statistically analyzed using one-way analysis of variance (ANOVA) and Duncan Multiple Range Test (Mahajan, 1997). Data from the test groups were compared with controls and differences at p<0.05 were considered significant.

RESULTS AND DISCUSSION

The phytochemical composition of the ethanolic extract of *Clerodendrum violaceum* leaves is shown in Table 1. Alkaloids and phenolics are the predominant phytochemicals in the extract.

In all the liver function indices evaluated, there was no significant difference (p>0.05) across the groups on day 3 post-infection. On day 8, there was a significant decrease (p<0.05) in serum albumin

Table 1: Phytochemicals of the ethanolic extract of *Clerodendrum violaceum* leaves

Phytochemicals	Concentration (%)
Alkaloids	3.71±0.03
Tannins	1.02±0.05
Phenolics	2.77±0.02
Glycosides	0.07±0.00
Saponins	0.10±0.01
Flavonoids	0.36±0.02
Steroids	0.30±0.02
Triterpenes	0.10±0.02
Anthraquinones	0.59±0.03

Values are means of 3 determinations±SD

Table 2: Concentrations of selected serum biomolecules in experimental animals

Groups (Days post-infection)	Total Bilirubin (μmol L ⁻¹)	Conjugated Bilirubin (μmol L ⁻¹)	Albumin (g L ⁻¹)	Globulin (g L ⁻¹)
(Day 3)				
A	8.25±0.46 ^a	0.33±0.17 ^a	39.25±0.52 ^a	25.50±0.76 ^a
B	9.00±0.42 ^a	0.35±0.11 ^a	38.50±0.53 ^a	26.00±0.88 ^a
C	8.50±0.35 ^a	0.43±0.10 ^a	38.00±0.42 ^a	27.50±0.76 ^a
D	8.25±0.52 ^a	0.33±0.10 ^a	39.00±0.46 ^a	25.75±0.75 ^a
E	8.50±0.53 ^a	0.35±0.11 ^a	39.00±0.42 ^a	25.30±0.86 ^a
(Day 8)				
A	8.75±0.52 ^a	0.40±0.17 ^a	39.20±0.46 ^d	25.15±0.53 ^b
B	10.25±0.46 ^a	0.45±0.11 ^a	40.00±0.42 ^d	24.50±0.53 ^b
C	30.25±0.52 ^d	2.48±0.30 ^d	24.50±0.74 ^a	17.25±0.75 ^a
D	22.50±0.67 ^c	1.45±0.27 ^c	31.50±0.53 ^b	19.00±0.73 ^a
E	19.25±0.33 ^b	1.00±0.20 ^b	34.25±0.61 ^c	18.25±0.61 ^a
(Day 14)				
A	9.00±0.42 ^a	0.35±0.11 ^a	39.50±0.67 ^c	25.70±0.61 ^c
B	9.25±0.52 ^a	0.43±0.19 ^a	40.00±0.42 ^c	25.50±0.53 ^c
C	35.25±0.69 ^d	3.43±0.30 ^d	23.00±0.68 ^a	7.50±0.53 ^a
D	20.25±0.52 ^c	0.98±0.23 ^c	33.50±0.67 ^b	18.75±0.86 ^b
E	14.00±0.80 ^b	0.65±0.17 ^b	35.50±0.53 ^b	21.50±1.00 ^{b,c}

Values are means of 4 determinations±SEM. Values for each day along the same column with different superscript letters are significantly different (p<0.05). A: Normal control group; B: Extract control group; C: Infected untreated group; D: Infected extract-treated group; E: Infected chloroquine-treated group

and globulin concentrations while there was significant increase (p<0.05) in serum total and conjugated bilirubin concentrations in infected untreated animals, all of which persisted to day 14, compared to the uninfected groups (Table 2). Treatment of infected animals with extract was able to ameliorate significantly (p<0.05) the alterations in serum albumin, total and conjugated bilirubin concentrations on days 8 and 14 while it was only able to significantly reverse (p<0.05) the decrease in serum globulin concentration on day 14, comparing favourably with chloroquine treatment.

There was significant decrease (p<0.05) in the activities of all the liver enzymes studied in the infected untreated animals with corresponding increase in the activities of the enzymes in the serum on days 8 and 14 compared to the uninfected groups (Fig. 1-4). Treatment of infected animals with extract was able to ameliorate significantly (p<0.05) these alterations, comparing favourably with chloroquine treatment in most cases. It is noteworthy that administration of extract to uninfected mice showed no significant effect (p>0.05) on the parameters studied both in the serum and liver compared to the uninfected untreated control except that it significantly increased (p<0.05) liver GPT activity, although without any significant alteration (p>0.05) in serum GPT activity.

Histopathological studies revealed that there was marked aggregation of inflammatory cells around the central vein of the liver in all infected groups on day 8, especially the infected untreated and infected extract-treated groups (Fig. 5). This increased in the infected untreated group on day 14 while there was improvement in the treated groups, with the infected chloroquine-treated group showing a better resolution (Fig. 6). The uninfected groups showed no aggregation of inflammatory cells.

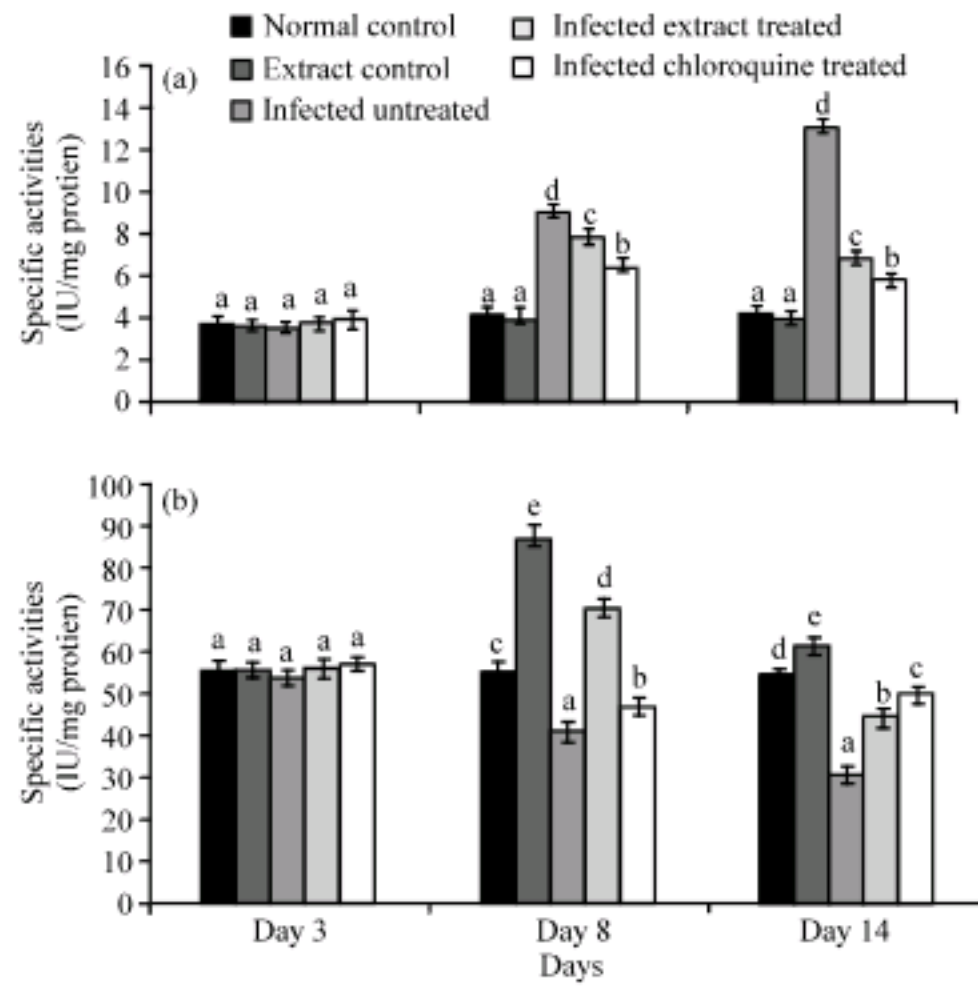


Fig. 1: GPT activities in serum (a) and liver (b) of experimental animals. Values are means of 4 determinations \pm SEM. For each day, bars with different letters are significantly different ($p < 0.05$)

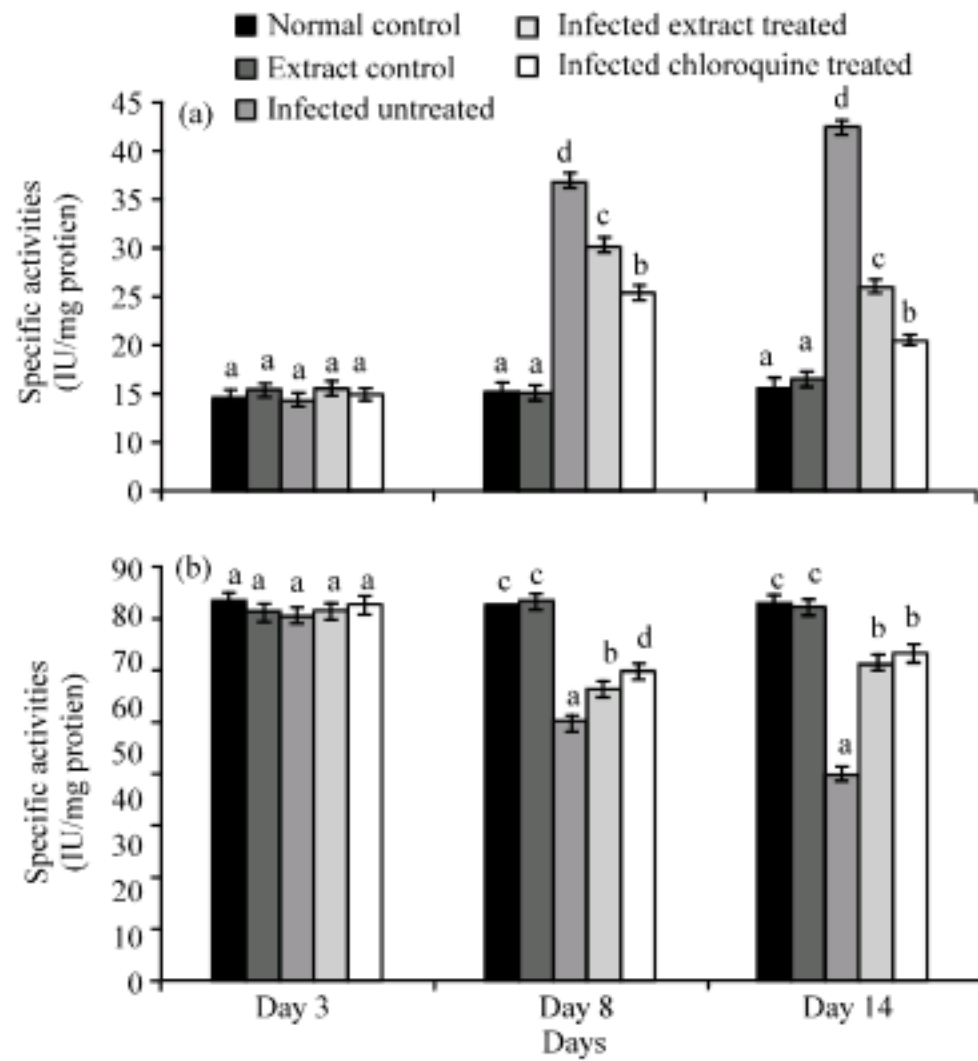


Fig. 2: GOT activities in serum (a) and liver (b) of experimental animals. Values are means of 4 determinations \pm SEM. For each day, bars with different letters are significantly different ($p < 0.05$)

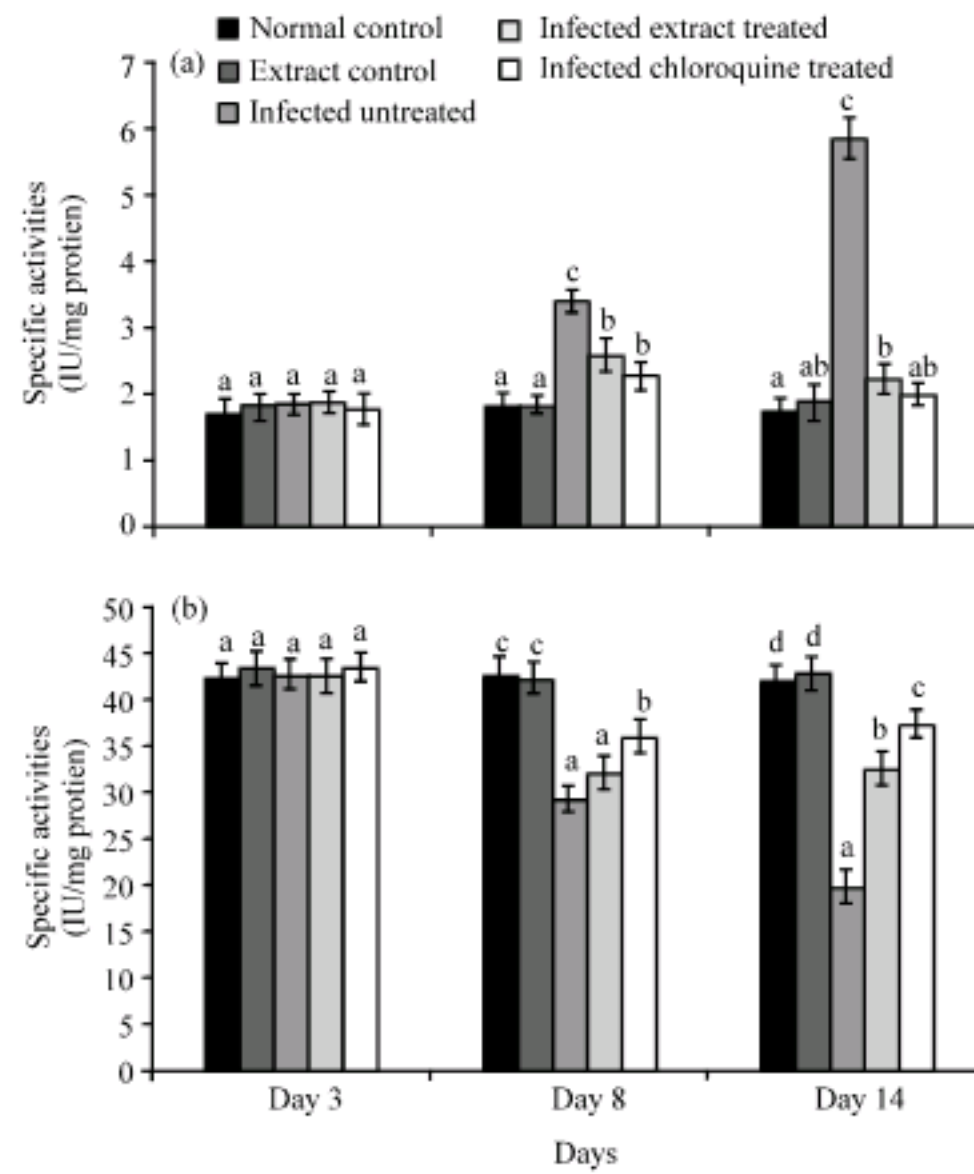


Fig. 3: ALP activities in serum (a) and liver (b) of experimental animals. Values are means of 4 determinations \pm SEM. For each day, bars with different letters are significantly different ($p < 0.05$)

It is of great importance to investigate the effect of the ethanolic extract of *Clerodendrum violaceum* leaves on liver function indices because the liver is the key organ regulating homeostasis in the body. It is involved in almost all the biochemical pathways related to growth, fight against disease, nutrient supply, energy provision and reproduction (Ward and Daly, 1999). Most liver pathological conditions cause only mild symptoms initially; hence it is vital that these pathological states be detected early (Sheila and Dooley, 2002). Liver function tests are therefore designed to give information about the state of the liver.

Increased serum bilirubin concentration (both total and conjugated) was observed in the infected untreated group. Bilirubin is the main bile pigment that is formed from heme catabolism. The causes of increased serum bilirubin concentration in severe malaria are multifactorial; these include intravascular haemolysis of Parasitized Red Blood Cells (PRBCs), hepatic dysfunction and possibly an element of microangiopathic haemolysis associated with disseminated intravascular coagulation. The incidence of jaundice in malaria has been reported to range from 20-31.7% and it is predominantly haemolytic rather than hepatic, as indicated by preeminence of unconjugated bilirubinaemia (Mishra *et al.*, 1992; Wilairatana *et al.*, 1994). While most malaria cases induce unconjugated bilirubinaemia due to haemolysis, conjugated bilirubinaemia may predominate due to liver dysfunction (Anand *et al.*, 1992; Warrel and Francis, 1992; Mishra *et al.*, 2003; Kochar *et al.*, 2003). This may result from alteration in blood flow through the liver as parasitized red blood cells adhere to endothelial cells, blocking the sinusoids and obstructing the intrahepatic blood flow (Kochar *et al.*, 2003). This leads to impairment of bilirubin transport and disturbance of hepatocyte microvilli which in turn leads to bile stasis (Anand *et al.*, 1992; Mishra *et al.*, 1992; Ashan *et al.*, 1993; Srivastava *et al.*, 1996;

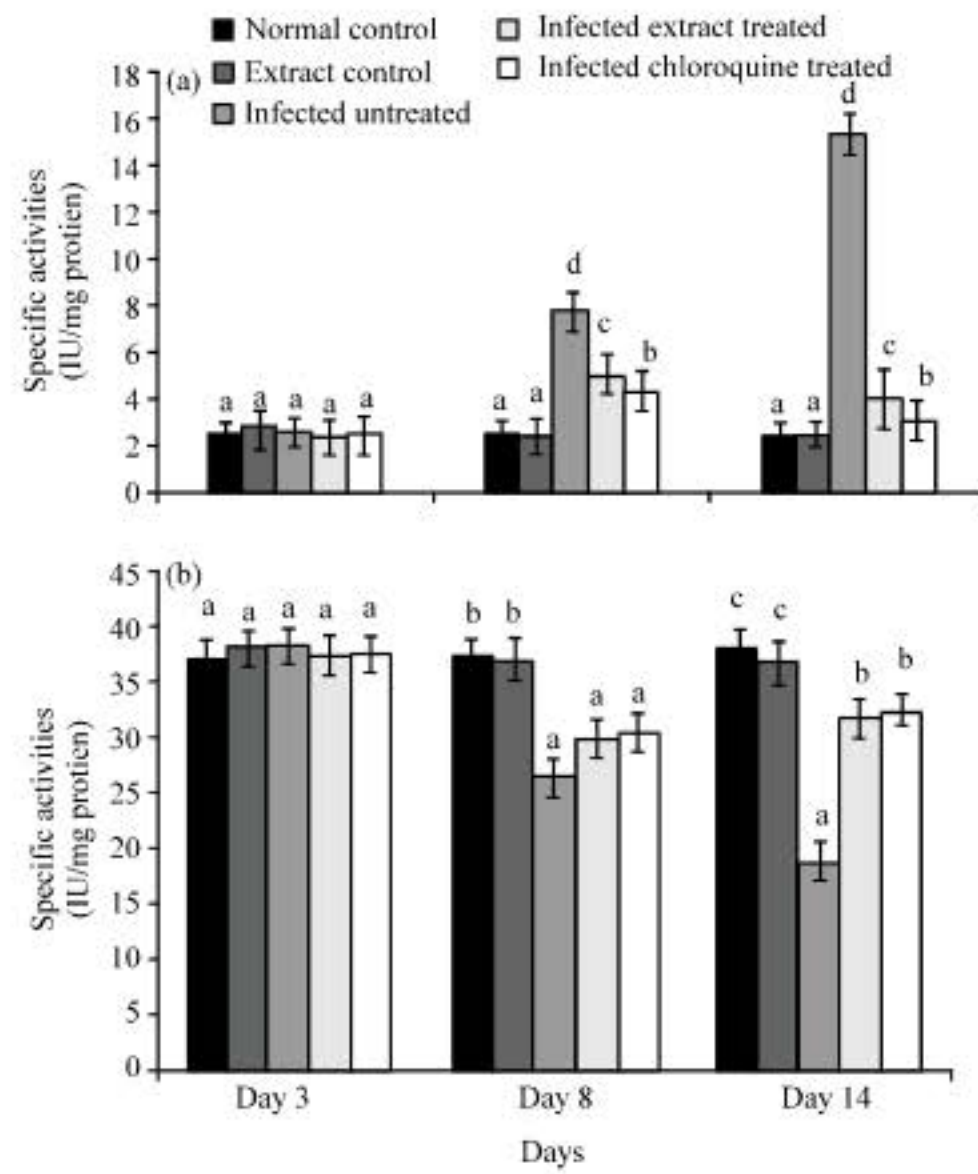


Fig. 4: GGT activities in serum (a) and liver (b) of experimental animals. Values are means of 4 determinations \pm SEM. For each day, bars with different letters are significantly different ($p < 0.05$)

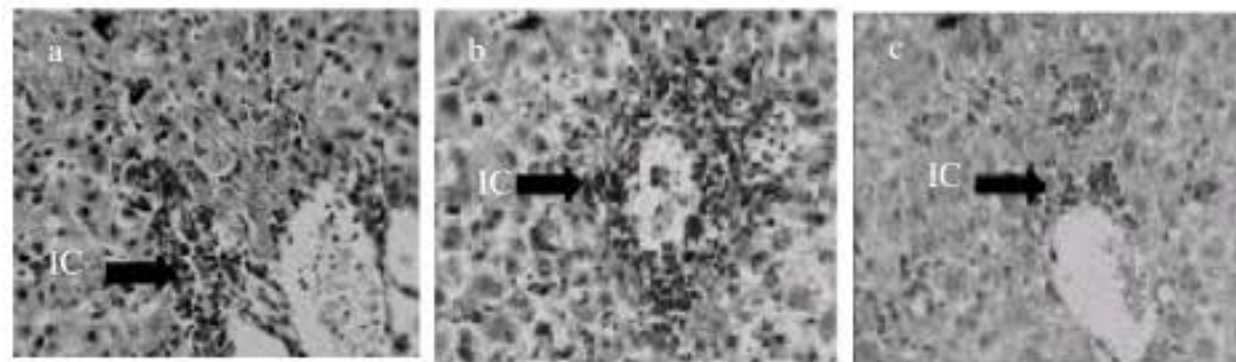


Fig. 5: Photomicrographs of the liver of *P. berghei* infected mice on day 8 (Magnification x400). a: Infected untreated group; b: Infected extract-treated group; c: Infected chloroquine-treated group. IC: Inflammatory cells

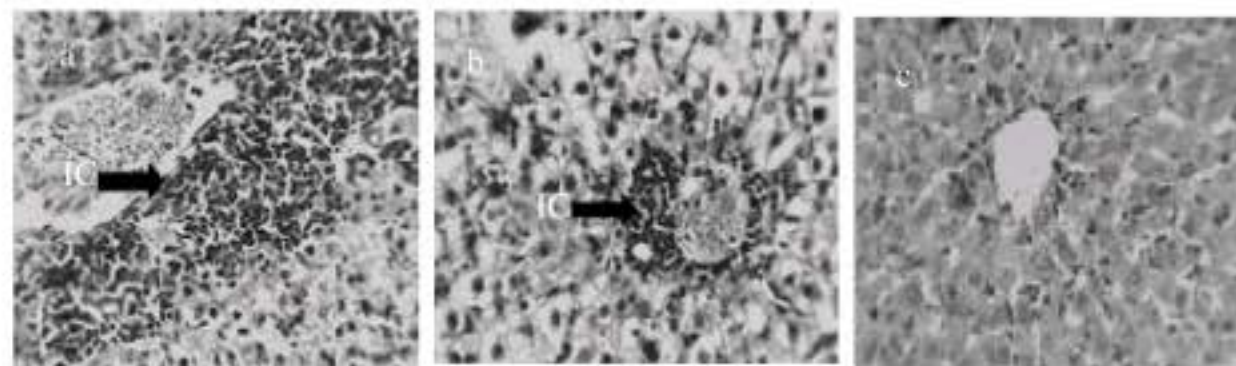


Fig. 6: Photomicrographs of the liver of *P. berghei* infected mice on day 14 (Magnification x400). a: Infected untreated group; b: Infected extract-treated group; c: Infected chloroquine-treated group. IC = Inflammatory cells

Premaratna *et al.*, 2001). Thus, increased serum conjugated bilirubin level in the infected untreated group observed in this study indicates impairment of hepatic bilirubin excretion which involves, as one of its processes, an active energy-dependent secretion of conjugated pigment across the canalicular membrane (Rosen *et al.*, 1967). The reduced serum albumin and globulin concentrations in infected untreated mice also observed in this study correlates positively with earlier reports by other workers (Premaratna *et al.*, 2001; Adeosun *et al.*, 2007). This still indicates hepatic dysfunction since albumin is synthesized by the liver. Reduced serum albumin level causes reduced plasma amniotic pressure. Administration of *Clerodendrum violaceum* leaf extract to infected mice was able to ameliorate these anomalies to a considerable extent, suggesting that the extract may be hepatoprotective.

The observed decrease in activities of all the liver enzymes with corresponding increased activities of the enzymes in the serum of infected untreated animals correlates positively with earlier reports (Maegraith, 1981; Premaratna *et al.*, 2001; Kochar *et al.*, 2003; Adeosun *et al.*, 2007). These are a direct indicator of hepatocyte dysfunction associated with malaria induced in the animals. Maegraith (1981) reported that centrilobular liver damage is one of the factors involved in hepatic dysfunction in acute malaria infection, leading to hyperbilirubinaemia which is a direct consequence of the impaired drainage capacity of the liver. Organ congestion and biliary stasis during malaria leads to loss of the membranous enzymes such as ALP and γ -Glutamyl Transpeptidase (GGT) to the Extracellular Fluid (ECF) (WHO, 1990; Jarikre *et al.*, 2001). This would explain the observed increase in activities of these enzymes in the serum of infected untreated animals with a corresponding decrease in the liver. Alkaline phosphatase is a marker enzyme for the plasma membrane and endoplasmic reticulum (Wright and Plummer, 1974). It is often employed to assess the integrity of the plasma membrane (Akanji *et al.*, 1993). Serum GGT measurement is principally used to diagnose and monitor hepatobiliary disease. GGT permits differentiation of liver diseases from other conditions in which serum ALP activity is elevated because serum GGT levels are usually normal in those diseases. Increased activities of both enzymes in the serum with corresponding decrease in the liver suggest that the integrity of the plasma membrane of the hepatocytes in the infected untreated mice has been compromised. Moreover, anaemia and interference with microcirculation within the liver associated with malaria causes hypoxia which compromises active transport and the cell contents ooze into the ECF through widened pores. This accounts for the increase in the activities of the intracellular enzymes (GOT and GPT) in the serum with corresponding decrease in the liver of the infected untreated mice. Administration of the extract to infected mice was still able to ameliorate these anomalies to a considerable extent, suggesting that the extract may be hepatoprotective. The cellular inflammation caused by the infection, as indicated by liver infiltration by inflammatory cells in infected untreated animals, was also appreciably resolved by the extract.

Administration of the extract to uninfected mice effected no alterations in the parameters studied both in the serum and liver compared to the uninfected untreated control except that it increased liver GPT activity without corresponding alteration in serum GPT activity. This is probably as a result of induction of enzyme synthesis due to a defensive mechanism by the organ to offset the stress imposed by the exogenous substance (Malomo *et al.*, 1993, 1995). This probable induction was also visible in the infected extract-treated group on day 8 which had higher liver GPT activity compared to the infected chloroquine-treated and uninfected untreated groups. The observed increase however was considerably reduced after treatment was withdrawn, suggesting that the induction was caused by extract treatment and the effect is reversible.

In conclusion, the repeated administration of the ethanolic extract of *Clerodendrum violaceum* leaves was able to considerably reverse the alterations caused by malaria infection in all parameters studied and is relatively non-toxic. Further studies on *Clerodendrum violaceum* as a source of antimalarial remedy are indicated on the basis of these results.

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