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VERNONIA AMYGDALINA, A LOCAL ANTI MALARIAL LEAVES EXTRACT INHIBIT LIPID PEROXIDATION AND EXHIBIT HEPATOPROTECTIVE AND NEPHROPROTECTIVE EFFECTS RESULTING FROM ARTESUNATE ADMINISTRATION IN RATS

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ABSTRACT

With the common practice in poor Africa endemic area to take local antimalaria herbs in combination with orthodox medicine and the increasing resistance to anti-malarial drugs, this study investigates the roles of methanol leaves extract of *Vernonia amygdalina*, in combination with artesunate. 24 male wistar rats weighing 190g were randomly grouped, administered therapeutic doses of artesunate and extract. Plasma total protein, urea, creatinine. Alanine. amino transferase (ALT), Alkaline phosphatase (ALP) and Gamma glutamyl transferase (GGT) were determined using international standardized methods. Percentage inhibition of lipid peroxidation, Malondialdehyde, (MDA), reduced glutathione (GSH) levels were determined spectrophotometrically. Results shows that extract conferred above 50% cell protection as it inhibited lipid peroxidation ,decreases MDA level significantly ($P \leq 0.05$) and increases GSH level significantly ($P \leq 0.05$) compared with controls, Artesunate alone and combined treatment of artesunate and extract. Plasma total protein concentrations were significantly ($P \leq 0.05$) increased in all treatment groups . Artesunate alone significantly increased ($P \leq 0.05$) plasma ALT, ALP and liver GGT activities while the combined treatments showed a modulatory effect as these activities were significantly decreased ($P \leq 0.05$) nearly to control levels .Also, artesunate elicit significant ($P \leq 0.05$) increases in plasma urea and creatinine concentrations which were attenuated to control levels in the combined treatment.Results are indicative of extract ability to inhibit lipid peroxidation, boost GSH formation, increases antioxidant status, shows hepatoprotective and nephroprotective effects.

Keywords: Anti- malarial, lipid peroxidation, hepatoprotective, nephroprotective, antioxidants.

INTRODUCTION

In the developing countries of tropical Africa, Asia and Latin America, Malaria constitutes one of the major public health problems. It was estimated that over (350-500) million people are infected by malaria parasite world-wide where among the four species of Plasmodium parasites; *P. falciparum, P.vivax, P.ovale, P. malariae*, the *P. falciparum* is the most virulent parasite and is highly present in all malaria endemic regions of the world especially in Africa. (Miller *et al*., 1996; Singh *et al*., 2004; WHO, 2005; Kumar and Srivastava, 2005; Hilou *et al*., 2006; Mbatchi *et al.,* 2006). The greater virulence of *P. falciparum* is associated with its tendency to infect a large proportion of the red blood cells, while patients infected with this species exhibit ten times the number of parasites per cubic millimeter of blood than patients infected with the other three malaria species (WHO, 2005).

Many strains of *P. falciparum* have developed resistant to some of the drugs used to treat or prevent malaria, however the alarming rate at which *Plasmodium falciparum* has developed resistance to chloroquine, artesunate and other synthetic antimalarial drugs makes it necessary to search for more effective anti malarial compounds (Mbatchi *et al*., 2006). In Africa and other countries where malaria is endemic, traditional medicinal plants are frequently used to treat or cure malaria, while it is well known that the convectional anti malarial such as quinine and artemisininis were obtained from plants. However, since malaria treatment evolved originally from traditional folkloric medicines obtained from plant preparations as herbs by various traditional healers, this treatment method still remain a promising source of new anti-malarial compounds (Ouattara *et al*., 2006; Chukwujekwe *et al*., 2009) as the use of indigenous plants have played important roles in the treatment of malaria by

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serving as good sources of novel anti plasmodia compounds whose natural products have been a good source for drug development (Mbatchi *et al*., 2006; Hilou *et al*., 2006; Challand and Wilcox, 2009).

Also with the advent of combination therapy involving artemisinins-based combinations with other anti malaria drugs, as currently approved by WHO (Looarwsuwan, 1996; Ridley, 2002) to ward off resistance. Certain bioactive agents which may serve as anti malarial drugs of plants origin abounds and are still yet to be discover while the continuous screening of these plants especially in Africa for effective malaria drugs may still be the best means or lead to either right combinations or single dosage of anti malarial drugs for overcoming resistance.

Although international efforts have been under way for decades to produce a vaccine, so far without success in order to immunize against *P. falciparum* resistance while combination of anti malarial agents has been introduced as a response to widespread drug resistance the advantages of combination therapy should be balanced against the increased chance of drug interactions (Giao and Vries, 2001).

During the last decade most of the pharmacokinetic and metabolic pathways involving anti malarial drugs have been elucidated, including the roles of the cytochrome P 450 (CYP) enzyme complex. In this way it is pertinent to note that a number of drugs currently in clinical use exert their pharmacological activities at least in part by increasing oxidative stress in the parasitized erythrocytes while ROS are often produced by the host immune system adds to the overall oxidative burden of the parasitized cell (Becker *et al*., 2004) .

Interestingly, based on the principle of oxidative stress associated with most anti malarial drugs action (Taylor and White, 2004), the present study explore the possibilities of using extract of known anti malarial medicinal plants as an agent for a possible means of an attempt to overcome drug resistance in malaria chemotherapy and in view of the common practice in poor Africa endemic area to take local anti malarial herbs in combination with orthodox medicine when privileged to have such drugs and with the increasing resistance to anti-malarial drugs, this study investigates the roles of methanol leaves extract of *Vernonia amygdalina*, a local anti malarial in combination with artesunate, a combinative therapy using artemisinins based drugs since treatment failures have been recorded with artemisinins combination treatment (ACTs) in some endemic regions especially in Africa, hence the justification for this study.

Vernonia amygdalina is a perennial herb belonging to the *Asteraceae* family. It is popularly known as bitter leaf, an under shrub of variable height with petiolate green leaves of about 6mm diameter. The leaves are usually bitter and are very popular soup vegetable in Nigeria (Iwu, 1982; Igile *et al*., 1995). The plant is found in Savannah regions and through central and south tropical Africa. All parts of the plant (leaves, stem and roots) are said to have medicinal uses such as cure of tonsillitis, fever, malaria, diabetes, pneumonia, jaundice, anaemia, stomach problem and ascaris (Iwu, 1982). Extracts of the plant have been used in various folk medicines as remedies against helminthes, protozoan and bacterial infections with few scientific support for these claims. In this study the combinative effect of the methanol extract of *Vernonia amygdalina* on artesunate activities as a possible model to study the potential effects of non-synthetic bioactive agents of medicinal plants with known anti malarial drug was examined.

MATERIALS AND METHODS

Materials

Materials and laboratory equipment used in this study includes: Cages, micropipette, test tubes, hand gloves, cotton wool, distilled water, electric blender, refrigerator, Artesunate tablets, *Vernonia amygdalina* leaves, Chemical Weighing balance, water bath, centrifuge, spectrophotometer, beakers, homogenizer, conical flask, cuvette, measuring cylinders, separating funnels, pH meter, thermometer, bottles, syringes, test tube racks etc.

Reagents

Methanol, sodium carbonate, sodium hydroxide, ascorbic acid, phosphate buffer, Tris HCl, potassium chloride, ferrous sulphate, Tris buffer , distilled water, normal saline, laboratory kits for determination of total protein, Alkaline phosphatase, Alanine aminotransferase, gamma glutamyl transferase activity, Trichoroacetic acid (TCA), Thiobarbituric acid (TBA) etc. All other chemicals were of the highest quality grade available obtained from British Drug house (BDH) and Sigma Chemical Company, USA.

Plants material and preparation of extract

Fresh healthy leaves of *Vernonia amygdalina* were collected within the premises of the Botanical garden Ladoke Akintola University of Technology, Ogbomoso Oyo State .The leaves were identified and confirmed by Dr. A.T.J Ogunkunle of the Botany Unit of the Department of Pure and Applied Biology of the same Institution with herbarium voucher number LHO 283 deposited. The leaves were air dried at room temperature for two weeks on the ceramic slabs in the laboratory and powdered. The powdered leaves was weighed and kept in a well labeled transparent plastic container. Methanol extract was prepared using 300g of the powdered leaves soaked in 3liters of methanol (95%v/v) and kept for 3 days in the dark cupboard. The colored methanol filtrate solution obtained from the mixture was decanted into a

clean dry breaker. The filtrate was concentrated by placing it in a water – bath at 40^0C to obtained the dry methanol extract.

Experimental Animals

Twenty four healthy male Wistar Albino rats obtained from the animal house of the Department of Biochemistry, University of Ibadan were used for this study. All animals were fed on rat pellets and maintained under standard laboratory conditions for two weeks before the start of the experiment. They were allowed food and water ad libitum before and during the experiment and randomly selected into four groups A, B, C, and D with six animals in each group. Handling and treatment of the animals throughout the experiment were based on the regulations and guard lines for handling laboratory animals by our institution which conform with international standards.

Experimental design and administration of test substances

Artesunate was administered at 2mg/kg body weight orally as its standard therapeutic dose for three days and this was applied to the administration of the extract based on the average weight of the experimental animals (190g). Hence four treatment groups (A, B, C, and D). were identified based on the experimental design and treated as follows; The control (Group A), given 0.1ml normal saline intravenously two times in a day morning and evening for three consecutive days).

Group B; administered orally with methanol extract of *Vernonia amygdalina* (in 0.1ml corn oil containing 0.38mg of extract twice a day for three days) while, 0.1ml containing 0.38mg of standard drug (Artesunate) was administered orally to the rats in Group C twice a day for three days.

Group D (combinative therapy or treatment) was also administered with 0.1ml of each drug and extract separately and simultaneous through oral means of route of administration, twice in a day for three consecutive days. The groups and the agents administered are shown below:

Group A: Control rats received only normal saline solution

Group B: Received only methanol extract at therapeutic dose

Group C: Received only artesunate at therapeutic dose

Group D: Received both artesunate and extract at therapeutic dose.

Collection of Blood Sample and preparation of plasma and tissue homogenates

All the animals in each group were sacrificed at the end of the treatment period by cervical dislocation. Blood was collected directly from the heart into plain and well-labelled sample bottles containing heparin anticoagulant and were centrifuged at 4000rpm for 5 minutes to obtain plasma for analysis of biochemical indices. One gram of the liver sample was homogenized in KCl [10 mM] phosphate buffer (1.15%) with ethylene-diamine tetra acetic acid (EDTA, pH 7.4) and centrifuged at 12, 000 rpm for 60 min. The supernatants were used to assay for reduced glutathione and malondialdehyde concentrations.

Biochemical indices

Plasma proteins, urea and creatinine concentrations were estimated by the principles and procedures of Lowry *et al*. (1951), Naito (1984) and Murray (1984) using diagnostic kits. Enzyme assays, Alanine Aminotransferase (ALT), Alkaline Phosphatase (ALP) and Gamma glutamyl transferase (γGT) were determined using the method of Wroblwski *et al*. (1956), Burtis (1999) and Szasz (1969) based on the standardized methods by the International Federation of Clinical Chemistry.

Antioxidants indices

Percentage inhibition of lipid peroxidation was determined using the method of Ruberto *et al*. (2000). Ferrous sulphate induces lipid peroxidation and measured lipid peroxide formed with ascorbic acid as standard were determined Spectrophotometrically. Malondialdehyde levels and reduced glutathione (GSH) determinations were carried out by the methods of Varshney and Kale (1990) and Beutler *et al*. (1963).

Statistical Analysis

Statistical analysis were based on the Duncan`s experimental analysis with mean standard deviation of sample analyzed using the student T test and $p< 0.05$ i.e. 95% level of significance or confidence limit (Bliss, 1967).

RESULTS AND DISCUSSION

Reports of chloroquine resistant strains of *P. falciparum* are being documented in all regions of the world where malaria is endemic while resistance to anti-malaria drugs other than Chloroquine such as Pyrimethamine /sulfadoxine, Mefloquine, Quinine, Halofantrine and artemisinins based drugs such as artesunate are occurring at an alarming rate such that WHO now recommends protocols, and change their drug policy significantly with the introduction of Artemisinins based combinative therapy ACTs. While artemisinins are one of the few classes of drugs useful to treat severe malaria that is resistant to Chloroquine, its compounds include artemether, artesunate and dihydroartemisinin which are sesquiterpene lactone with a peroxide bridge and their anti malarial activity has been attributed to their peroxide moiety (Klayman *et al*., 1985) that generates free radicals which damages the parasite membrane (Muller *et al*., 2004), while their possible toxicity such as neurotoxicity

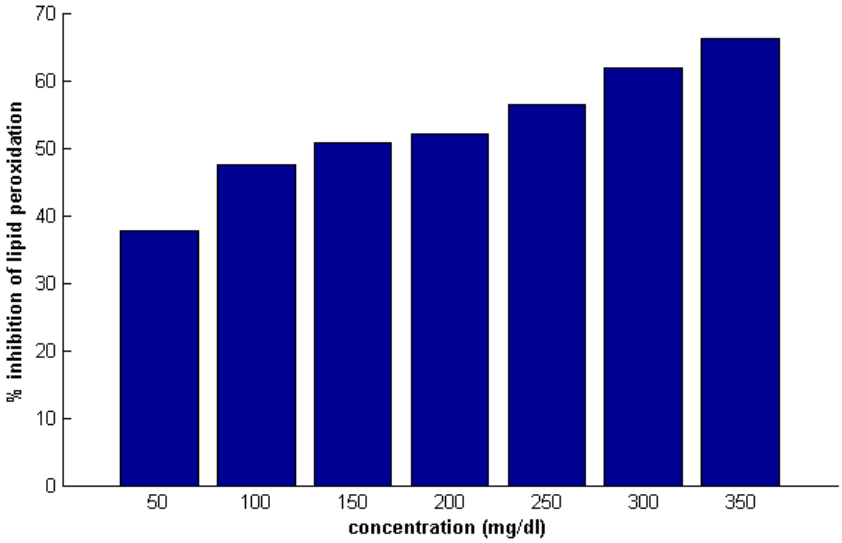

Fig. 1. Percentage Inhibition of lipid peroxidation by methanol extract of *Vernonia amygdalina* at various concentrations.
Values are given as mean and standard deviation of six determinations
P value (P≤ 0.05) as the level of significance.
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P value (P≤ 0.05) as the level of significance.

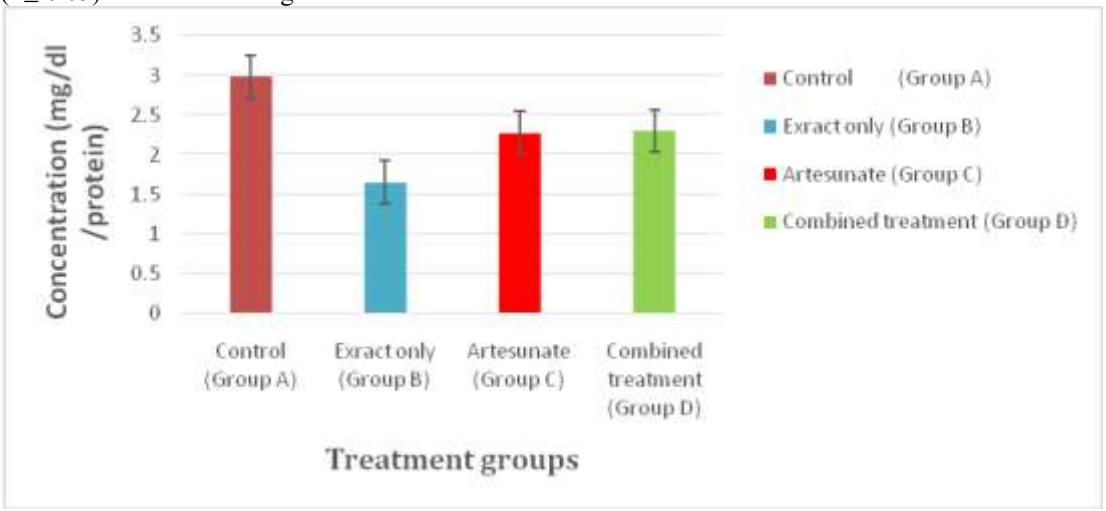

Fig. 2. Malondialdehyde (MDA) levels in the liver of various treatment groups.
Values are given as mean and standard deviation

and reproductive toxicity has been reported (Woedenbag *et al*., 1994; Li *et al*., 2005; Clark *et al*., 2008).

Medicinal plants have formed the basis of health care throughout the world right from the ancient times, the development of novel anti malarial drugs as a tool for combating malarial drug resistance is the reigning trend in malaria chemotherapy, hence search for new drugs with better and cheaper substitutes from plant origin is a natural choice especially in poor populations of malaria endemic regions in Africa. However studies have shown that the medicinal values of these plant lies in some bioactive substances present in them that produce a definite physiological action on human body (Ames and Forward, 1994), hence investigating them in a relation to most claims by the traditional healers and users is imperative in order to determine their potentials as sources of new anti malarial agents (Farombi, 2003).

In this study co-administration of artesunate and methanol extract of *Vernonia amigdalina* leaves used and known locally in Nigeria and across West Africa as a potent anti malarial plant was examined with the aims of validating or recognition of ACTs and as a means to source for

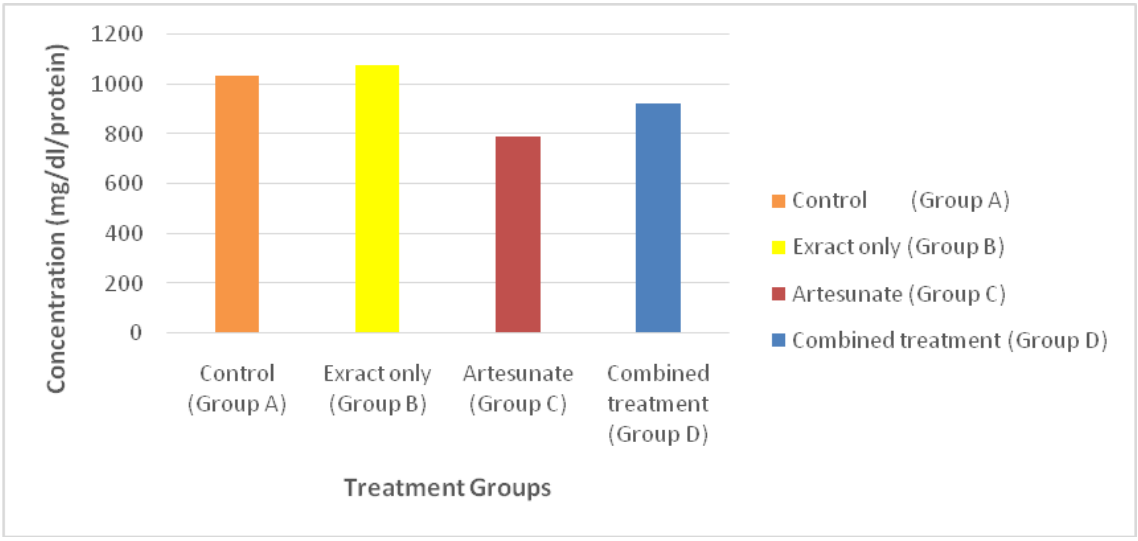

Fig. 3. Reduced Glutathione (GSH) Concentrations in the liver of various treatment groups.
Values are given as mean and standard deviation of six determinations
P value (P≤ 0.05) as the level of significance.

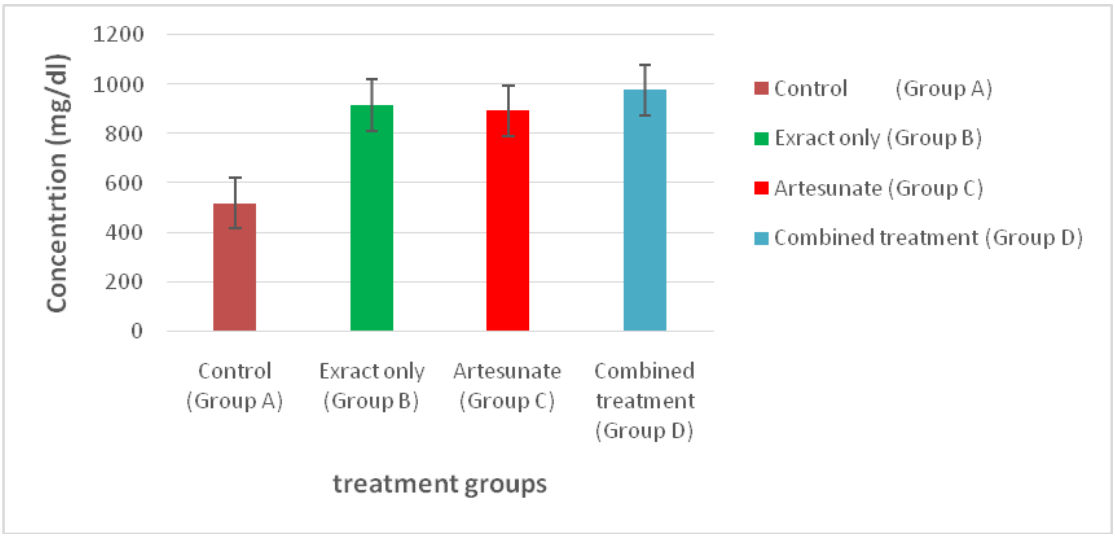

Fig. 4. Plasma protein concentrations of various treatment groups.
Values are given as mean and standard deviation of six determinations
P value (P≤ 0.05) as the level of significance.

indigenous plant based anti malarials whose bioactive agents may be useful and could be combined with artesunate probably as a common practice among poor African countries which depends solely on these herbs and often combined them with synthetic drugs when they are privileged to have them or whenever they are available or affordable.

Numerous studies have suggested that oxidative stress take part in the pathogenesis of thrombocytopenia associated with malaria as a result of the cumulative effects of free radicals and ultimately the chain process of lipid peroxidation whose continuous reactions leads to the formation of Malondialdehyde (MDA) and conjugated di -ene compounds which are cytotoxic and mutagenic damaging cell membranes (De Vries *et al*., 1997; Taylor and White, 2004; Ejiofor *et al*., 2006). However, one of the important marker for evaluation of natural herbal products that can be harnessed as an anti malarial is the ability of such agents or compounds to arrest the process of lipid peroxidation (Marnett, 1999). In Figure 1, Ferrous sulphate -induced lipid peroxidation in the liver homogenates was inhibited in a concentration –dependent manner by the extract and maximally at 350µg/ml by

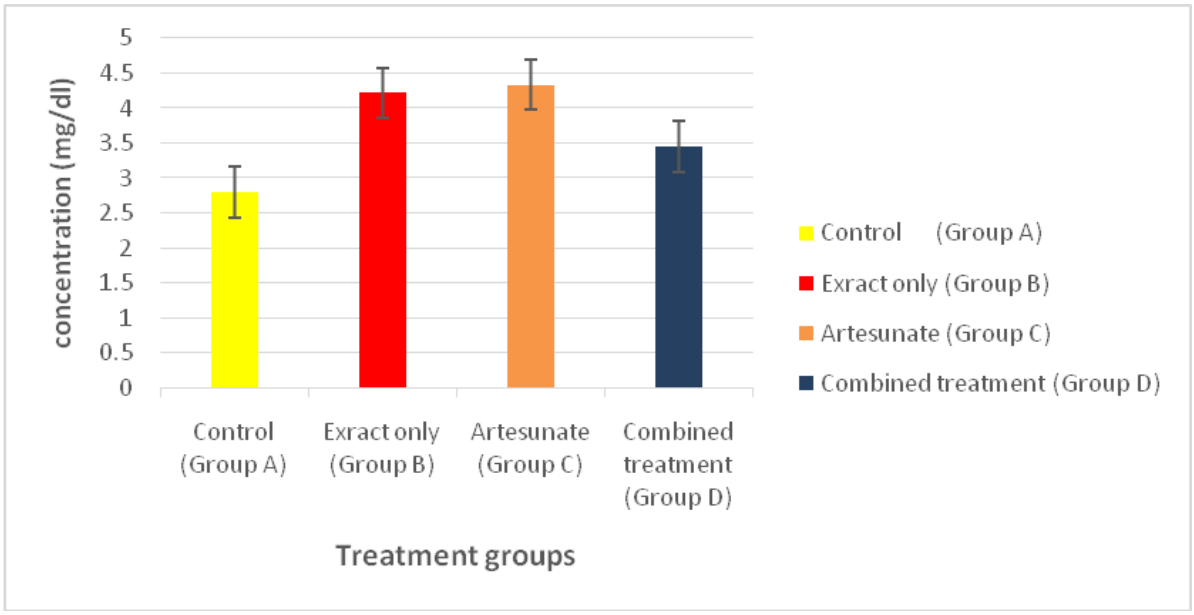

Fig. 5. Plasma urea concentrations of various treatment groups.
Values are given as mean and standard deviation of six determinations
P value (P≤ 0.05) as the level of significance.

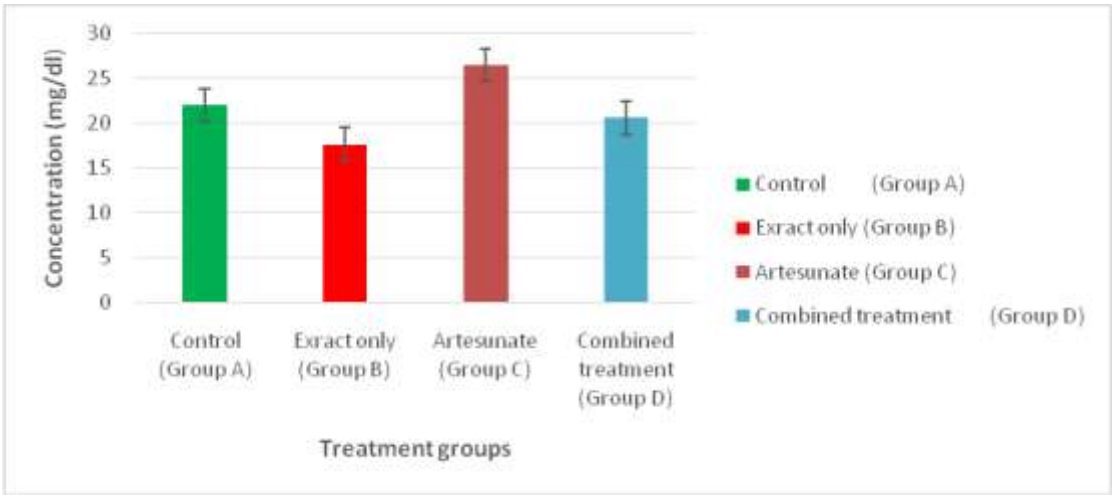

Fig. 6. Plasma Creatinine concentration of various treatment groups.
Values are given as mean and standard deviation of six determinations
P value (P≤ 0.05) as the level of significance.

66.19% an indication that the extract convered 50% cellular protection. Likewise in Figure 2, the extract alone elicit significant decreases ($p<0.05$) in the level of MDA (group B) compared with the controls and the artesunate group. The extract also modulates artesunate activity in bringing the MDA to control level when the combined treatment (group D) is compared with the standard drug artesunate (group C). The ability of the extract to inhibit MDA formation and lipid peroxidation is an indication of its potential to inhibit a variety of acute or chronic patho physiological processes associated with diseases (Ames *et al*., 1993; Adedosu *et al*., 2015).

Reduced glutathione (GSH) is a tri peptide of glycine, glutamic acid and cysteine, it is the most abundant intracellular small molecule thiol, an important antioxidant with roles in detoxification of variety of electrophilic compounds and peroxides via catalysis by glutathione S-transferases (GST) and glutathione peroxidases (Gpx) preventing damage to important cellular components caused by oxidants and free radicals (Pompella *et al*., 2003). A deficiency of GSH put the cell at risk of oxidative damage especially with oxidative stress associated with most anti malarial hence an imbalance of GSH level observed in a wide range of

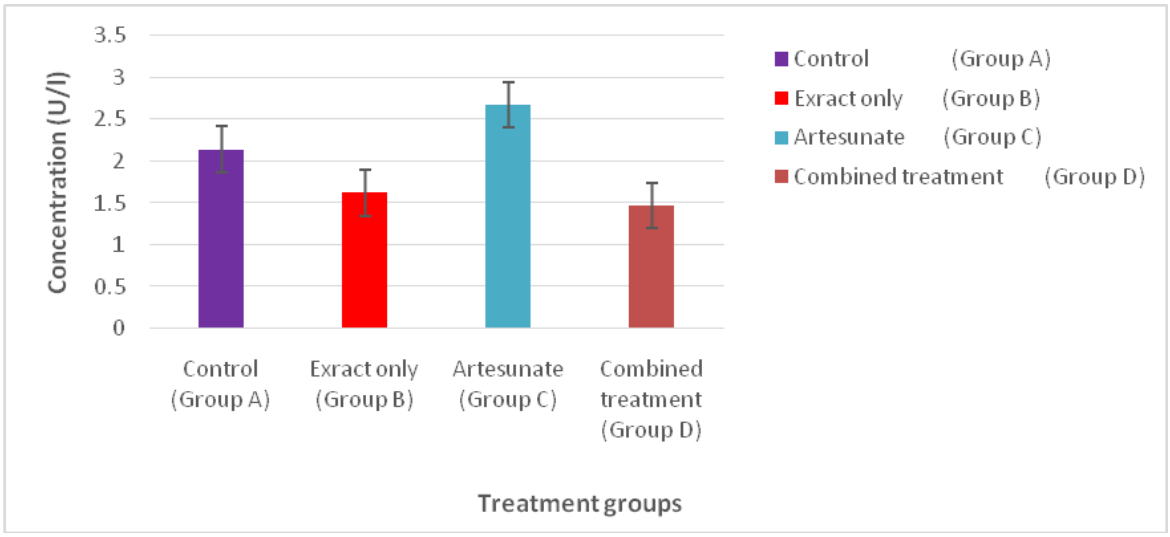

Fig. 7. Plasma ALT activities of various treatment groups.
Values are given as mean and standard deviation of six determinations
P value (P≤ 0.05) as the level of significance.

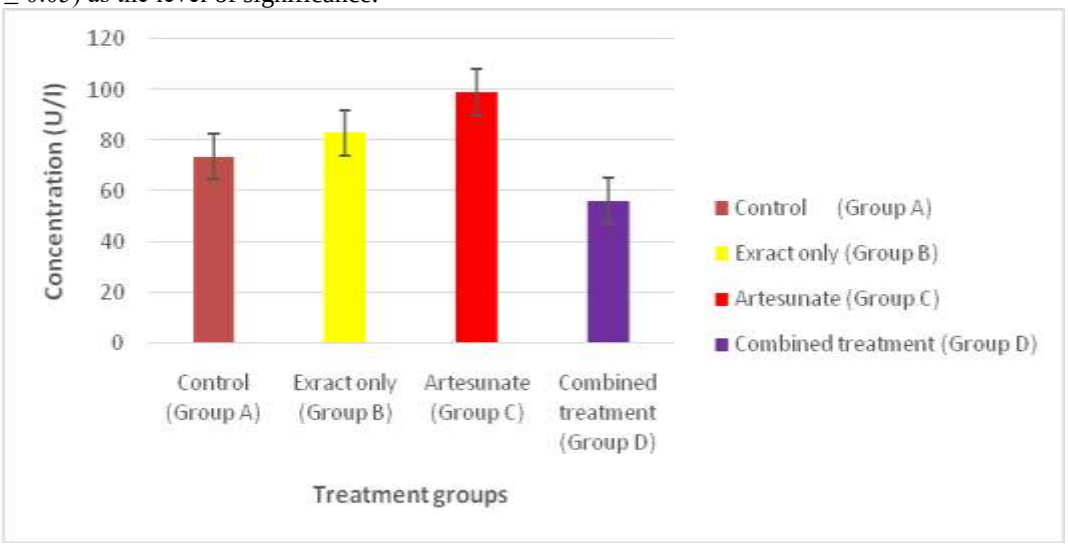

Fig. 8. Plasma ALP activities of various treatment groups.
Values are given as mean and standard deviation of six determinations
P value (P≤ 0.05) as the level of significance.

pathologies including malaria, diabetes, cancer, neurodegenerative disorders, cystic fibrosis, HIV and aging serves as a measure of cellular toxicities (Pastore *et al*., 2003). In this study *Vernonia amygdalina* methanol extract boost GSH levels as it significantly increase ($p<0.05$) GSH concentrations compared with other treatment groups (Fig. 3). The standard drug, artesunate however elicit a significant decrease ($p<0.05$) in GSH concentrations suggestive of overproduction of free radical associated with the mechanism of the drug action which may be implicated with diminished hepatic glutathione GSH concentrations ,as to combat the over production of the free radicals ,hepatic GSH stores might have been exhausted. However, with the combined treatment of the extract and artesunate, this shows significant increases in GSH level compared with the standard drug alone, suggestive of the extract ability to boost body's antioxidant status, protects ,detoxifies and modulates the activity of the drug (Mitchell *et al*., 1973).

In Figure 4, a significant increase ($P\leq0.05$) in plasma total protein concentrations in all the treatment groups compare to controls were observed. However, the combined treatment (artesunate + extract) has the highest increase in plasma protein concentration when compared with other treated groups. Behaviour exhibited by the combined treatment might not be unconnected with their ability to increase certain tissue protein concentrations which might

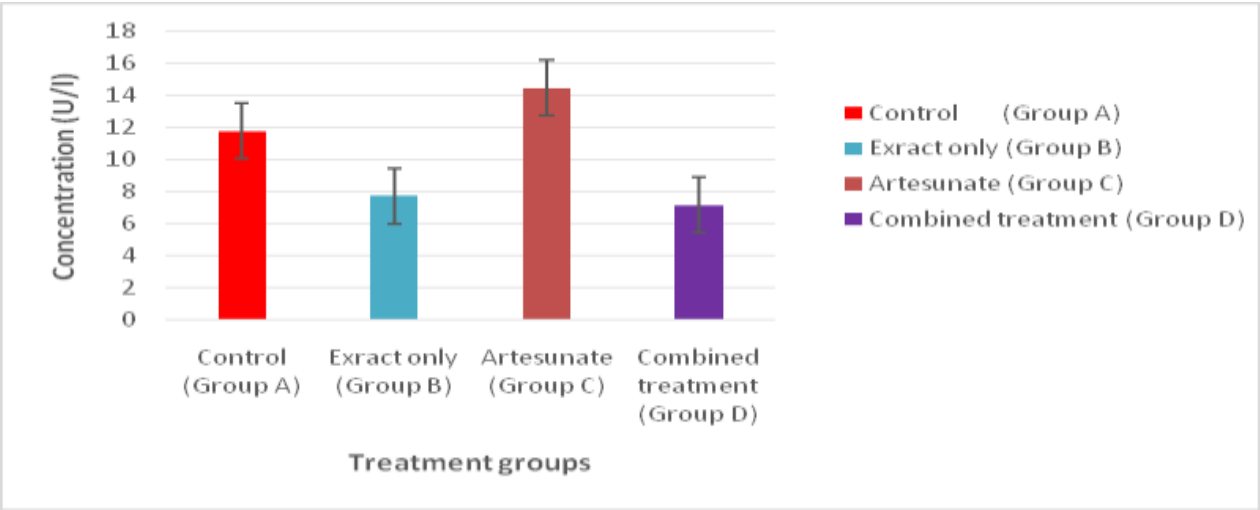

Fig. 9. GGT activities in the liver of various treatment groups.
Values are given as mean and standard deviation of six determinations
P value ($P \leq 0.05$) as the level of significance.

have been reduced due to their degradation by malaria parasites.

Also, in the current study, the concentrations of plasma urea and creatinine were examined as obtained in Figures 5 and 6 in various treatment groups. Significant elevation of plasma levels of urea and creatinine in Group C ,may explain the presence of strong correlation between nephrotoxicity and oxidative stress associated with the drug in the treatment of malaria (Li *et al*., 2006). The elevated H_2O_2 and O_2^- production alters the filtration surface area and modifies the filtration coefficient; both factors could decrease the glomerular filtration leading to accumulation of urea and creatinine in the blood. The combined treatment group reduces plasma urea at nearly control level while similar trends were obtained in creatinine clearance with the combined treatment showing the highest creatinine clearance thus showing a modulatory effect on the drugs action.

Administration of artesunate alone resulted in the significant increases ($P \leq 0.05$),in the plasma activities ofAlanine Aminotransferase and Alkaline phosphatase (Fig. 7 and 8) which are membrane bound enzymes found primarily in the liver with small amountsof ALP produced by cell lining intestines,the placenta and the kidney(in the proximal convulated tubules).This increases may be due to lipid peroxidation of biomembranes which causes leakage of cellular components into the blood (Matsuo *et al*., 1989). However rats treated with the drugs and the extract showed significant decreases in plasma levels of these enzymes , the results of this effects are suggestive of the extract capabilityto accelerate parenchymal regeneration,thus protecting against membrane fragility and subsequent decreasing leakage of marker enzymes into the circulation. That the extract has the potential to protect the liver and the kidney from artesunate - dependent damage as revealed in this report by modulating artesunate activities may be interpreted on the basis of the antioxidant properties of the extract and their constituent bioactive agents. If artesunate toxicity were dependent on radical-generating mechanisms, one would expect that an antioxidant might protect the organs from such oxidative damage which might be responsible for the anti malarial potency of the plant as shown from this study.

Similarly, the results of the activities of gamma- glutamyl transferase (Fig. 9) also indicated the modulatory potential of the extact on the drug action.

The results above suggested that the methanol extract of *Vernonia amygdalina* have anti-oxidant activities with potential protective role on the liver and the kidney in administration with artesunate and is able to modulate the effect of the drug as an anti malarial probably as a result of oxidative stress associated with its mode of action, an indication that the extract bioactive agents may be useful in drug design, template or as food supplements for prevention or combating resistance in malarial chemotherapy, hence the wide spectrum of phytochemicals ‘bottled up’ in this leaf extract, could help in preventing man from the present burden of malaria.

CONCLUSION

While clinical studies would definitely be needed to establish the safety of combining the extract with artesunate, the present study suggests that such combination may actually protect the tissues from the likely dangers associated with prolonged artesunate administration however, further research must be carried

out to identify or isolate the active compounds responsible for these effects which may be helpful in drug design and development towards eradication of resistance to malaria drugs while the local usage of the extract as anti malarial in poor endemic countries could be encouraged as more has to be done, to transform the large volume of research already done on the plant into practical, readymade nutraceuticals or phytherapeutics so that mankind may begin to effectively utilize the plant for maximal benefits especially towards malaria treatment.

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