



Antioxidant activity of the methanol stem bark extract of *Uapaca togoensis* (pax) in mice exposed to *Plasmodium berghei* NK65

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ABSTRACT

Background & Aim: Oxidative stress has been linked to the development of systemic complications in malaria infection. Recent approaches in treatment of malaria suggest that the control of oxidative stress in infected patients may be of therapeutic advantage. The stem bark and leaves of *Uapaca togoensis* are used locally in the treatment of various diseases including malaria. This study aimed to evaluate the antioxidant activities of the methanol stem bark extract of the plant in *Plasmodium berghei* infected mice.

Experimental: Mice were inoculated intraperitoneally with 0.2 ml of parasitized erythrocytes and parasitemia level assessed after 72 h by the preparation of thin blood films stained with Geimsa stain. The mice were divided into five groups of six mice each. Groups I and V were administered with distilled water (10 ml/kg) and chloroquine (5 mg/kg) orally for four days. The extract at doses of 250, 500 and 1,000 mg/kg were orally administered to groups II, III and IV, respectively, for the same period. Mice were sacrificed under light chloroform. Blood was collected by cardiac puncture and centrifuged at 2,500 rpm for 15 minutes to obtain the serum. The serum was then analyzed to determine the levels of glutathione (GSH), superoxide dismutase (SOD), catalase (CAT) and lipid peroxidation product (malondialdehyde - MDA).

Results: Oral median lethal dose of the methanol stem bark extract of *Uapacatogoensis* was estimated to be greater than 5,000 mg/kg. Administration of the extract to *P. berghei* infected mice produced a significant ($p < 0.05$) increase in superoxide dismutase, reduced glutathione and catalase levels in. However, a significant ($p < 0.05$) decrease in lipid peroxidation activity in the parasitized mice was observed.

Recommended applications/industries: The plant possesses antioxidant property which can be exploited in the management of oxidative stress caused by malaria.

1. Introduction

Malaria is an ancient life-threatening infectious disease that has affected humans for many years. Malaria is caused by a single-celled parasite belonging to the

genus *Plasmodium* (WHO, 2017). Several species of *Plasmodium* parasite have been identified notably of which five are known to cause human malaria including *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae*

and *P. knowlesi* (Tcherniuk *et al.*, 2015). However *P. falciparum* malaria is more deadly than malaria caused by other species of the parasite, and has been responsible for more than 90% of the world's malaria mortality (WHO, 2018). Infants, children under 5 years of age, pregnant women, patients with HIV/AIDS, as well as non-immune migrants and travelers are mostly at risk of malaria (WHO, 2018).

The role of oxidative stress during malaria infection is not yet fully understood (Agbafor *et al.*, 2015). Oxidative stress results from an imbalance between the generation of reactive oxygen species and endogenous antioxidant systems (Chanda and Dave, 2009). Studies suggest that the generation of reactive oxygen species and reactive nitrogen species (ROS and RNS) associated with oxidative stress is implicated in the pathogenesis and development of systemic complications caused by malaria (Guha *et al.*, 2006; Ojezele *et al.*, 2017). Malaria complications including anemia, jaundice and pre-eclampsia have been linked to oxidative stress damage caused by the parasite (Fabbri *et al.*, 2013; Sarr *et al.*, 2017). Malarial infection has been found to decrease the levels of antioxidant enzymes and other anti-oxidants such as catalase (CAT), glutathione (GSH) peroxidase, superoxide dismutase (SOD), albumin, glutathione, ascorbate and plasma tocopherol. Malaria severity has also been found to be directly proportional to the level of lipid peroxidation - an indication of membrane damage which is associated with increased malondialdehyde levels (Asagba *et al.*, 2010; Adil *et al.*, 2013). Antioxidants which are compounds that are capable of deactivating or stabilizing free radicals before they attack cells, have become of interest for the treatment of many kinds of diseases including cancer, depression and malaria inclusive (Adedapo *et al.*, 2014).

Medicinal plants have been found to be important sources of antioxidants (Rice-Evans, 2004). Studies have shown that these natural antioxidants obtained from plants such as *Glycyrrhiza glabra*, *Camellia sinensis*, *Daucus carota* and *Ocimum sanctum* have the capacity to increase the antioxidant capacity of the plasma and reduce the risk of certain diseases (Nigam and Sodhi, 2014). The plant *Uapaca togoensis* of the family Euphorbiaceae is an evergreen tree that usually grows up to about 20 meters tall. The stem is around 20 cm in diameter with roots. Its fruits are ellipsoid in shape, around 25 mm in diameter and 20 mm long,

green in color ripening to yellow and clustered on twigs below the leaves (Bretler, 2013). The plant is distributed in the Savanna regions of Senegal, Cameroun and sometimes in Sierra Leone. It is also found in West Africa and Central Africa where they occur mainly in the rainforest zones (Kadiri *et al.*, 2013). Various parts of the plant have been known to be useful in traditional medicine for the treatment of pneumonia, cough, fever, rheumatism, vomiting and epilepsy (Kone *et al.*, 2007). Previous phytochemical studies on this plant revealed the presence of steroids, triterpenes cardiac glycosides, saponins, tannins, flavonoids and alkaloids (Omachi *et al.*, 2015). Reports have shown that *Uapaca togoensis* leaf, stem bark and fruit extracts possess numerous pharmacological activities including; antibacterial (Kone *et al.*, 2007), larvicidal (Azokou *et al.*, 2013), antiplasmodial (Olorukooba *et al.*, 2018), antifungal and antimicrobial activities (Omachi *et al.*, 2015; Seuquep *et al.*, 2016). Compounds isolated from the fruits of *U. togoensis* namely; β -amyryl acetate, 11-oxo- α -amyryl acetate, lupeol, pomolic acid, futokadsurin B, arborinin, and 3-O- β -D-glucopyranosyl sitosterol have demonstrated strong cytotoxic activity on many drug-resistant and sensitive cancer cell lines (Kouete *et al.*, 2015). Recent biochemical advances have been focused on antioxidants and their potency in minimizing the damaging effects of free radicals, as well as their roles in potentiating drug efficacy (Aja *et al.*, 2015).

There has been no documented report on the antioxidant activities of the methanol stem bark extract of *Uapaca togoensis*. Therefore, this study was primarily designed to assess the ability of the plant extracts to ameliorate or lower the oxidative stress caused by malaria infection.

2. Materials and Methods

2.1. Plant collection

The plant material *Uapaca togoensis* (stem bark) was collected in a compound in Edumoga District, Okpokwu Local Government Area, Benue State, Nigeria in September, 2014. It was identified and authenticated by Malam M. D. Musa in the Herbarium section of the Department of Biological Sciences Ahmadu Bello University, Zaria and compared with an existing voucher specimen. Voucher specimen number 1279 was obtained for future reference.

2.2. Extraction

The stem barks were washed and shade dried for two weeks till a constant weight was obtained. The dried stem barks were size reduced to powder using a mortar and pestle. The powdered (2 kg) material was then soaked in 70% w/v methanol in a maceration chamber with intermittent shaking for 72 hours. This was followed by filtration using a filter paper (Whatman No. 3). The filtrate was kept in an evaporating dish to evaporate and dryness. The methanol extract was stored in an air tight container in a desiccator.

2.3. Phytochemical screening

Preliminary phytochemical screening was carried out using the method as described by Trease and Evans (2002).

2.4. Parasite inoculation

The parasite *Plasmodium berghei* NK 65 was obtained from Nigeria Institute of Medical Research (NIMR), Lagos, Nigeria. The parasites were kept alive by continuous intraperitoneal injection of 0.2 ml of infected blood containing about 1×10^7 *P. berghei* parasites into new groups of mice every 5-10 days. Each mouse used in the experiment was inoculated intraperitoneally with 0.2 ml of infected blood containing about 1×10^7 *P. berghei* parasitized erythrocytes. This was prepared using the method as described by David *et al.* (2004).

2.5. Animals

The study was carried out using Adult Swiss albino mice (18-22 g) of both sexes obtained from the Animal House of the Department of Pharmacology and Therapeutics, Ahmadu Bello. They were maintained on standard animal pellets and water *ad libitum*. Permission and approval for animal studies were obtained from the Ahmadu Bello University, Zaria Committee on Animal Use and Care. Experiments performed on the laboratory animals were in accordance with the criteria outlined in the Guide for the Care and Use of Laboratory Animals by the National Institute of Health (Publication No. 80-23, 2011).

2.6. Drug and extract administration

Chloroquine (standard drug) and the methanol extract used in the study were orally administered with the aid of a stainless metallic feeding cannula.

2.7. Determination of oral median lethal dose (LD50)

The oral median lethal dose (LD₅₀) of the extract was estimated in mice using the method of Lorke (1983). The study was carried out in two phases. The first phase involved the oral administration of different doses of the extract (10, 100 and 1,000 mg/kg) to groups of three mice each. The animals were observed for manifestation of physical signs of toxicity such as diarrhea, writhing, convulsions, decreased motor activity, decreased respiration and death over 24 hours. Following the first phase, a second phase was initiated in which 4 animals were each orally administered with graded doses of the extract (1,200, 1,600, 2,900 and 5,000 mg/kg) respectively. Observations for signs of toxicity and death was carried out for 24 hours after which the LD₅₀ value was determined by calculating the geometric mean of the lowest dose that caused death and the highest dose for which the animal survived as follows:

$$LD_{50} = \sqrt{\text{Highest non lethal dose} \times \text{Lowest lethal dose}}$$

2.8. Animal grouping, inoculation and extract administration

Thirty (30) mice of both sexes were used. The mice were injected intraperitoneally with standard inoculums of 0.2 ml containing approximately 1×10^7 *P. berghei* infected erythrocytes on the first day. Seventy-two hours later, parasitaemia was confirmed using thin blood films prepared from the cut tail tip of the infected mouse and stained with Giemsa stain (1%), then, viewed with the microscope at x 100 magnification. The mice were divided into five groups namely; I, II, III, IV and V consisting of six mice each. The negative control (I) was treated daily with 10 ml/kg distilled water, while the positive control group (V) was treated with 5 mg/kg body weight of chloroquine, an antimalarial drug. Groups II, III and IV were administered with the extract at doses of 250, 500 and 1,000 mg/kg respectively. The extract and drug were administered as single oral daily dose for four days. On the seventh day mice were starved overnight and sacrificed under chloroform anesthesia and blood collected.

2.9. Preparation of serum

Blood was collected from each mouse into sterile plain bottles and centrifuged at 2,500 rpm for 15

minutes at room temperature to obtain the serum which was used for the analysis of SOD, CAT, GSH and MDA levels.

2.9.1. Determination of malondialdehyde levels

Lipid peroxidation levels were measured by the thiobarbituric acid (TBA) reaction using the method of Ohkawa *et al.* (1979). One hundred and fifty microliter (150 μ L) of the supernatant from the serum was diluted to 500 μ l with deionized water. 250 μ L of 1.34% thiobarbituric acid were added to all the tubes, followed by the addition of equal volume of 40% trichloroacetic acid. The mixture was shaken and incubated for 30 minutes in hot boiling water bath with a temperature > 90°C. The test tubes were then cooled to room temperature. The amount of malondialdehyde (MDA) formed was measured as the intensity of the pink-colored complex formed spectrophotometrically at 532 nm (in this assay absorbance at 532 nm is taken as a measure of MDA or lipid peroxidation). The MDA level was calculated using the formula below:

$$\text{MDA (nmol/L)} = \frac{\text{absorbance} \times 100}{1.56}$$

2.9.2. Determination of superoxide activity

Superoxide dismutase was assayed as described by Misra and Fridovich. About 0.5 ml of serum and 1.5 ml of phosphate buffer pH 7.8 were added into a test tube and mixed thoroughly. The mixture was then centrifuged for 5 minutes. 0.2 ml of the supernatant was transferred into another test tube. 2.5 ml of phosphate buffer and 0.5 ml of adrenaline (0.011 ml epinephrine) were added to the test tube containing the supernatant. The absorbance of the test at 480 nm wavelength was read at interval of 30 seconds for 3 minutes against the blank.

2.9.3. Determination of catalase activity

Catalase activity was measured using Aebi's method (1974). Exactly 10 μ L of serum was added to a test tube containing 2.8 mL of 50 mM potassium phosphate (buffer pH 7.0). The reaction was initiated by adding 0.1 mL of freshly prepared 30 mM hydrogen peroxide (H_2O_2) and the decomposition rate of H_2O_2 was measured at 240 nm for 5 minutes on a spectrophotometer. One unit of CAT activity represented the amount of enzyme that destroyed 1 μ mole H_2O_2 /min and was calculated using the below formula:

$$\text{Activity (unit/ml enzyme)} = \frac{3.45}{\text{min}} \times 0.1$$

Where 3.45 = 3.45 micromoles of hydrogen in reaction mixture producing a decrease in absorbance at 240 nm

Min = Time in minutes required for the absorbance at 240 nm to decrease

0.1 = Volume (mL) of enzymes

2.9.4. Determination of glutathione activity

To 150 μ l of serum (in phosphate - saline buffer pH 7.4), 1.5 mL of 10% trichloroacetic acid was added and centrifuged at 1,500 g for 5 minutes. One (1) mL of the supernatant was treated with 0.5 mL of Ellman's reagent and 3 mL of phosphate buffer (0.2M, pH 8.0) and absorbance was read at 412 nm. The quantity of GSH was obtained from the graph of a GSH standard curve plotted by taking absorbance at 412 nm on the Y-axis and concentration on the X-axis and expressed as mg/dl (Ellman, 1959).

2.10. Statistical analysis

Experimental data in triplicates were expressed as Mean \pm SEM (Standard Error of Mean). Results were subjected to statistical analysis using one-way analysis of variance (ANOVA) to determine the differences between groups. Differences between means at 5% level ($p < 0.05$) were considered significant.

3. Results and discussion

Malaria remains a global health problem that greatly affects the underdeveloped and poverty-stricken countries (Ibrahim *et al.*, 2012). The Sub-Saharan Africa region has continued to suffer from a disproportionately high incidence of the disease (WHO, 2018). During malaria infection, free radicals are generated due to the activities of the plasmodium parasite in the hosts' erythrocytes. These free radicals interact with antioxidants (hosts defense system) to inactivate the antioxidant enzymes producing a state of oxidative stress (Opajobi *et al.*, 2018). The biological system tries to protect itself against the damaging effect of these activated species by the actions of free radical scavengers and chain terminator antioxidant enzymes such as SOD, CAT and reduced GSH system (Weydert and Cullen, 2010; Onyesom *et al.*, 2015).

The result from this study revealed an increase in lipid peroxidation levels (MDA) and decrease in the activities of superoxide dismutase (SOD), catalase

(CAT) and the concentrations of reduced glutathione (GSH) in parasitized untreated mice when compared to the extract treated mice. This suggested the presence of an oxidative environment and stress in the parasitized untreated mice which could have occurred because of toxic effect of elevated free radicals produced by immune system as well as synchronized release of O^{2-} during haemoglobin degradation by plasmodium parasite (Erel *et al.*, 1997).

Measurement of malondialdehyde (MDA) levels were used as an index for lipid peroxidation as studies have shown a positive correlation between increase in lipid peroxidation and oxidative stress (Cabral *et al.*, 2011). SOD is a chain breaking enzymatic antioxidant which inhibits the damaging phase of reactive oxygen species (Valko *et al.*, 2007). The enzyme employs superoxide anion as a substrate converting it to hydrogen peroxide which is subsequently broken down by CAT (Koracevic *et al.*, 2011). CAT and GSH (also classified as preventive antioxidants) act by inhibiting the initial production of free radicals including reactive oxygen species (Valko *et al.*, 2007). They protect tissues from oxidative stress damage by decomposing highly reactive hydrogen peroxide to oxygen and water (Chelikani *et al.*, 2004).

A decrease in the level of antioxidants observed in serum of *Plasmodium* infected rodents has been suggested to be an indication of their increased utilization due to oxidative stress (Ogbuehi *et al.*, 2014; Agbafor *et al.*, 2015; Opajobi *et al.*, 2018). Administration of the methanol stem bark extract of *Uapaca togoensis* produced a significant ($p < 0.05$) dose dependent decrease in serum MDA levels compared to the negative control (distilled water treated) group (Figure 1). The reduction in MDA levels produced by the 1,000 mg/kg dose of extract was comparable to that exhibited by the standard drug (Chloroquine, 5 mg/kg). The ability of the extract to reduce MDA levels suggests that the extract reduced oxidative stress, since an increase in lipid peroxidation has been linked to cause oxidative stress (Momoh *et al.*, 2015). In comparison with the negative control (distilled water treated group), a significant increase in lipid peroxidation was observed. This shows that *P. berghei* malarial parasite infection induced oxidative stress in the mice.

The extract at 500 and 1,000 mg/kg, significantly ($p < 0.05$) increased serum CAT levels compared to the negative control (Figure 2).

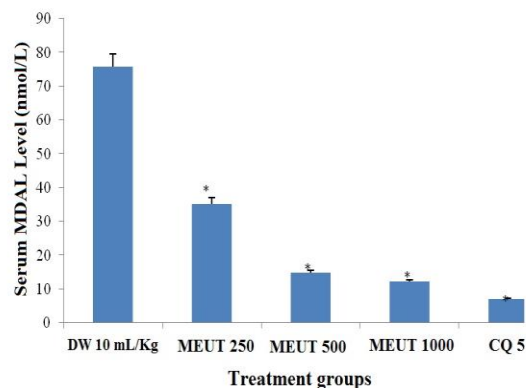


Figure 1: Effect of methanol stem bark extract of *Uapaca togoensis* on serum malondialdehyde levels in *Plasmodium berghei* infected mice. Values are presented as Mean \pm SEM; Data analyzed using one way ANOVA followed by Dunnett's Post-hoc test; DW = Distilled water; MEUT = Methanol stem bark extract of *Uapaca togoensis*; CQ = Chloroquine; MDA = Malondialdehyde; * = $p < 0.05$ compared to DW group; n = 6.

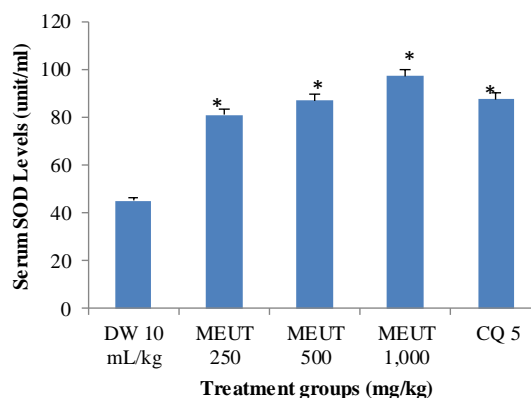


Figure 2: Effect of methanol stem bark extract of *Uapaca togoensis* on serum superoxide dismutase levels in *Plasmodium berghei* infected mice. Values are presented as Mean \pm SEM; Data analyzed using one way ANOVA followed by Dunnett's Post-hoc test; DW = Distilled water; MEUT = Methanol stem bark extract of *Uapaca togoensis*; CQ = Chloroquine; SOD = Superoxide dismutase; * = $p < 0.05$ compared to DW group; n = 6.

Similarly, a significant ($p < 0.05$) dose dependent increase in serum GSH and SOD levels were also observed on administration of the methanol stem bark extract of *Uapacato goensis* compared with the negative control (Figures 3 and 4). The increase in levels of serum SOD, CAT and GSH activities by the methanol extract suggests that the extract could scavenge free radicals. This also suggests that the extract may be

exerting its protective actions through the inhibition of the proliferation of *Plasmodium berghei* parasite and free radicals.

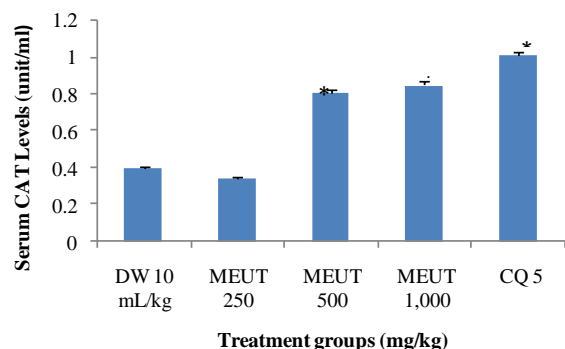


Figure 3: Effect of methanol stem bark extract of *Uapaca togoensis* on serum catalase levels in *Plasmodium berghei* infected mice. Values are presented as Mean \pm SEM; Data analyzed using one way ANOVA followed by Dunnett's Post-hoc test; DW = Distilled water; MEUT = Methanol stem bark extract of *Uapaca togoensis*; CQ = Chloroquine; CAT = Catalase; * = $p < 0.05$ compared to DW group; $n = 6$.

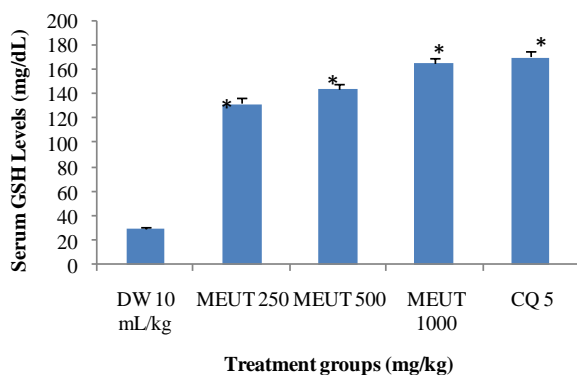


Figure 4: Effect of methanol stem bark extract of *Uapaca togoensis* on serum glutathione levels in *Plasmodium berghei* infected mice. Values are presented as Mean \pm SEM; Data analyzed using one way ANOVA followed by Dunnett's Post-hoc test; DW = Distilled water, MEUT = Methanol stem bark extract of *Uapaca togoensis*; GSH = Glutathione; CQ = Chloroquine; * = $p < 0.05$ compared to DW group, $n = 6$.

According to Iribhogbe *et al.* (2013), antioxidant micronutrients can potentiate erythrocyte membrane stabilization. Also, Agbafor *et al.* (2015) in their study further postulated that the antiplasmodial activity of *Hyptis Spicigera* and *Cytopogon Citratus* may be due to the ability of the extracts to reduce the effects of free radicals observed in *P. berghei* infected mice by stabilizing the erythrocyte membrane from the harmful

manifestation of the free radicals generated from parasite degradation of host hemoglobin. Thus, the methanol stem bark extract of *Uapacatogoensis* may be acting through a similar mechanism.

Studies have proposed that the antiplasmodial/antimalarial activity of some medicinal plants including *Piper betle* (Al-Adhroey *et al.*, 2011), *Dodonaea angustifolia* (Amelo *et al.*, 2014) and *Alchornea laxiflora* (Okokon *et al.*, 2017) may be due to their antioxidant activity. Thus, the antioxidant activity exhibited by the methanol extract of *Uapaca togoensis* may also contribute to its antiplasmodial activity. Preliminary qualitative phytochemical screening of the extract revealed the presence of glycosides, steroids, triterpenes, saponins, tannins, flavonoids and alkaloids. According to David *et al.*, (2004), saponins, flavonoids and tannins have been suggested to act as primary antioxidants or free radical scavengers that can counteract the oxidative damage induced by the malaria parasite. Although the exact mechanism of *Uapacatogoensis* action needs to be elucidated, the presence of these phytochemicals singly or in combination may be responsible for its antioxidant and antiplasmodial activities.

4. Conclusion

Plasmodium berghei malarial infection resulted in a marked depletion of the host serum antioxidant enzymes while elevating the levels of lipid peroxidation (observed in the form of its product MDA). Treatment of infected mice with *Uapacatogoensis* stem bark extract reduced lipid peroxidation and oxidative stress probably by improving antioxidant defense system. This was shown by the increased serum levels of CAT, GSH and SOD and a reduction in MDA levels. The plant may thus be useful in the treatment malaria and management of oxidative stress.

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