IMMUNOREGULATORY PROPERTIES OF N-HEXANE EXTRACT OF *OSMUNDASTRUM CINNAMOMEUM* IN TREATMENT OF *PLASMODIUM BERGHEI* INFECTION IN MICE

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ABSTRACT

Malaria control is threatened by the emergence of drug-resistant parasites and there is an urgent need for the development of antimalarial agents with novel mechanisms of actions. This study evaluated the anti-plasmodial and immune-modulatory activities of N-hexane leaf extract of Osmundastrum cinnamomeum in a mice model. Chloroquine-resistant Plasmodium berghei infected mice were separated into six treatment groups and treated orally with 0, 50, 100, 200 and 400 mg/kg of extract, water and combination of dihydroartemisinin/ piperaquine (DHAP), respectively. Parasitological activities and survival rates were monitored for 30 days' post infection. Phytochemical composition of O. cinnamomeum was analysed by gas chromatography-mass spectrometry (GC-MS) methods. Levels of TNF-α and IL-10 were assessed using enzyme-linked immunosorbent assay (ELISA). Leaf extract of O. cinnamomeum is rich in terpenoids, saponins and cardiac glycosides. The extract showed significant (p<0.05) antiplasmodial effect in the treated groups relative to parasitemia (23.68 %) in the untreated control on day 13. Parasitaemia was significantly higher in the DHAP group (9.83 %) on day 30 compared to extract treatment of 50 mg/kg (2.09 %) and 100 mg/kg (1.83 %). Significantly low level of TNF-α (28.82 pg/ml) and conversely, high expression of IL-10 (79.04 pg/ml) were recorded in the 50 mg/kg test group. There was a significantly higher survival rate of animals in the same group (50 mg/kg). In conclusion O. cinnamomeum demonstrated potential activity to suppress parasite and also prime the immune system against malaria infection in mice. Therefore, O. cinnamomeum may be used as a potential adjunctive therapy in the treatment of malaria infection.

Keywords: *Osmundastrum cinnamomeum*, *Plasmodium berghei*, Immunomodulation, Inflammatory cytokines

INTRODUCTION

Malaria is a leading cause of death among children in Sub-Saharan Africa, accounting for 40 % mortality in 2016 among Nigerian children (WHO, 2017). More than 90 % of the Nigerian population is at risk of stable endemic malaria and about 50 % of the total population have at least one episode of malaria across age groups (Aladesemipe *et al.*, 2013). Despite several attempts made to control the disease, success of drug treatments has been threatened by the emergence of drug resistant strains. Hence, the urgent need for an alternative therapy with

novel mechanisms of action for both prophylaxis and chemotherapy.

Malaria infection triggers a cascade of immune responses in the host; however, parasite ligands, host receptors and signaling pathways responsible for these interactions remain controversial (Wu *et al.*, 2014). During infection, cytokines play a dual role of controlling parasite growth and exacerbating pathology. These opposing effects have been attributed to the timing of cytokine expression as well as imbalance in the expression of inflammatory and anti-inflammatory cytokines (Dodoo *et al.,* 2002). Inflammatory cytokines, TNF-α and IFN-γ mediate parasite inhibition and killing, but high levels of TNF-α have also been associated with severe malaria syndromes such as cerebral malaria. In addition, high levels of the anti-inflammatory cytokine IL-10 or high IL-10/TNF-α ratios reduce the risk of severe malarial anemia (Kabyemela *et al.,* 2013).

History has shown that plants are important sources of medicine for the treatment of human diseases (Nostro *et al.*, 2000; Bako *et al.*, 2005). Quinine and artemisinin are anti-malarial drugs extracted from *Cinchona* spp. (Family: Rubiaceae) and *Artemisia annua* (Family: Asteraceae) respectively (Willcox and Gilbert, 2004). Curative properties of medicinal plants have been attributed to the presence of secondary metabolites which includes alkaloids, sterols, tannin, saponin, phenol and flavonoid (Bero *et al.*, 2009). Chinese people consume fern dishes made from a variety of ferns such as *Pteridium aquilinum, Callipteris esculenta, Matteuccia struthiopteris, Osmunda japonica* and *Osmundastrum cinnamomeum* (Liu *et al.*, 2012). Some of the bioactive compounds present in different edible ferns have been reported to possess antitumor, antifungal, antibacterial and anti-inflammatory properties; as well inhibitory effect on platelet aggregation (Liu *et al.*, 2012).

Osmundastrum cinnamomeum commonly called cinnamon fern is found growing in swamps, bogs and moist woodlands. It is a deciduous herbaceous plant which produces separate fertile and sterile fonds. It is a perennial fern belonging to the Family: Osmundaceae (Royal Fern Family) and one of the major contributors

of fern food market (Liu *et al.*, 2012). This study was designed to investigate the efficacy of *O. cinnamomeum* as a novel treatment for malaria. In light of this, anti-plasmodial and immune-modulatory properties of the plant were evaluated in *Plasmodium berghei* (ANKA strain) infected mice.

MATERIALS AND METHODS

Plant Collection and Extraction: The leaves of the plant, *O. cinnamomeum* were collected from Omu-Aran, Kwara State, Nigeria. The plant was identified and authenticated at the Department of Plant Biology, University of Ilorin. The leaves were air dried at room temperature and then crushed into fine powder using electric mill. The extraction of the plant components was done using the method of Nostro *et al.* (2000) with little modification. Powdered plant weighing 540 g was soaked in N-hexane solvent for five days in a jar, which was covered with foil paper and allowed to fractionalize. After five days, the extract was decanted, followed by filtration using muslin cloth (20 μm). The filtrate was then concentrated using rotary evaporator and the extract was separated from the solvent. The extract was dried, weighed and transferred into air-tight glass container and refrigerated at 4^0 C for later use.

Phytochemical Screening of the Extract: Phytochemical screening was done to test for the presence of alkaloids, saponins, flavonoids, tannins, phlobatannins, polyphenols, terpenoids, steroids, anthraquinones and glycosides following the method of Tiwari *et al.* (2011).

Measurement of Plant Composition using Gas Chromatography-Mass Spectrometry (GC-MS): The chemical composition of the extract was determined using Agilent 19091S-433HP-5MS coupled to an ionization flame detector (FID) and equipped with 30 m by 250 mm by 0.25 mm pH column. The compounds were separated on the column packed with 5 % phenylmethyl. The column temperature was initially held at 35 °C for 3 minutes with injection volume of 1.0 μL and then programmed to rise at the rate of 5° C per

minute to 280° C over a total runtime of 62 minutes at a split mode of ratio 50:1. The heater was set at 300° C, whereas the detector (mass spectrometer) temperature was maintained at 250° C. Carrier gas, helium was at a velocity of 44.3 cm/s and pressure of 11.604 psi. Ionization mode was electron impact at a voltage of 70 eV. The identification of the chemical components was done by comparison of the mass spectral with those of National Institute of Standards and Technology (NIST) Mass Spectrometry Data Center (Library). The relative amounts of the chemical components were expressed as percentages, obtained by FID peak-area normalization.

Preparation of Extract and Drugs: Stock solution of 1000 mg/kg was prepared by dissolving the extract in distilled water. The prepared stock solution was then stored in an air-tight container in the refrigerator at 4° C. Subsequently, 0, 50, 100,200 and 400 mg/kg concentrations to be administered to the experimental animals were prepared from the stock solution by serial dilution. Piperaquine and dihydro-artemisinin were also prepared according to the standard dosage of 18 mg/kg for piperaquine and 4 mg/kg for dihydro-artemisinin. Piperaquine was prepared with distilled water while dihydro-artemisinin with dimethyl sulfoxide (DMSO) and distilled water in the ratio 1:10.

Antimalarial Efficacy Testing: Animal studies were carried out in accordance with the institution's ethical standard. All experiments were conducted using a complete randomized design of six treatments, each replicated thrice. Study animals (average weight of 21 ± 1.0 g) were inoculated with 1×10^7 infected erythrocytes and separated into 6 treatment groups of 5 mice per group. The animals were fed *ad libitum*. The curative potential of the crude extract was evaluated using the method described by Ryley and Peters (1970). Treatment commenced three days after infection and the test groups were administered 50, 100, 200 and 400 mg/kg of extracts, while the negative and positive controls were administered distilled water and combination of

piperaquine plus dihydro-artemisinin respectively for four consecutive days. Blood was obtained from the tail of each mouse and thin smear was made on a microscopic slide, air-dried, fixed with 100 % methanol and stained with 10 % Giemsa for 10 minutes. The stained slides were rinsed with water and air-dried. The number of infected erythrocytes per 1000 erythrocytes were counted using microscope. Percentage parasitaemia was calculated using the formula: % parasitaemia = number of infected erythrocytes ÷ total number of erythrocytes x 100.

Collection of Blood for Measurement of Cytokine Proteins in Serum: Blood samples were collected by retro-orbital bleeding from mice in each group before and after treatment on Day 3, 6 and 13 respectively. Blood was also obtained from a set of uninfected, untreated mice to serve as baseline. The blood samples were collected into eppendorf tubes, allowed to clot for 30 minutes at 4^0 C and later cold centrifuged at -6^0 C at a speed of 12000 rpm for 25 minutes to separate the serum from plasma. The blood plasma and serum were separated using micro-pipette and the sera were stored in the refrigerator at -80^0 C for subsequent analysis. The levels of IL-10 and TNF-α were measured using commercial BosterImmunoleader and Bioscientific ELISA kits following the manufacturers' instructions and as reported by Raza *et al.* (2014). The range of cytokine detection was 15.6 – 1000 pg/ml

Survival Test for the Infected Animals: The infected animals were observed daily for 30 days to determine the mean survival rates for treated infected mice as well as non-treated infected mice.

Statistical Analysis: Data collected from cytokine expression and parasitaemia were analyzed using one-way analysis of variance. Statistical significance was defined as p≤0.05 using SPSS version 21.0. The differences between the groups were separated using Duncan post-hoc test. GraphPad Prism software was used for preparation of Kaplan-Meier

survival curves, parasitaemia curves and cytokine graphs.

RESULTS

The result of the preliminary phytochemical screening of N-hexane extract of *O. cinnamomeum* revealed that the plant is rich in terpenoids, flavonoids, tannins, saponins and cardiac glycosides (Table 1).

Table 1: Phytochemical screening showing presence of plant components

Samples	*Osmundastrum cinnamomeum* n-hexane
Steroid	+
Terpenoid	++
Tannin	+
Polyphenols	-
Saponin	++
Alkaloids	-
Flavonoids	+
CardiacGlycoside	++
Anthraquinone	-
Phlobatannins	-

++ Strongly present, + Present, - Absent

Gas chromatography identified majorly triterpenoids in the N-hexane extract of *O. cinnamomeum* D:A-friedoolean-6-ene (16.25 %) and olean-12-en-3-one (15.08 %) compounds were found to be abundant (Table 2).

Table 2: Gas chromatographic identification of bioactive components

Peak No	Retention Time	% Relative Abundance*	Compound Names
1.	14.88	2.89	phytol
2.	15.08	7.03	α-Amyrin
3.	15.33	1.71	Ethyl 9-hexadecenoate
4.	17.92	2.52	2-Monopalmitin
5.	19.15	15.08	Olean-12-en-3-one
6.	19.92	16.25	D:A-Friedoolean-6-ene

Other compounds were detected at relatively much lower concentrations.

The result of *O. cinnamomeum* N-hexane extract on parasitaemia showed that low parasitaemia levels were observed in all test groups from day 3 to 6, with highest peak being 1.24 % on day 6, compared to the controls. Parasitaemia consistently rose to 8.78 % in the negative control group on day 8, peaking at 23.68 % on day 16 prior to mortality. For the test groups, 50 and 100 mg/kg had significantly lower ($p<0.05$) parasitaemia level of 2.09 and 1.83 % compared to the positive control group which had a higher percentage of 9.83 % on day 30 post-infection (Figure 1).
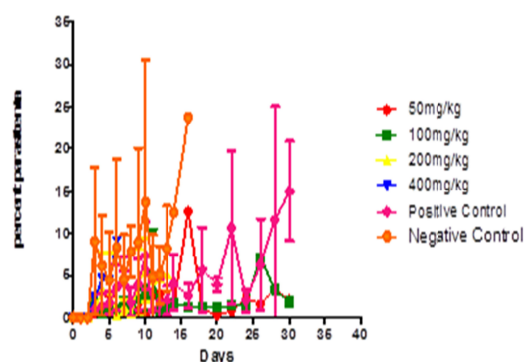

Figure 1: Comparison of level of parasitaemia between groups using Graphpad prism

The positive control and the 100 mg/kg test group showed steady and high survival rates of 95.46 and 95.00 % respectively from day 6 to 8. Survival rates of 80.00, 85.00 and 90.91 % were recorded in the negative control, 50 and 200 mg/kg groups respectively. At day 14 to 30, there was no change in survival rate in the test groups and the positive control, a steady survival rate of 78.46 % for 50 mg/kg, 82.701 % for 100 mg/kg, 60.606 % for 200 mg/kg and 95.46 % for positive control were observed. The 400 mg/kg and negative control had the least survival outcome. Continuous decline was recorded in the negative control, with survival rate of 40 % on day 13 prior to total mortality (Figure 2).
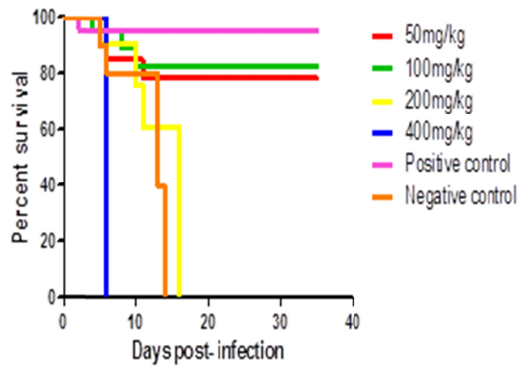
Figure 2: Survival percentage of *Plasmodium berghei* infected mice treated with *Osmundastrum cinnamomeum*

On day 3, 50 mg/kg group showed highest TNF-α expression of 21.26 pg/ml. Other test groups, 100, 200, 400, positive and negative controls expressed TNF-α levels were 12.93, 11.10, 18.00, 11.71 and 16.16 pg/ml respectively. As the treatment progressed to day 6, the level of TNF-α in 50, 100 and negative control groups considerably increased to 102.92, 238.31 and 58.06 pg/ml respectively compared to all other test groups, whereas the level decrease significantly in 200 mg/kg and positive control groups to 5.37 and 7.13 pg/ml respectively. Subsequently, at day 13 level of TNF-α decreased significantly in the 50 and 100 mg/kg groups to 28.82 and 88.57 pg/ml respectively, while that of 200 mg/kg and positive control groups increased significantly to 100.42 and 84.45 pg/ml respectively (Figure 3).
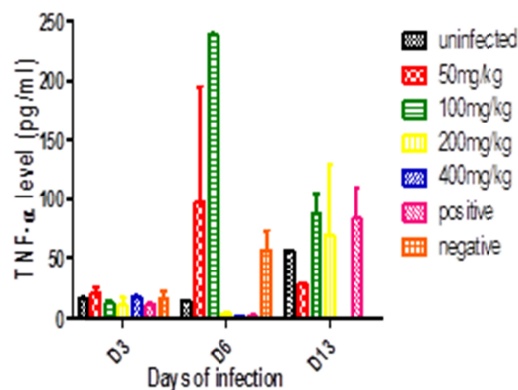

Figure 3: TNF-α levels in *Plasmodium berghei* infected mice treated with N-hexane extract of *Osmundastrum cinnamomeum*

There was no significant difference in the IL-10 levels of 50, 100, 200, 400 mg/kg, positive control and negative control group of mice at 3.90, 4.73, 7.53, 5.35, 4.02 and 3.52 pg/ml respectively in day 3 before inoculation. On day 6, 100 and 200 mg/kg test groups showed slight increase in the level of IL-10 at 9.31 and 13.74 pg/ml respectively. However, at day 13, 50,100, 200 mg/kg and positive control groups showed a significant increase in the level of IL-10 at 79.04, 67.54, 173.39 and 55.78 pg/ml respectively (Figure 4).
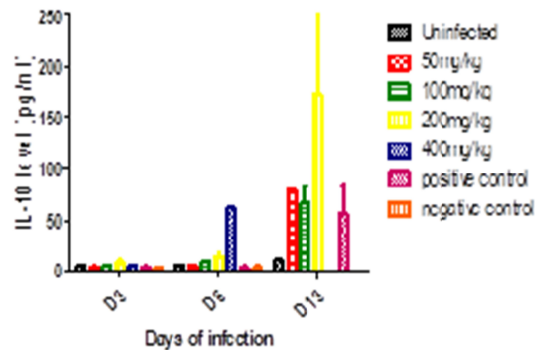

Figure 4: IL-10 levels in *Plasmodium berghei* infected mice treated with N-hexane extract of *Osmundastrum cinnamomeum*

DISCUSSION

In this study, phytochemical screening carried out on the N-hexane extract of *O. cinnamomeum* showed the presence of terpenoids, saponins, flavonoids and cardiac glycosides. These metabolites have been reported to have anti-plasmodial and immunomodulatory effects on malaria parasite (Belay, 2008; Kaur *et al.,* 2009; Al-Adhroey *et al.,* 2011). Flavonoids have been reported to possess anti-oxidant property and are capable of inhibiting inflammatory disorders (Eri *et al.,* 2000; Ram *et al.,* 2012). Thus, *O. cinnamomeum* may be a primary source of potential lead molecules in the drug discovery of compounds with antioxidant properties. Flavonoids present in *A. annua* conferred immunomodulatory effect in subjects afflicted with parasitic and chronic diseases. Consequently, administration of artemisinin in combination with flavonoids has been reported to be effective in the treatment of malaria

(Ferreira *et al.,* 2010). Therefore, flavonoids present in the N-hexane extract of *O. cinnamomeum* may have exerted absorptive effect that enhanced the anti-plasmodial effect of the extract in mice.

Furthermore, saponins are immunostimulatory adjuvants composed of triterpene, steroidal or alkaloid nuclei associated to carbohydrate moieties. Saponins exert therapeutic actions on the immune system by modulating the expression of IL-2, IL-4, IL-10 and IFN-Y in damaged endothelial cells (Hu *et al.*, 2016). Saponins isolated from *Radix glycyrrhizae* or *Radix achyranthes* have been reported to significantly enhance immune responses in mice (Kong *et al.,* 2004). Therefore, the presence of saponins in the N-hexane extract of *O. cinnamomeum* may be responsible for the expression of TNF-α and IL-10, thereby conferring immunity to *P. berghei* infection in the test groups.

GC-MS analysis showed the presence of high percentage of triterpenoids in the extract is an indication that the plant is endowed with terpenoids which could work synergistically with other metabolites to produce antimalarial and anti-inflammatory effects. These compounds have been reported to possess anti-inflammatory, anticancer and antioxidant functions (Volkman, 2006). D:A-Friedoolean-6-ene and D:A-friedoolean-4-en-2β have been identified as a constituent of the flowers of *Rhododendron arboretum* and root back of *Tripterygium wilfordii,* respectively (Gautam *et al.*, 2016; Yang *et al.*, 2001). D: A- friedoolean-6-ene was also isolated from the stem bark of *Mallotus philippensis* and reported to possess anti-tumour-promoting activity (Tanaka *et al.*, 2008). Olean-12-en-3-onehas also been identified as a constituent of *Vellozia graminifolia* (Branco *et al.*, 2004). This explained the anti-plasmodial and immune-modulatory properties of the *O. cinnamomeum* used in this study.

In this study, significant lower level of parasitaemia was observed in the test groups 50 mg/kg and 100 mg/kg. This is probably responsible for the higher survival rates in the two groups at the end of the study. Total mortality observed in negative control group was an indication that *O. cinnamomeum* had immunological and plasmocidal effects on the treated infected groups. The potential of *O. cinnamomeum* to mediate parasite clearance, regulate cytokine expression and improve survival outcome in infected mice suggests that the plant may exert dual mechanism of action, in terms of immunomodulatory and plasmocidal properties and may thus be a potential adjunctive candidate for the management of uncomplicated malaria.

IL-10 has emerged as a key immunoregulator during infection with viruses, bacteria, fungi, protozoa, and helminths, ameliorating the excessive Th1 and CD8 T cell responses that result in overproduction of IFN-Y and TNF-α and immunopathological outcomes associated with infections of *Toxoplasma gondii, Trypanosoma* spp.*, Plasmodium* spp. (Moore *et al.,* 2001; Couper *et al.,* 2008). IL-10 inhibits the activity of Th1 cells such as NK cells, and macrophages which are required for optimal pathogen clearance but nonetheless contribute to tissue damage. In consequence, IL-10 can both impede pathogen clearance and ameliorate immunopathology (Couper *et al.,* 2008).The potentials of 50 and 100 mg/kg doses of O. *cinnamomeum* to hinder parasite growth, culminating in low parasitaemia and prolonged survival may be attributed to the timely expression of pro-inflammatory cytokine (TNF-α) during the early phase of infection and a consequential modulation of its expression by the secretion of IL-10 as infection progressed further. Low level of IL-10 production is reportedly associated with enhancement of TNF-α production, followed by increased IFN-γ production (Niikura *et al.*, 2011) consequently leading to immunopathological outcomes (Wu *et al.*, 2014). Andargie and Ejara (2015) reported that excessive or mistimed IL-10 production can inhibit pro-inflammatory response to *Plasmodium* spp., *Leishmania* spp., *T. cruzi* and lymphocytic choriomeningitis virus, to the extent that pathogens escape immune control, resulting in either rapidly fatal or chronic non-healing infections.

Poor survival outcome and high parasitaemia observed in 400 mg/kg test group may be due to excessive IL-10 secretion during

early phase of infection on day 6, inhibiting pro-inflammatory response (TNF-α expression) necessary for parasite clearance. Also, total mortality seen in the untreated control before day 14, in contrast to high survival rates in 50 and 100 mg/kg groups, might be due to the higher parasitaemia level of 23.68 % in the control and immunopathological response generated due high TNF-a expression. High TNF/IL-10 ratio is reportedly responsible for severe malarial anaemia (SMA) via erythrophagocytosis and depressing erythropoiesis (Boeuf *et al.*, 2012).

There were significant differences (p<0.05) in the cytokine expression levels (both IL-10 and TNF-α), as infection progressed from day 6 to 13. This showed that malarial infection triggers differential cytokine expression patterns during early and late phase of infection. Ademolue *et al.* (2017) reported that differential cytokine expression during clinical malaria is based on parasitaemia levels and transmission intensity.

Ferreira *et al.* (2005) reported the presence of terpenes and polyphenols in *Artemisia annua* and these metabolites were reported to show beneficial effects in treatment of cardiovascular disease, cancer, and most importantly parasitic diseases such as malaria. Terpenes were also significantly abundant in the extract of *O. cinnamomeum* investigated in this study. Artemisinin, the recommended first line treatment for treatment of uncomplicated malaria is a major derivative of *A. annua* (WHO, 2010) and this evidence suggests that *O. cinnamomeum* may be a potential source of lead antimalarial derivatives. There are accumulating evidence on the synergistic effects of terpenes and polyphenols with anticancer drugs and with anti-parasitic drugs, besides the facts that some polyphenols have curative effect. Based on documented evidence on the anti-malarial properties of *A. annua* (Elfawal *et al.,* 2012), *O. cinnamomeum* can be mirrored as a potential source of novel chemotherapy based on the similarities in phytochemical composition such as the presence of terpenes.

Conclusion: The results obtained from this study indicate that N-hexane extract of *O.*

cinnamomeum possesses promising anti-plasmodial and immunoregulatory potentials. The extract contains active metabolites such as saponins, flavonoids, steroid and terpenes, which have been reported in literature, to be responsible for the curative properties of medicinal plants. Pro-inflammatory and anti-inflammatory cytokine expression at the early and late phases of infection may be attributed to the modulatory advantage conferred by the extract in regulation of immune response. GC-MS analysis identified some major compounds such as; olean-12-en-3-one, α-amyrin, triterpeniods, which may have mediated parasite clearance, improved survival and enhanced protective immunity in infected mice. Therefore, this plant can be used as a potential adjunctive therapy in the treatment of malaria infection.

ACKNOWLEDGEMENTS

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