IN VIVO AND *IN VITRO* EVALUATION OF THE INHIBITORY EFFECT OF SOME MEDICINAL PLANT EXTRACTS ON HAEMOZOIN CONCENTRATION

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ABSTRACT

The resistance of current drugs against malaria parasite is increasing, thus the need for evaluation of the haemozoin (HZ) concentration in malaria parasite as a novel strategy for malaria control. Haemozoin load in the blood of patients was measured after taking antimalarials or plants extracts. The tested plant extracts were established to reduce HZ concentration in vivo. Haemozoin was extracted from the blood samples of all the malaria positive patients studied by centrifugation and the concentration analyzed spectrophotometrically at 400 nm wavelength. Comparative anti-malaria activity of some conventional drugs: Maldox, Halfan, Artecxin, Amatem, Mefloquine (quinolines) and Malmed, the leaf and stem back extracts of Sarcocephalius latifolius and Alstonia boonei, containing potent pyhytochemicals including tannins, flavonoids, saponins, alkaloids, was evaluated to establish the most effective agent for haemozoin reduction and subsequently, malaria therapy. Each agent was administered to patients in each malaria episode, and the absorbance of haemozoin produced determined at 4000 nm wavelength. Packed cell volume (PCV) was estimated to establish the proportion of red blood cells before and after haemozoin production, using microhaematocrit reader. All the chemical antimalarial drugs used effected reduction in haemozoin concentration. However, Mefloquine (Quinolines) showed the highest activity with significant difference of 0.01 ($p<0.05$). The plant extracts similarly exerted significant reduction in the haemozoin concentration. Nevertheless, Alstonia boonei extract was the most effective in haemozoin reduction at 0.00 significant level ($p<0.05$). Of all the therapeutants (chemical and plant extracts) tested, Alstonia boonei stem back extract most significantly reduced haemozoin production ($p<0.05$), indicating its potential for use in novel anti-plasmodium and anti-malaria drug formulation.

Keywords: Haemozoin, Antimalarial agents, Haemoglobin degradation, *Plasmodium falciparum*

INTRODUCTION

Haemozoin is a disposal product formed from the digestion of haemoglobin present in the erythrocytes by blood-feeding malaria parasites by means of its lysate enzymes such as plasmepsins, falcipains and dipeptidylpeptidase. During malaria infection, the parasite ingests

abound 5mM of haemoglobin as a source of amino acids. As it grows, free toxic haeme, which is capable of generating oxygen radicals are released together with globulin (protein component) after haemoglobin catabolism in an acidic lysosome like organelle. The digestion of haemoglobin releases toxic monomeric α-haematin called haeme, which is capable of generating oxygen radicals (Lew *et al.,* 2003).

A haeme is a prosthetic group that consists of reactive iron which forms a unique iron-oxygen coordinate bond found in the centre of a heterocyclic porphyrin ring, as a result of this, it is also called iron protoporphyrin, or ferriprotoporphyrin (fePPTX), which is toxic to the cells of the malaria parasites *Plasmodium falciparum* (Pagola *et al.,* 2000). For the malaria parasite to survive it therefore detoxifies the toxic haeme by secreting haeme polymerase enzyme which converts the toxic haeme into non-toxic, insoluble and chemically-inert ß-haematin crystals called haemozoin, otherwise called the malaria pigment (Egan *et al.,* 2001). The formation of haemozoin is thus known as bio crystallization (Bennett *et al.,* 2004). The primary function of haemozoin is to prevent oxygen radical-mediated damage to the parasite. Another vital function of haemozoin is essentially the removal of the reactive iron, haeme out of solution in the oxygen rich acidic environment of the vacuolar compartment where haemoglobin degradation occurs (Sallusto, 2001).

Many chemically used drugs are thought to act by inhibiting the formation of haemozoin in the food vacuole (Buller, 2004). This prevents the detoxification of the haeme released in this compartment and kills the parasite. The best understood examples of such haematin bio crystallization inhibitors are quinolines drugs such as chloroquine and mefloquine. These drugs bind to both free haeme and haemozoin crystal and therefore block the addition of new haeme units into the growing crystals (Dedios, 2003). Haemozoin formation during malaria infection results in the reduction of packed cell volume (PCV) of the patients due to the consumption of red blood cells by the merozoites. This reduction gives rise to anaemia which is one of the symptoms of severe malaria (Clarke, 2002). Hence, evaluation of packed cell volume of patients following malaria treatment in each episode of malaria attaches is an essential step in assaying for the effectiveness of the target drug.

Haemozoin formation is an excellent anti-malarial drug target, since it is a process that is essential for the survival of the malaria parasites (Gligorijevic, 2006). The drug target haematin is host-derived and largely outside the genetic control of the parasites, which makes the development of drug resistance more difficult (Jani *et al.,* 2006). Therefore anti-malaria drugs to be used or produced for therapeutic purposes are those that act by inhibiting the formation of haemozoin in the food vacuole of the parasite. This prevents the detoxification of the haeme released in this compartment, and thus, killing of the parasite (Pisciotta, 2006).

Each day, malaria disease claims the lives of over 3000 people, 90% of whom are children in sub-Sahara Africa (WHO, 1998). Among the more than 120 *Plasmodium species* that infect reptiles, birds, and mammals, four protozoan species of *Plasmodium* infect humans and *P. falciparum* is more common, causing over 90% of the deaths (Ekpeyong and Eyo, 2006). In tropical geographic areas such severe *Plasmodium falciparum* malaria morbidity and mortality affect all ages from children to adults (Kazmi and Pandit, 2001). Malaria is endemic throughout the regions of Nigeria. The World Health Organization (WHO) estimated that malaria mortality rate for children under five in Nigeria is at 729 per 100,000. The Ministry of Health of Nigeria further indicated that malaria was responsible for one out of ten deaths in pregnant women and costs the Federal Government of Nigeria over one billion naira annually (PRCU, 2005).

Increased multi-drug resistance of malaria parasites especially *Plasmodium falciparum* to conventional orthodox antimalarials in Nigeria has necessitated the search for a cost effective toxic free and highly active compound from natural sources for the management of malaria and associated anaemia. Quinine extracted from the bark of the

cinchona tree, has been used as an antimalarial agent in Nigeria for several years.

There is increasing resistance of malaria parasites to chloroquine, the cheapest and commonly used drug for malaria in Nigeria. Chloroquine, a quinine derivative took the pride of place in malaria control for several years, and the fear of the emergence of chloroquine – resistant strains of malaria parasites has led to the use of Artemisin, an Indian product whose active ingredient is derived from *Artemisia annua.* However, little has been done to further develop Nigerian local plants with antimalarial activity or enhance the activities of traditional or folkloric medicine in the advancement of the search for malaria cure.

Traditional medicine has nevertheless remained the most affordable and easily accessible source of treatment in the primary healthcare system of resource poor communities; the local therapy therefore, is the only means of medical treatment in such communities. The use of medicinal plants in the treatment of diseases has generated renewed interest in recent times, as herbal preparations are increasingly being used in both human and animal healthcare systems. The study observed that in spite of the advancement in modern medicine and healthcare programmes, many people in developing countries including Nigeria still rely on traditional healing practices and medicinal plants for their daily healthcare needs. Most of the antimalarial drugs currently in use were not developed on the basis of rationally selected targets, but by investigation of traditional medicinal plants (quinine and artemisinin), synthesis of analogues (CQ, mefloquine, primaquine, atovaquone), chemical modification of an active natural product (arteether, artemether, artesunate), or by assaying drugs that were used against other infectious pathogens (antifolates, antibiotics) (Fidock *et al.,* 2004). The herbal remedies are often prepared by pounding either the fresh or dried parts of the plants followed by either soaking or boiling in water, and the infusions or decoctions administered by drenching.

Sarcocephalius latifolius (African peach), of the family Rubiaceae, is a multi-stemmed tree or shrub up to 12 m. Reports of its medicinal value include its effectiveness in the treatment and management of malaria, constipation, dysmenorrhoea, abscesses, vomiting and threatened abortions stomach disorder, cough, fever and jaundices. The aqueous root bark extracts of *S. latifolius* demonstrated protection against liver toxicity induced by CCl_4 in a dose dependent manner and this was linked to the phytochemical constituents. The terminal event in the attack on the liver by CCl_4 was the production of highly reactive radicals leading to lipid peroxidation and inhibition of calcium pump of the microsome giving rise to liver lesions (Gamzi *et al.,* 1999). Several secondary metabolites have been shown to have wide ranges of antimicrobial activities. Similar, *S. latifolius,* was observed as one of the sixty-four extracts assayed from twenty-one plants used in the Malian traditional medicine that were found to be significantly active against the intracellular forms of Leishmania major. It was further showed to exhibit antiplasmodial activity against *P. falciparum* (Abreu and Pereira, 2001).

Alstonia boonei de Wild (Apocynaceae) is a large deciduous tree up to 45 m tall, which consists of 50 species widely distributed in Africa and beyond. It is called, Egbu in Igbo and is widely used by the natives for the treatment of various ailments. The stem bark of *A. boonei* has anti-inflammatory, analgesic and antipyretic activities and is commonly used against malaria (Olajide *et al.,* 2000). An infusion of the bark is used as antivenom for snake bites. It is also used in treating painful menstruation and rheumatic conditions (Azuzu and Anaga, 1991). Other documented scientific reports validating the medicinal values of *A. boonei,* include, the presence of phytochemicals such as alkaloids which possess analgesic, antispasmodic, antiplasmodial and bactericidal effects; flavonoids, potent water-soluble antioxidants and free radical scavengers which prevent oxidative cell damage and have strong anticancer activity as well as lower the risk of heart diseases. Saponins are capable of neutralizing some enzymes in the intestine that can become harmful, building the immune system and promoting wound healing. Tannins hasten the healing of wounds and inflamed

mucous membrane. Cardiac steroids are widely used in the treatment of congestive heart failure. They help in increasing the force of contraction of the heart (Okwu, 2004).

The daily increasing high rate of mortality and morbidity in the sub-Sahara Africa and especially in Nigeria as a result of multi-drug resistant malaria parasites has reached an alarming proportion. This has therefore led to a search for an alternative therapeutic regimen for effective management of malaria parasitemia especially in the malarious areas of the south-east Nigeria, the site of this research, which aims at extracting haemozoin from the blood samples containing malaria parasites, screening for, and comparing both conventional antimalarial drugs and plant extracts for antimalarial activity.

MATERIALS AND METHODS

Study Location: The study was carried out for a period of 14 months at the Bishop Shanahan Hospital (BSH), Nsukka, Enugu State, Nigeria. This hospital serves the health needs of the local community and beyond and is basically a point of healthcare for the poor and underprivileged. People from nearby rural and peri-rural communities including the border towns of Kogi and Benue states, especially those who cannot afford high hospital bills of the private and teaching hospitals in the states troop in here for attention and medicare. One of the major concerns here for which routine tests and treatment is sought is malaria, a serious scourge in this area beset with malnutrition, poverty and attendant anaemia, unhygienic conditions and drug abuse.

Ethical Issues: The approval to undertake the study was granted by University of Nigeria ethical committee. Anther approval was obtained from Bishop Shanahan Hospital Management Board to under take the study. Written consents of willingness to participate in the study as subject were obtained from 48 participants listed for the study. All listed subject were symptomatic malarial cases managed by BSH. All ethical issues involving the identity, compensation and management of the subjects followed the approved guidelines.

Plant Collection: An ethnobotanical survey of Nigerian medicinal plants commonly used in folkloric medicine for the management of malaria carried out among traditional practitioners and herbalist who both treat and sell dried and fresh plant materials at Nsukka Local Government Area, Enugu State, reveal that *S. latifolius* and *A. boonei* were commonly used singly or in combination for the treatment of malaria in the locality. This informed the selection of these plants for the study. Both plant were collected from the Botanical Garden, University of Nigeria, Nsukka and identify to species level by the curator. Voucher specimens SL-2134 and Ab-2135 were stored in the herbarium of the Department of Plant Science and Biotechnology, University of Nigeria, Nsukka.

Plant preparation and Extraction: The fresh leaves of *S. latifolius* and fresh stem back of *A. boonei* were used for the investigation. Five hundred grammes (500g) of *S. latifolius* leaves were washed and boiled in 450 cm^3 of water for 45 minutes. Three hundred grammes (300g) of the stem bark of *A. boonei* was thoroughly washed and pulverized in a mechanical home blender and soaked in 150 cm^3 of water for 6 days as practised by the traditional healers. The resulting concoctions were separately filtered and store in sealed plastic 1 litre bottles pending administration. All extracts were freshly administered.

Phytochemical Screening: Aqueous extracts of the tested plants were subjected to phytochemical analysis for the qualitative determination of phytochemical constituents using standard procedures (Sofowora, 1983).

Determination of the Effect of Selected Conventional Antimalarial Drugs and Plant Extracts on Haemozoin Concentration: The effect of conventional chemical antimalarial agents, Maldox, Halfan, Mefloquine, Amatem, Arteccin, Malmed was evaluated on 48 individuals by administration of the drugs to

each malarial patient during each malaria episode according to the prescription of the physicians in attendance. Patients were strictly followed up during the 14 months study and monitored for malaria symptoms during which prescribed doses of the different drugs were given at different malaria episodes which varied among patients: some came down with malaria faster than others; some within 2 weeks and others within 1 – 4 months intervals depending on patients' resistance to malaria attack, exposure to mosquito bites and effectiveness of each test drug on individual patient. Criteria used for drug administration included the classical malaria symptoms: fever, malaise, pains on the joints, severe weakness and in minor cases vomiting; marked reduction in PCV and presence of haemozoin in patients' blood samples. However, effort was made to administer all test drugs to each patient at different malaria episodes and the PCV and haemozoin concentration estimated simultaneously at 24 hours interval for 96 hour post administration. To ensure compliance, patients were not allowed to take drugs home but encouraged to take them in the presence of the physician and the investigators.

Administration of Plant Extract Concoctions: Concoctions of the test antimalarial plant extracts, were administered to the same malarial patients in subsequent episode of malaria (with 4 weeks interval after administration of chemotherapeutants), following examination of their blood smears for malaria parasites and evaluation of haemozoin level. Consequently, a cup (50 cm^3) of *S. latifolius* concoction was administered orally thrice daily (morning, midday and night) to patients for 5 days with concurrent estimation of their PCV and haemozoin concentration. Furthermore, patients were each monitored for a period of 2 months after which their blood samples were collected and screened for malaria parasites. All those who came down with malaria based on established criteria were subjected to the next regimen of *A. boonei* concoction; consequently, 50 cm^3 of the concoction was given by oral administration to patients twice daily (morning and night) for 3 days. Haemozoin concentration and patients' PCV were estimated following each administration at 24 hourly intervals for 96 hours. The malarial patients without the antimalarial agents served as a control experiment for both the PCV and haemozoin concentration determination.

Collection of Blood Samples: Approximately 2ml of venous blood samples were collected from malarial symptomatic patients at the Hospital and dispensed into ethyl diamine-tetra acetic acid (EDTA) vacutainer tubes.

Screening for Malaria Parasites

The test was carried out using standard Giemsa staining techniques, by thin and thick smears. The working solution was prepared by diluting 1 ml of standard Giemsa stain with 19 ml of phosphate buffer (pH 7.2). The solution was prepared prior to use (John and Petri, 2006). The test was carried out by examination of thick and thin Giemsa stained blood smears, prepared in duplicates.

Preparation of thin film: Thin films (smears) of blood samples were made on clean, greaseless slides and fixed by immersing in absolute ethyl alcohol for 30 seconds. The films were allowed to air dry, immersed in Giemsa working solution for 30 minutes and then further immersed in phosphate buffer for 10 seconds, after which they were air-dried in a vertical position. After drying, the slides were then examined at x 40 objective lens before re-examination in oil immersion (x 100 objective lens). The percent of infected RBCs was determined by enumerating the number of infected RBCs in relation to the number of uninfected RBCs. A minimum of 500 RBCs was counted. Percent infected RBCs = Number of infected RBCs ÷ Total number of RBCs counted × 100.

Preparation of thick films: Duplicate slides of prepared thick blood films were allowed to air dry completely for 1 hour or more depending on the thickness of the film. The films were immersed in phosphate buffer solution for 30

seconds and air dried in vertical position. The microscopic examination was done as described above. The number of parasites/µl of blood was determined by enumerating the number of parasites in relation to the standard number of WBCs/µl (8000). Number of parasites per µl of blood = Number of Parasites × 8000 ÷ Number of WBCs counted.

Qualitative evaluation of parasitemia: A modified qualitative three point scale of classification from CDC (2003) five point scale classification of parasitemia was adopted thus: + (1) 10 – 10,000 parasites/µl of blood = mild parasitemia, ++ (2) 10,001 – 100,000 parasites/µl of blood = severe parasitemia and +++ (3) > 100,000 parasites/µl of blood = very severe parasitemia.

Determination of Packed Cell Volume (PCV): To determine the level of haemoglobin degradation before and following administration of drug, packed cell volume (PCV), which is the amount or proportion of red blood cells present in a given sample of blood was assayed using the Microhaematocrit Reader. Approximately 5 ml of blood samples were collected from patients using sterile syringes and needles and dispensed into EDTA vacutainer tubes (Rubio *et al.,* 2001). An aliquot of the blood samples was pipetted into capillary tubes by capillary movement and centrifuged for 5 minutes using haematocrit centrifuge after which the capillary tubes containing the sediment samples were placed in a Microhaematocrit Reader and the packed cell volumes (PCV) read.

Haemozoin Extraction: A 2ml EDTA blood samples were centrifuged for 5 minutes using bucket centrifuge (Model 80-2). The supernatant was discarded and the pellets suspended in normal saline (NaCl) and further centrifuged for 5 minutes and the supernatant discarded. About 0.5ml of phosphate solution, pH 7.6 was added to each tube and vigorously shaken mechanically for 2 seconds to haemolyse the erythrocytes. The tubes were then kept on ice for 10 minutes to avoid excess haemolysis and then centrifuged for 5 minutes before discarding the supernatant. Approximately 1 ml of Tris buffered solution of pH 7.2 was dispensed into the pellets in the tubes, centrifuged for 10 minutes, and the supernatant discarded. The insoluble pellets were re-suspended in 0.5ml of 2.5% Sodium dodecyl sulphate solution (SDS), buffer with Tris buffer solution, pH 7.8 and kept at room temperature for 1 hour before centrifuging for 10 minutes. The supernatant was again discarded and the pellets once more re-suspended in 0.5ml of 2.5% Sodium dodecyl sulphate (SDS) solution buffered with Tris buffer solution pH 7.8 and kept at room temperature for 1 hour. The suspension was then centrifuged for 10 minutes, and the supernatant discarded before harvesting the SDS insoluble pellets (haemozoin) as previously described (Orji, 2001).

Determination of Haemozoin Concentration by Spectrophotometry: The weight of extracted haemozoin was determined using the Mettler weighing balance and the various masses obtained recorded. The concentration of haemozoin was calculated by completely dissolving known masses of haemozoin (in mg/ml) in 0.5ml of diluted sodium hydroxide, and the solution of haemozoin analyzed spectrophotometrically using Spectrophotometer S23A, at 400nm wavelength (Bohle *et al.,* 2005).

Statistical Analysis: Data were analysed by one-way or two way analysis of variance where appropriate at 95% Confidence intervals between means.

RESULTS

Phytochemical composition of Tested Plant Extracts: Analysis of the phytochemical constituents of the test plant extracts *S. latifolius* and *A. boonei* indicated the presence of alkaloids, saponins, tannins, steroids, terpennoids, flavonoids, cardiac glycosides with steroidal ring. However, there was absence of anthroquinones in both plants (Table 1). Highly active compounds including alkaloids, tannins, and flavonoids were more in abundance in *A. boonei* than in *S. latifolia.*

Haemozoin Extraction: A brown crystalline pigment, haemozoin in form of insoluble crystals in the sodium dodecyl sulphate (SDS) solution used for the extraction was obtained from all the malaria positive blood samples at different levels of malaria parasitemia.

Table 1: Phytochemical constituents of *S. latifolia* and *A. boonei* aqueous extracts

Phytochemicals	*S. latifolia*	*A. boonei*
Alkaloids	+	++
Saponins	+	+
Tannins	+	+++
Anthroquinones	-	-
Steroids	+	+
Terpenoids	+	+
Flavonoids	+	++
Cardiac glycosides with steroidal ring	+	++

Legend: *+ = moderate, ++ = abundant, - = absent*

Parasitemia, PCV and Haemozoin Concentration: There was significant reduction in the number of patients corresponding with increased parasitemia. Patients with mild parasitemia (19) being significantly higher than those with very severe parasitemia (13) (Table 2). Negligible and insignificant increase in the PVC of patients corresponding to increasing levels of parasitemia was observed. Proportionate increase in haemozoin concentration with increase in the level of malaria parasitemia among subjects was observed (Table 2).

Effect of Administered Conventional Antimalarial Drugs on Haemozoin Concentration: All the antimalarial medications administered to the malarial patients showed a decrease in haemozoin concentration at 400nm wavelength at 24 hourly intervals with simultaneous increase in patients' PCV values. The differences were statistically significant at 0.00 ($p<0.05$)**.** However**,** mefloquine belonging to quinoline group exerted the highest decrease on the haemozoin concentration. The difference was statistically significant ($p<0.05$). Nonetheless, Maldox a combination of sulfadoxine and pyrimethamine showed the lowest reduction in haemozoin concentration at significant level of 0.02 ($p<0.05$) (Figures 1 and 2).

Extracts of Administered *Sarcocephalius latifolius* Leaf and *Astonia boonei* Stem Bark on the Haemozoin Concentration in the Malaria Patients at 24 hour Intervals: The two anti-malarial test plant extracts, *S. latifolius* leaf extract and *A. boonei* stem back extract exerted appreciable decrease on the haemozoin concentration at 24 hour intervals. The differences were statistically significant ($p<0.05$). However, *A. boonei* extracts caused the highest decrease in haemozoin concentration with a significant increase in the PCV values ($p<0.05$) (Figures 3 and 4).

Comparative effect of Mefloquine and Alstonia boonei on Haemozoin Concentration in the Malaria Patients: Comparison of the most effective medicine, mefloquine and the plant extracts, in haemozoin reduction is presented in Figure 5. *A. boonei* nevertheless exerted much higher decrease in haemozoin concentration than the medicine, mefloquine ($p<0.05$).

DISCUSSION

Haemozoin formation is reckoned a vital step in the survival of *Plasmodium falciparum*, the malaria parasite. Haemozoin, variously identified as a black-brown by-product of haemoglobin degradation by the process of biocrystallization is a unique diagnostic tool in the malaria parasite identification and thus a laboratory marker of malaria infection. Its formation has therefore become a subject of intensive scientific research as interest in the search for efficient therapeutic regimen for malaria broadens. Due to its pivotal role in the survival of the malaria parasite, haemozoin formation by *P. falciparum* is therefore considered an essential target in the malaria drug discovery, and remains the ultimate goal of novel malaria research.

In spite of the stringent global efforts to fight and eliminate malaria, it is still recorded as one of the greatest human killers, causing almost one million deaths per year (mainly small children in Africa) and 300-400 million infections annually (WHO, 2010).

Table 2: Parasitemia, packed cell volume (PCV) and haemozoin concentration of malaria patients in Bishop Shanahan Hospital, Nsukka

Parameters	Number of patients	Blood PCV	Haemozoin concentration mg/ml	Absorbance
Parasitemia		**+ (Mild)**		
Sub total	19	7.17	0.79	12.57
Mean	-	0.38 ± 0.01	0.04 ± 0.01	0.66 ± 0.03
Parasitemia		**++ (Severe)**		
Sub total	16	6.03	1.31	12.33
Mean	-	0.38 ± 0.01	0.08 ± 0.004	0.77 ± 0.01
Parasitemia		**+++ (Very severe)**		
Sub total	13	5.05	1.9	10.5
Mean	-	0.39 ± 0.02	0.15 ± 0.01	0.81 ± 0.01

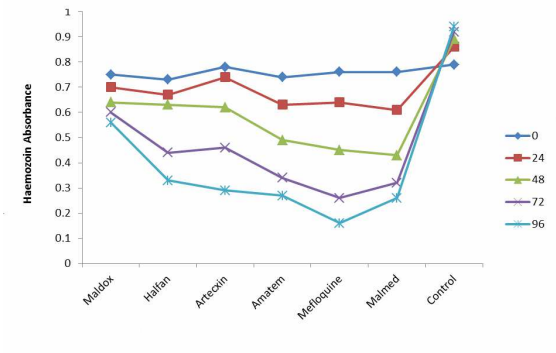

Figure 1: Effects of test anti-malarials on haemozoin concentration of patients in Bishop Shanahan Hospital, Nsukka

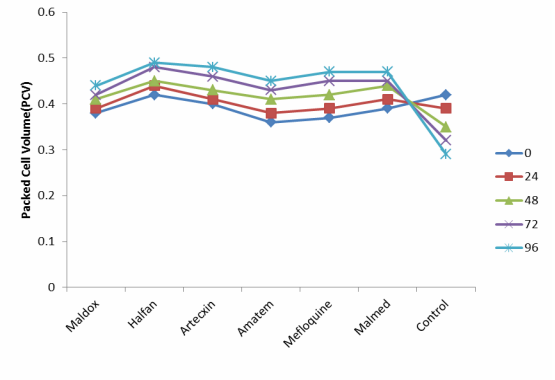

Figure 2: Effects of test anti-malarials on PCV of patients in Bishop Shanahan Hospital, Nsukka

Several approaches have been envisaged by Nigerian government in their roll back malaria strategy, and these include the mosquito larval control (vector control) strategies, indoor residual spraying (IRS), intermittent preventive treatment in infants (IPTi) and using drugs to protect infants. Currently, the cornerstone of malaria control across the globe and especially in Nigeria is the exploitation of effective and inexpensive drugs particularly those from plant sources. Hence, inhibition of haemozoin biocrystallization in the *Plasmodium* using natural products of plant origin which compares favourably with currently used antimalarial drugs (Mepacrine, Fansidar, Maloprim, Halofantrin *(Halfan),* and Artemisinins) might be the answer to the age-old quest for malaria eradication and the roll back malaria (RBM) objective in Nigeria.

This study therefore attempted the extraction of haemozoin from the blood samples of malaria patients, as well as elucidation of the antimalarial activity and/or comparative inhibitory effect of some indigenous plant extracts on haemozoin formation in *Plasmodium falciparum,* the malaria parasite. The evidence presented in the study, and especially the clinical trial data, clearly validates the claim outlined here, on the haemozoin extraction as well as the inhibitory effect of the elucidated chemical antimalarials and some esteemed natural plant extracts: *S. latifolia* and *A. boonei* on haemozoin formation in malaria parasites. Malarial pigment has long fascinated malariologists, with Meckel first having observed pigment in the blood and spleen during an autopsy of an insane person, though he failed to make the connection to malaria (Meckel, 1847). In his description of blood film examinations from 70 patients, William Osler later related the pigment to malaria and instituted routine blood smear analyses to diagnose malaria in febrile

patients at Johns Hopkins Hospital (Osler, 1889). Haemozoin formation in malaria parasite is therefore observed as a unique diagnostic tool and/or surrogate marker of malaria infection. Results of this study finds credence in the reports on haemozoin extraction and is further supported by the reports which identified haemozoin as a brown pigment in malaria infected blood samples (Orji, 2001; Urban and Roberts, 2003).

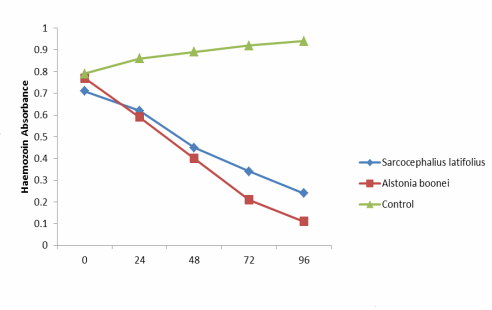

Figure 3: Effects of *Sarcocephalius latifolius* leaf extracts and *Alstonia boonei* stem bark extracts on haemozoin concentration of patients in Bishop Shanahan Hospital, Nsukka

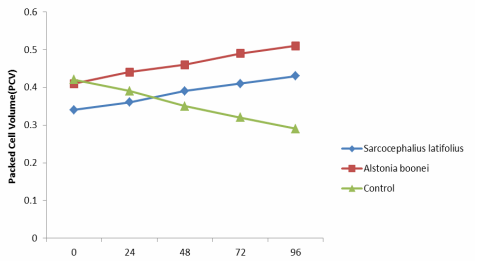

Figure 4: Effects of *Sarcocephalius latifolius* leaf extracts and *Alstonia boonei* stem bark extracts on PCV of patients in Bishop Shanahan Hospital, Nsukka

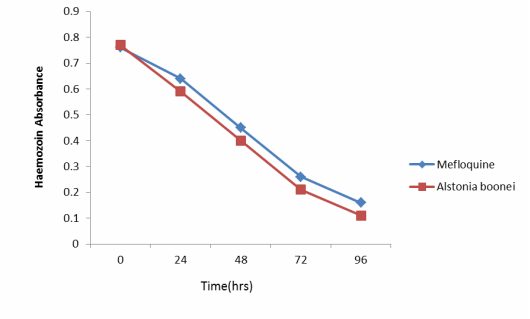

Figure 5: Comparison of effect of mefloquine and *Alstonia boonei* on haemozoin concentration of patients in Bishop Shanahan Hospital, Nsukka

Reduction in packed cell volume of malaria infected people was observed in the study, and this could be attributed to the consumption of red blood cells by the actively feeding merozoites in malaria infected persons during haemozoin formation. The dogma concerning haemoglobin degradation is that the parasite is reliant on the amino acids in haemoglobin for incorporation into its own amino acid pool. It was further indicated that the most conclusive evidence of this reliance on haemoglobin degradation is that the specific inhibition of haemoglobinases is fatal for the parasite (Francis *et al.,* 1997). Recently, Ginsburg reported that the parasite invades an erythrocyte containing 300 mg/ml protein, 95% of which is haemoglobin at a concentration of 5mM; this correlates to 30 pg (or 0.5 mol) of haemoglobin per cell. The parasite ingests more than two-thirds of the haemoglobin (20 pg), yet only uses 3 pg for its own amino acids the resultant effect of this therefore is acute anaemia in infected individual.

A direct relationship between haemozoin production and haemoglobin depletion or reduction in packed cell volume established in this study is thus confirmed by similar findings in which reduction of packed cell volume (PCV) of malaria infected individuals, with resultant anaemia, a symptom of severe malaria was reported (Clark, 2002). Orji further opined that haemoglobin catabolism is switched on during the ring stage in the food vacuole of *P. falciparum* where Fe^{3}-porphyrin, a by-product of haemoglobin digestion, is incorporated into β-haematin, the principal pigment of haemozoin (Orji *et al.,* 1994). In addition, haemozoin was identified as a by-product of haemoglobin degradation during malaria infection, and attributed haemozoin production to haemoglobin digestion by *P. falciparum* (Liu *et al.,* 2006). This study therefore provides clinical evidence of the association of haemozoin formation with anaemia, the major health impact of malaria, and the view is further supported by the findings on the impact of haemozoin production on malaria and related anaemia, as well as the observation that haemozoin was able to inhibit differentiation and maturation of human monocyte derived

dendritic cells in patients with malaria during malaria outbreak in the United States of America in 1990 (Palucka *et al.,* 1998).

Malaria parasites detoxify haeme by forming crystalline haemozoin. Many clinically-used drugs are thought to act by inhibiting the formation of haemozoin in the parasite food vacuole; this prevents the detoxification of the haeme released thereby causing parasite death due to haeme build up. The formation of haemozoin by *P. Falciparum* is therefore an essential process in the survival of malaria parasite. This assumption pre-supposes that an efficient control strategy against the parasite must necessarily be directed towards inhibition of the process. Many clinically-used drugs have been reported to exert inhibitory effect on haemozoin formation by malaria parasites, thereby preventing the detoxification of the haeme released in the food vacuole which is otherwise toxic and lethal to the parasite. Therapeutic doses of quinoline drugs such as chloroquine and mefloquine were reported to inhibit haemozoin production in human erythrocytes, and the inhibition caused monomeric haeme to accumulate and kill the parasites (Jani et al., 2006; Pisciotta, 2006). This view is further buttressed by the reports of the present study which highlighted the inhibitory effect of the test chemical therapeutants: Maldox (sulfadoxine and pyrimethamine), Halfan (halofantrine), Malmed (amodiaquine and artesunate), Mefloquine (quinolines), Artecxin (dihydroarteminisin) and Amatem (artemether and lumefantrine). The effectiveness of Mefloquine, a quinoline drug in exerting inhibition on haemozoin formation observed in this report is attributed to the activity of its functional group, which is reckoned as the best haematin bio-crystallization inhibitor, and therefore effective in the management of chloroquine resistant malaria (Wood and Eaton, 1993). The study similarly indicated the remarkable inhibitory activity of Amatem, a derivative of arteminisin, also known to inhibit haemozoin formation. Artecxin of the group dihydroarteminisin and piperaquine phosphate and trimethoprin, were also observed to significantly reduce haemozoin concentration in malaria infected persons. The arteminisin drugs produced from the plants arteminisia have previously been shown to significantly reduce the malaria pigment (haemozoin) produced by malaria parasite (Orji *et al.,* 1994). The monoalkylated (HA) and dialkylated (HAA) haeme derivatives of artemisinin have been implicated in binding to PfHRP II (*P. falciparum* histidine-rich protein II), and thereby inhibiting haemozoin formation. Artemisinins therefore owe their antimalarial activity to the presence of an endoperoxide bridge, since deoxyartemisinin, which lacks the bridge, is devoid of antimalarial activity. Several studies have also suggested that the haeme-promoted cleavage of the peroxide in artemisinin, leading to the formation of C-radicals which alkylate some proteins of the malaria parasite, also contributes to its antimalarial action. In addition, specific reactions of artemisinin with TCTP (translationally controlled tumour protein), inhibition of the SERCA (sarcoplasmic /endoplasmic-reticulum Ca^{2+}-ATPase) orthologue (PfATP6) of *P. falciparum* and inhibition of *P. falciparum* cysteine proteases have also been suggested to contribute to the drug's activity (Eckstein-Ludwig *et al.,* 2003). The observed inhibitory activity of these drugs is ultimately linked to their ability to bind to both free haeme and haemozoin crystals, and thereby blocking the addition of new haeme units onto the growing crystals (haeme polymerization). The resultant effect therefore was a remarkable increase in the average packed cell volume (PCV) of the patient's blood.

The increasing resistance of *Plasmodium falciparum* to quinoline-based drugs and the absence of an effective vaccine against malaria have made the management of malaria in endemic areas of Nigeria highly problematic, and therefore the development of newer and more potent pharmacophores from natural products of plant origin has become crucial to the control and management of malaria. Consequently, novel drug targets and pathways controlling the unique life cycle of the parasite, such as haemozoin formation was studied and the inhibitory effect of *S. latifolia* and *A. boonei* on the product formation elucidated. Comparative evaluation of the activity of both

test chemical antimalarials and plant extracts on haemozoin reduction indicated the efficacy of the test plant extracts, notably *A. boonei* over the chemical therapeutants in the reduction of haemozoin formation. This was observed as a significant increase in the PCV values of all patients administered the concoction, thus highlighting their inestimable value in folkloric medicine as highly efficient and cost effective anti-malarial agents.

Several scientific reports lend credence to the antimalarial efficacy of *S. Latifolia*. Alkaloids present in *S. Latifolia* are known to have numerous beneficial pharmacological effects. *S. latifolius* was observed to display antiplasmodial activity against *P. falciparum.* Phytochemical investigation of the root extract of *S. latifolius* led to the isolation of the new indole alkaloids -methylstrictosamide aglycone and ethylstrictosamide aglycone, together with strictosamide, angustine, nauclefine, angustidine, angustoline, ethylangustoline, naucleidinal, -epi-naucleidinal, quinovic acid-3 beta-O-beta-D-fucopyranoside, quinovic acid-3 beta-O-alpha-L-rhamnopyranoside, scopoletin, and beta-sitosterol. Strictosamide displayed moderate antiplasmodial activity against *P. falciparum* (Guede *et al.,* 2005). Furthermore, *S. latifolius* was reported to have significant antimalarial properties. These findings corroborates with the results of this study on the efficacy of the plant resulting from its phytochemical constituents, chiefly alkaloids, tannins and flavonoids.

The various species of Astonia are highly rich in alkaloids, steroids and triterpenoids, and phenolic compounds which contribute to their toxicity. The antimalarial efficacy of the root back extract of *A. boonei* was previously reported (Sofowora, 1983). However, their relationship to haemozoin reduction was not studied Result of this study, establishing the remarkable efficacy of *A. boonei* in haemozoin reduction and consequently its unique antimalarial activity is in consonance with similar on the anti-malaria efficacy the plant. The high activity of *A. boonei* on malaria parasite which has been well elucidated in this study and also cited in scientific literature could be alluded to its chemical constituents including alkaloids, terpenes and steroids; over 90% of the isolated chemical constituents are alkaloids (Kweifo-Okai *et al.,* 1995). *In vitro* antiplasmodial activity of the alkaloids against both drug sensitive and resistant strains of *P. falciparum* and *P. berghei* in mice was well reported. Though study on the specific class of alkaloid implicated was not done, however, all the known classes have been reputed to exert good activity on malaria parasites (Kirandeep *et al.,* 2009). This study observed that, *Alstonia boonei* was the most efficacious inhibitor, exerting a significant decrease in the haemozoin concentration from the initial average absorbance of 0.77 to 0.11, with a correspondingly high increase in the packed cell volume (PCV), making it a choice antimalarial agent among all the therapeutants (chemical and plant extracts) tested, thereby underscoring its potential for use in anti-malaria drug formulation as previously demonstrated (Majekodunmia *et al.,* 2009).

Conclusion: The emergence of multi-drug resistant strains of the malaria parasites especially in the sub-Saharan Africa is reducing the therapeutic arsenal for the treatment of malaria at a rate that is barely balanced by the development of novel effective drugs. Haemozoin formation was in this study established as an attractive target for developing antimalarial drugs; its reduction by antimalarial drugs particularly, the plant extracts *A. boonei* was similarly ascertained. The inhibitory activity of test anti-malaria agents, chemicals and plants on haemozoin formation, was therefore demonstrated: all the therapeutants tested exhibited varying degrees of activity in terms of reduction in haemozoin concentration, with Mefloquine showing the highest decrease among the chemical agents, while *Alstonia boonei* stem back extract, was the most efficacious among all the test therapeutants. The potential use of *Alstonia boonei* stem back extract as an effective antimalarial is therefore evident from the study in consonance with its use in folkloric medicine, therefore should be further exploited the search for a novel natural source of therapeutant in the antimalarial drug discovery.

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