PRECLINICAL ANTIMALARIAL EVALUATION OF A BI-HERBAL COMBO OF *ENANTIA CHLORANTHA* AND *NAUCLEA LATIFOLIA* (BHE BATCH 4) IN MICE INFECTED WITH *PLASMODIUM BERGHEI* (NK65)

BY

EKPEMANDU NGOZI EIZABETH

LSC1906487

DEPARTMENT OF BIOCHEMISTRY

FACULTY OF LIFESCIENCES

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APRIL, 2024.

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EKPEMANDU NGOZI ELIZABETH

LSC1906487

A PROJECT SUBMITTED TO THE DEPARTMENT OF BIOCHEMISTRY, FACULTY OF LIFE SCIENCES, UNIVERSITY OF BENIN, BENIN CITY. IN PARTIAL FUFILMENT OF THE REQUIREMENTS FOR THE AWARD OF BACHELOR OF SCIENCE (B.Sc.) DEGREE IN BIOCHEMISTRY

APRIL, 2024.

CERTIFICATION

This is to certify that the project titled "Preclinical antimalarial evaluation of a bi-herbal combo of *Enantia chlorantha* and *Nauclea latifolia* (BHE batch 4) in mice infected with *Plasmodium berghei* (NK65)" was carried out and written by **EKPEMANDU NGOZI ELIZABETH** with Matriculation Number **LSC1906487** in the Department of Biochemistry in partial fulfillment of the requirements for the award of Bachelors of Science in Biochemistry.

_____ _____

DR. N. ERHUNSE DATE

(PROJECT SUPERVISOR)

_____ _____

DR. S.I OJEABURU DATE

(PROJECT COORDINATOR)

_____ _____

PROF. E.C. ONYENEKE DATE

(H.O.D BIOCHEMISTRY)

_____ _____

EXTERNAL SUPERVISOR DATE

DEDICATION

This project is dedicated to Almighty Yahweh, my source and sustainer, who preserved me through it all

ACKNOWLEDGEMENT

I want to appreciate the Almighty God for his Faithfulness, Grace and Love towards me and my family.

A big thank you to my lovely parents Mr. and Mrs. EKPEMANDU and my beloved siblings for all their love and support towards me.

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ABSTRACT

Enantia chlorantha and *Nauclea latifolia* are plants utilized as traditional medicine in Nigeria for the treatment of malaria. *Enantia chlorantha* is able to confer antiplasmodial activity *in vitro* due to the fact that it possesses protoberberine alkaloids and flavonoids. In this experimental research, a hydroethanol extract was obtained from the barks of *Enantia chlorantha* and *Nauclea latifolia.* For the antimalarial study, twenty-five male Swiss albino mice weighing an average of 22 g, were randomly distributed into 5 groups labelled; group 1; Negative control (NC) which received normal saline after infection, group 2; positive control (PC) which had infected mice that received 25 mg/kg chloroquine after infection, group 3 which received 50 mg/kg body weight of BHE batch 4_A after infection, Group 4 which received 100 mg/kg body weight of BHE batch 4_B after infection and group 5 which received 250 mg/kg body weight of BHE batch 4_C after infection. Treatment was done 3 hours after infection and 3 days thereafter. Smears were made from the lateral vein of each mice for determination of percentage parasitemia. Infection was with 2 x 10^4 *Plasmodium berghei*. Mice administered 250 mg/kg body weight of BHE batch 4 showed a significant decrease in percentage parasitemia as compared to the negative control group and this suggest that the bi-herbal combo has an impressive antimalarial activity.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.0 INTRODUCTION

Malaria is a long-standing public health issue that has killed people for thousands of years. We must understand the background of the conflict between people, the malaria parasite, and the mosquito vector in order to fully comprehend the difficulties in controlling malaria (Moss *et al.,*2008). In Africa and certain Asian countries, malaria is the most prevalent disease with the highest number of native cases. Worldwide, the fatality rate from malaria ranges from 0.3% to 2.2%, and in tropical locations, the rate can reach up to 30% in cases of severe malaria (White *et al*., 2014).

Plasmodium is a genus of unicellular parasite that causes malaria, an infection spread through vectors. Obligate intracellular parasites known as *Plasmodia* can infect and multiply within erythrocytes following a phase of replication in the liver that is clinically quiet. Although *P. falciparum, P. malariae, P. ovale, and P. vivax* are the four species that are generally thought to cause natural infections in humans, *P. knowlesi* malaria has recently become more prevalent in South-East Asia and causes zoonotic malaria, leading medical professionals to believe that it is the fifth human malaria parasite. The deadliest type of human malaria, *P. falciparum*, is not just limited to humans as previously thought; new species have been detected in gorilla, bonobo, and chimpanzee populations, indicating a far more complex biological ancestry. These findings come from recent investigations conducted on wild-living apes in Africa. Though not as striking fresh information (Antinori *et al.,*2012). The vector is not only a carrier but also the host, where *Plasmodium* species reproduce sexually. The parasite's growth in the insect is necessary for its

transfer to the corresponding vertebrate host. The range of insect species that each parasite species can support for *Plasmodium*'s crucial development varies, but anopheline mosquitoes are the only vector by which the five *Plasmodium* species that cause malaria in humans are transmitted. Because of their exceptional genetic adaptability, *Plasmodium* species can swiftly develop resistance to treatments like antimalarials and shift host specificity in response to environmental changes (Sato, 2021).

Human populations in malaria-endemic areas have probably been driven to evolve and to select for certain distinct genetic variants due to the disease's severe negative pressure. For instance, patients with thalassemia and sickle-cell disease, two genetic illnesses that impact red blood cells, are frequently found in malaria endemic areas (Hedrick, 2011) and exhibit malaria resistance. The majority of people in Central and West Africa have the Duffy-negative blood type, which is another well-known example (Rosalind *et al.,*2011). This gives specialized resistance against infection by *P. vivax*, a specific species of *Plasmodium* (Kano *et al.,* 2018). It is thought that 42,000 years ago is when this feature first started to spread throughout the population (McManus *et al*., 2017), and *P. vivax* malaria is now uncommon among these areas where *P. falciparum* is common.

Even in this modern age with effective antimalarials and insecticide-treated bed nets, people in different parts of the world are still at risk of being hospitalized due to this disease and the amount of these malaria cases is mainly due to *P. falciparum* which occurs in economically poor Nations of African origin (Haldar *et al.,* 2018).

Human-infecting *Plasmodium* species have a life cycle that is comparable, with an early stage of development in the liver and subsequent growth in the host's blood. Additionally, they exhibit

comparable susceptibilities to some antimalarial medications, including artemisinin, quinine, and chloroquine, as well as the development of resistance to these medications. (Cibulskis *et al.,*2016)

1.1 LITERATURE REVIEW

1.2.1 MALARIA PARASITE LIFE CYCLE

The different types of *Plasmodium* species have a life cycle that is synonymous and complex (Klein, 2013). The life cycle of each malarial parasite that infects humans is differentiated by an endogenous asexual phase (schizogony) which takes place in vertebrates and an exogenous sexual phase (sporogony) with which replication occurs in several *Anopheles mosquito* species. The development of *Plasmodium* in vertebrates starts with the transformation of the sporozoite followed by the injection of the parasite stage to vertebrate host by the mosquito through the skin. This migrates to the liver where it invades hepatocytes and develops into merozoites, the stage that infects red blood cells to initiate the erythrocytic stage of the disease (Gueirard *et al.,*2010). In rodents, about 50% of the parasites survive a mosquito bite in the skin, and within 24 hours, about 10% grow in the dermis, epidermis, and immunoprivileged hair follicles, where they can live for weeks. Despite being frequently unsuccessful, the parasite developmental pathway in skin cells produces merozoites that are infectious to erythrocytes and are discharged by merosomes, as is usually seen in the liver. Consequently, in rodents with malaria, the skin serves as both the final destination for many of the injected parasite where they might be able to survive and develop into merozoites (Gueirard *et al.,* 2010).

Malaria infection starts when the sporozoites are introduced through a mosquito bite (Vanderberg *et al.,*2004). After entering the liver and invading hepatocytes, *Plasmodium* sporozoites multiply as hepatic schizonts, producing thousands of merozoites that are then discharged into the bloodstream. Certain liver parasites (hypnozoites) in *P. vivax* but not in *P.*

falciparum stay inactive, resuming replication and infection after a few weeks or months. *Plasmodium* parasites replicate asexually once erythrocytes enter the bloodstream. This results in adult schizonts, which burst to release merozoites, which then invade fresh erythrocytes. Instead, some blood stage parasites differentiate into male and female gametocytes, which are then activated to produce gametes when they are consumed by mosquitoes as a blood meal. After gamete fusion occurs in the midgut of the insect, the zygote becomes a motile ookinete that travels through the gut wall to become an oocyst, which gives rise to thousands of sporozoites. When sporozoites are transferred into a new human host by a mosquito bite, the life cycle is completed (Guilia *et al.,* 2015).

1.2.2 SYMPTOMS OF MALARIA

These include

(1) Fever

(2) Cold

(3) Headache

(4) Vomiting

(5) Diarrhea

(6) Dizziness

(7) Loss of appetite

(8) Body ache or joint pain

(9) Pale eyes

(10) Salty-tasting palms

(11) Weakness (Sixpence *et al.,* 2020)

1.3 MALARIA DISTRIBUTION

Malaria is estimated to cause 435,000 deaths per year and majority of these cases occurs in sub-Saharan Africa and affect children under 5 disproportionately. Modern advancement in releasing malaria burden in sub-Saharan Africa with the aid of distributing bed net, rapid clinical diagnostic, treatment responses and household level spraying appeared to slow down in 2017 and 2018 (WHO, 2018). Unprecedented efforts to combat malaria have been made over the past ten years, with increasing financial and political support as well as greater accessibility to both traditional and novel tactics and instruments. Malaria still poses a serious threat to public health, nevertheless, especially in Africa. Important issues that must be addressed immediately include the brittleness of many health systems, the emergence of medication and insecticide resistance, and, in particular, the anticipated decrease in financing and coverage of important interventions if they are not replaced when necessary (Alonso *et al.,* 2013). Complex interactions and feedback loops between humans, mosquitoes, parasites, their habitats, healthcare systems, and the execution of policies at a particular time all play a role in the transmission and management of malaria (Savi *et al.,* 2021). For instance, the biodiversity of malaria in West Africa has significant economic effects (Sachs *et al.,* 2002). The danger of contracting malaria rises when a person has the infection and is unable to generate wealth. Malaria control expenditures and country economic growth are negatively correlated (Jowett *et al.,* 2005).

1.4 PATHOPYSIOLOGY OF MALARIA

P. falciparum, P. ovale, P. vivax, P. malariae and P. knowlesi are the five plasmodium species that have the capability to affect humans (Fletcher *et al.,* 2013). During a blood meal, the female anopheles mosquito ingests gametes and these gametes are able to form sporozoites that replicate in the gut (Garcia, 2010). Saliva carrying sporozoites is discharged into the bloodstream of a human host during successive bloodmeals (Carlton, 2018). These Sporozoites enter the liver in

less than an hour, infiltrate hepatocytes, and then quickly proliferate to form merozoites. When an infection is active, microorganisms re-enter the blood and infect erythrocytes (Carlton, 2018).

Malaria parasite feed on hemoglobin within erythrocytes and progress from trophozoites that are not mature (ring stage) to mature trophozoites or gametocytes. The reproduction of mature trophozoites leads to the formation of schizonts, destruction of the integrity of the erythrocyte cell membrane, capillary endothelial adhesion, and cell lysis.

When peripheral blood is exposed to free heme, it promotes endothelial activation (Plato *et al.,* 2008). When malaria is not treated it can last for 2 to 24 months, that is about 2 months to 2 years. *P. ovale* and *p. vivax* can display a dormant type of schizogony where intrahepatic parasites that are inactive, also known as hypnozoites are able to remain inactive until reactivation months to years in the future. There are not many cases of *p. falciparum* that resurface years after exposure, despite the fact that hypnozoite parasite do not usually grow in the liver in the context of *p. falciparum* and *p. malariae* infections. Low levels of arginine, nitric oxide and increase in the enzyme activity of arginase, have been observed in cases of severe malaria cases of peripheral blood (Janka *et al.,* 2010).

Research has also shown that the parasite's arginase level may contribute to the decreased level of arginine in patients that are severely ill, thereby reducing the production of nitric oxide. The resulting low nitric oxide can lead to pulmonary hypertension and myocardial wall stress in children. Thus, possible treatment option are peripheral arginine and inhaled nitric oxide (Bangirana *et al*., 2018).

1.5 PATHOGENESIS OF MALARIA

Plasmodium invasion, biology, and host defense is crucial in order to comprehend the pathogenesis of malaria. Additionally, by comprehending *Plasmodium*'s life cycle, the effects of *Plasmodium* and host interactions may be clarified. The most severe clinical symptoms are caused by *Plasmodium falciparum* infections, which also have the best understood pathophysiology. Patients' clinical presentations vary depending on the patient's age, immunological condition, and the local malaria epidemiology. These factors are intimately linked to the malaria parasites. Those who are most vulnerable to malaria include pregnant women, who run the risk of anaemia and low birth weight neonates, and babies and young children (6–59 months), who can contract severe malaria. Older children and adults acquire a partial immunity through recurrent infections, lowering their risk of severe malaria in regions where the disease is year-round. Antimalarial medication and symptomatic care are the mainstays of malaria treatment, which is challenging due to the pathophysiological alterations in several organ systems implicated in severe malaria (Chen, 2023).

An important predictor of incidence of malaria in many locations is temperature (Pascual *et al.,* 2006) and the potent effects of climate-induced temperature shifts due to intervention and efforts on vector control (Siraj *et al*., 2014).

1.6 PREVENTION OF MALARIA

Proven malaria prevention techniques as well as additional strategies that are known to lower mosquito populations are included in these preventive measures. The particular techniques supported by the integrated approach are:

(1) Sleeping under long-lasting insecticidal nets (LLINs)

(2) Adding screens to windows, vents, and open eaves to keep mosquitoes out of houses

(3) Use of indoor residual spray (IRS)

(4) Modifying the layout of homes to keep mosquitoes out

(5) Managing larval sources

(6) Shutting windows and doors at dusk to keep mosquitoes out of houses

(7) Larviciding in large pools of stagnant water

(8) Topical and spatial mosquito repellents

(9) Use of mosquito coils

1.7 TREATMENT OF MALARIA

Treatment for patients suffering from malaria includes; hospitalization for patients with severe cases, supportive care, schizonticidal medications. Pediatric patients and naïve adults that are receiving active antimalaria treatment should remain in the hospital for at least 24 hours to make sure that the medication dosing is correctly timed and to trend parasitemia to evaluate treatment response. A high initial parasitemia is related with fluid imbalance, respiratory distress syndrome and a dysfunctional kidney (Fletcher, 2013).

Treatment of malaria involves a combination therapy that aims for both erythrocytic and hepatic forms (Delves *et al.,* 2012). The main antimalarials are hydroxychloroquine, chloroquine, primaquine, artemisinin-based combination therapy and atovaquone-proguanil. Both chloroquine and hydroxychloroquine are synthetic forms of quinine and are able to disrupt the erythrocytic stage of malaria by disrupting the parasite hemoglobin metabolism and increase intracellular pH (Ben-Zvi *et al.,* 2012). Chloroquine and hydroxychloroquine are treatment that

generally require two days treatment thereby allowing better tolerance and a brief time of admission. Chloroquine may be able to enhance gametogenesis resulting to it contributing to resistance and this is a major concern in the South Asia regions.

An active drug against all malaria parasite's lifecycle is Artemisinin. Atovaquone inhibits ATP production by targeting cellular electron transport chain; proguanil is able to enhance the effect of atovaquone by sensitizing parasitic mitochondria (Staines *et al.,* 2018).

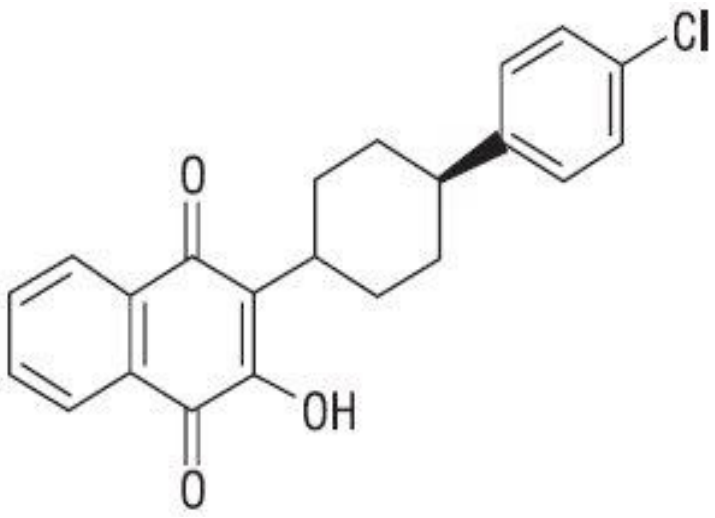

Figure 1: Chemical structure of Proguanil

1.8 ANTIMALARIAL DRUGS

Antimalarial drugs are very important and play a vital role in decreasing malaria morbidity and malaria mortality, but it should be noted that their role is mainly due to their effectiveness. This effectiveness is the probability of an antimalarial drug been employed as a successful treatment in individuals suffering from malaria parasites. This antimalarial drug effectiveness is facilitated

by health system quality, quality of drug, resistance of drug and patient's adherence to drug use that is, its influence on malaria burden varies through time and space (Rathmes *et al.,* 2020).

1.8.1 CHLOROQUINE

For more than 70 years, chloroquine has been used globally and it is a part of the model list of important medicines of world health organization (WHO). This drug has an established profile of being clinically safe and it is also cheap (Colson *et al*., 2019). Chloroquine has at times shown therapeutic effects in rheumatoid arthritis and lupus erythrocytes (Thome *et al.,* 2013). Chloroquine and its analogue hydroxychloroquine are classified by quick onset, reduced toxicity, a long duration of action and increased tolerance in humans (Titus *et al*.,1989). Chloroquine can be partly metabolized into mono-desethyl metabolite and is excreted through the kidneys.

The analogue hydroxychloroquine is created by replacing an ethyl group in chloroquine with a hydroxyethyl group and this leads to a larger volume of distribution and reduced level of toxicity in humans (Verbaanderd *et al*., 2017).

1.8.2 ARTEMISININ

This antimalarial drug is vastly utilized and is combined with other antimalarial drugs in the form of artemisinin-based combination therapy. This therapy is the first line of treatment for *P. falciparum* infection that is uncomplicated.

Artemisinin, a tetracyclic 1, 2,4-trioxane and contains an endoperoxide bridge, is the main pharmacophore of ACTs (Antoine *et al.,* 2014). This drug is gotten from herb sweet wormwood*; Artemisia annua.* Kelch protein, which is located on chromosome 13 (k13 propeller) of *P. falciparum,* has been shown to undergo mutation and this results to resistance to artemisinin. Considering the action of artemisinin, two main action has been brought to notice; firstly,

artemisinin has an unusual bridge which interacts with the iron of hemoglobin this has interrupted the process of detoxification and generates ROS (reactive oxygen species) that attack the parasites protein. Secondly, it may directly attack the mitochondria (Cragg and Newman, 2013).

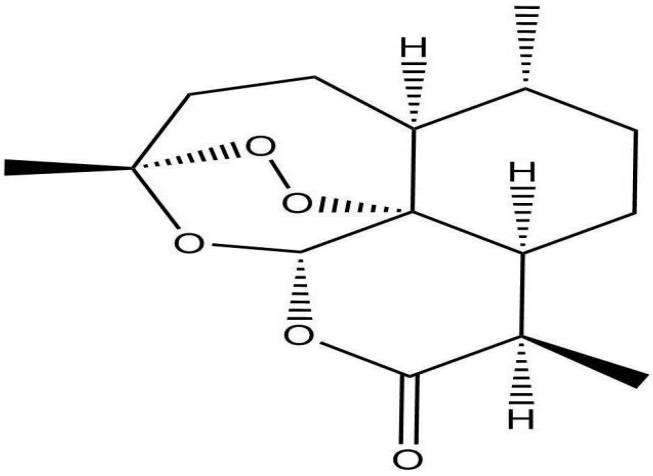

Figure 2: Chemical structure of Artemisinin

1.9 ANTIMARIAL DRUG RESISTANCE

The resistance of malaria parasite to antimalarial drugs is mediated through two processes which includes;

1. The rate at which *de novo* mutations conferring resistance seem to appear and are picked through drug utilization within an individual.
2. The spread of the resistant alleles to other individuals (Klein, 2013).

The establishment and spread of drug resistant plasmodium species are intimately tied at the molecular level, to the various forms that the parasite takes on and the range of the setting it inhabits, from human hepatocytes to the midgut and salivary glands of mosquitoes (Blasco *et al*., 2017). Malaria parasites have resistance to the parasite's life cycle and metabolism and partly distinct from those found in other microorganisms such as drug efflux, modification of the drug target, enzymatic degradation and drug modification as well as less common mechanism that relates to parasite's metabolism and life cycle (Mbengue *et al*., 2015).

1.10 *ENANTIA CHLORANTHA* AND ITS ANTIMALARIAL POTENTIAL

Enantia chlorantha is a dense forest tree found along the tropics of central Africa and the west coast of west Africa (Abubakar *et al.,* 2020). It is commonly known as African yellow wood and it belongs to kingdom plantae, has the order of Magnoliales and is from the Annonaceae family (Akpan *et al.,* 2021). In indigenous languages this plant is identified by several names such as Awopa, Osu pupa or Dokita igbo in Yoruba, Osumolu in Ikale, Kakerim in Boki, Erenba-vbogo in Benin (Hedin, 1929). The fluted stem carries leaves which are about 0.14-0.15 m long and 0.05-014 m broad. The dark brown bark covering the stem is thin on the outside meanwhile the inner back is brown above and creamy beneath (Tcheghebe *et al.,* 2016).

Several times the antimalarial activity of the plant has been proven by different studies because of the plant's aqueous extract ability to produce up to 70% *Plasmodium berghei* chemosuppression in a dose dependent manner in mice infected with *Plasmodium berghei* (Bassey *et al*., 2017). A similar outcome was observed by (Bassey *et al*., 2017) by obtaining 77.45% of *P. berghei* in adult Swiss albino mice treated with extract of the plant's stem bark

using a dose of 64.80 mg/kg. Also, alkaloids and flavonoids which are abundantly found in the stem have been seen to facilitate the antiplasmodial activity. An *in vivo* investigation of the antimalarial activity of total alkaloid extracted from *Enantia chlorantha's* stem bark confirmed that the alkaloid extract from the stem bark of *Enantia chlorantha* indeed exhibits a direct antiplasmodial activity against *P. berghei.*

Figure 3: *Enantia chlorantha* stem bark

1.11 PHYTOCHEMICAL CONSTITUENTS OF *ENANTIA CHLORANTHA*

Enantia chlorantha has several bioactive constituents that serves as evidence for its numerous medicinal properties. In a study carried out by Dawodu *et al.,* (2014) to ascertain this plants material and its solvent extract potential to determine the plant's proximate components and phytochemical constituents in aqueous and ethanol extract (Dawodu *et al.,* 2014) The proximate analysis revealed that *Enantia chlorantha* has a high percentage of crude fibre (72.25%) and

possesses a low ash content of about 2.48% with other 4 compound present; carbohydrate 6.29%, crude protein 10.78%, crude fat 3.78% and moisture content of about 3.85%.

The phytochemical screening revealed that flavonoids, saponins, alkaloids, phenols, reducing sugar and cardiac glycoside was present. The phytochemical active constituents have been said to be berberine-like alkaloids, saponins and tannins.

1.12 *NAUCLEA LATIFOLIA*

Nauclea latifolia smith (Rubiaceae) is commonly known as pin cushion tree or African peach and it is a strangling shrub that is native to tropical Asia and Africa (Grace and Grace *et al.,* 2017).

N. latifolia is an evergreen tree that possess several stems and has the ability to adapt very well in both the savanna woodlands and the tropical rainforest zone located central Africa and the West.

N. latifolia is largely distributed throughout the forest and tropical forests of Benin, Cameroon, Democratic Republic of Congo, Burkina Faso, Republic of Congo, Nigeria and Ghana (Lamidi *et al.,* 1993). In Nigeria, it is found in areas like Akwa Ibom, Abuja, Kontagora, Shaki, Cross River, Abakiliki, Enugu and other parts of the country. The generic name of the plant is gotten from the combination of the Greek words “sarco” which means fleshy and “cephalus” which means headed. This generic naming is given in reference to its flower (Balogun *et al.,* 2016). Amongst the igbo people of Nigeria, *N. latifolia* is commonly known as “Ubulu inu”; in the Northern part of the country or rather in Hausa, it is known as “Tafashiya” or tabashiya or marga or tuwon biri;

in the Western part of Nigeria, that is amongst the Yoruba, it is known as Egbesi (Arise *et al.,* 2012).

Figure 4: Fruit and leaves of *Nauclea latifolia*

1.13 ANTIMALARIAL POTENTIAL OF *NAUCLEA LATIFOLIA*

Nauclea latifolia stembark possesses alkaloids, flavonoids cardiac glycosides, saponins and tannins (David *et al.,* 2004). These secondary metabolites have been suggested to be responsible for the antimalarial activity of the plant. the antimalarial activity could have been facilitated by these metabolites singly of in synergy with each other. Alkaloids show antiplasmodial activity by shutting off protein synthesis in *Plasmodium falciparum* (David *et al.,* 2004). Flavonoids, tannins and saponins have been suggested to have the characteristics of free radicals or antioxidants and

are able to eliminate the oxidative damage caused by the malaria parasite (David *et al.,* 2004). The antioxidant activity might be due to the presence of vitamins and may represent another mechanism that contributes to *N. latifolia's* antimalarial activity (Lamien-Meda *et al.,* 2008). Flavonoids are known to be able to chelate with nucleic acid base pairing of malaria parasite and is also known to show a good amount of antiparasitic activity against various strains of malaria, trypanosome and Leishmania (Nwafor *et al*., 2008).

1.13 AIM OF STUDY

The study aimed at assessing the antimalarial potential of a bi-herbal combination of *Enantia chlorantha and Nauclea latifolia* (BHE Batch 4) in mice infected with chloroquine sensitive strain of *P. berghei.*

CHAPTER TWO

MATERIALS AND METHOD

2.1APPARATUS/ EQUIPMENT USED

Syringe (1ml)

Microscopic slide

Microscope

Measuring cylinder

Eppendorf tubes and rack

Lyophilizer/Freeze dryer

Refrigerator

Cotton wool

Hemocytometer

Plastic cages

Mortar and pestle

Hand gloves

Plastic mice cages

Weighing balance

Oral gavage

Rotary evaporator

2.2 CHEMICALS AND REAGENT

Immersion oil

Giemsa stain

Distilled water

Picric acid

Chloroquine

Chloroform

Methanol

Normal saline

2.3 EXPERIMENTAL ANIMALS

Male Swiss albino mice (25) of about 6-8 weeks old with an average weight of 22 – 25 g were gotten from the Department of biochemistry, University of Benin and were randomly distributed into plastic cages in a properly ventilated animal house of the department. The mice were kept in groups of 5 and were allowed to acclimatize to their new environment for one week before commencement of study.

The animals were given clean water and fed standard mice feed in clean plates and water bowls. The feed and water were changed daily and the mice cages were cleaned frequently to maintain a clean environment. These standard conditions were ensured for the mice throughout the experimental timeframe. Four (04) parasitized mice (mice infected with chloroquine sensitive (NK65) strain of *Plasmodium berghei)* were gotten from National institute of Medical Research (NIMR) Lagos and were used to infect the experimental mice.

2.4 4-DAY SUPPRESIVE ANTIMALRIAL STUDY

2.4.1 MEASUREMENT OF MICE BODY WEIGHT

Before sorting the mice into groups of individual cages, the measurement of each mice was taken to ensure they were up to the require weight of 20-25g for the experiment.

$$AVERAGE\ WEIGHT = \frac{total\ weight}{total\ number\ of\ mice}$$

2.5 EXPERIMENT LAYOUT

The mice were sorted into 19 groups of 5 mice (that is, total amount of mice used was 95). Apart from group 1 which remained uninfected throughout the period of the experiment, the mice in groups 2-19 were all infected with 2 x 10^4 *P. berghei* and given the following treatments 3 hr. after infection.

GROUP 1 (NC): Negative control received normal saline after infection.

GROUP 2 (PC): Positive control received 25 mg/kg body weight chloroquine after infection

GROUP 3 (BHE4_A): Receive 50 mg/kg body weight of BHE batch 4 after infection.

GROUP 4 (BHE4_B): Received 100 mg/kg body weight of BHE batch 4 after infection.

GROUP 5 (BHE4_C): Received 250 mg/kg body weight of BHE batch 4 after infection.

Treatment was done for a total duration of 4 days and mortality was computed for a duration of 30 days.

2.6 DETERMINATION OF PERCENTAGE OF PARASITEMIA

To estimate the percentage of parasitemia, the slides were carefully placed under a microscope using x100 objective with the aid of immersion oil. The percentage of parasitemia was calculated using the formula below;

$$\textbf{Percentage(\%):} \frac{\textit{number of infected cells}}{\textit{total number of red blood cells}} \boldsymbol{x\ 100}$$

When the percentage of parasitemia of the parasitized mice from NIMR was checked, parasitemia was determined to be 16%. Parasitemia was measured two days later to be 26%. Blood was taken from the mouse by cardiac puncture. Cells were counted by the help of a hemocytometer after which the blood was diluted with normal saline to give each mouse 2 x 10^4

parasitized red blood cell in an injection volume of 0.2 ml. Infection of experimental mice was carried out by injecting 200 μl of 2 x 10^4 parasitized red blood cell intraperitoneally.

2.7 METHODOLOGY

2.7.1 SAMPLE EXTRAT PREPARATION

The samples *Enantia chlorantha* (5 g) and *Nauclea latifolia* (45 g) were both placed in a Winchester bottle in which 40% ethanol and distilled water was already added. The samples were shaken at regular intervals for 72 hr. The extract was thereafter filtered using muslin cloth. Concentration was done using a rotary evaporator followed by a freeze dryer to obtain a powdered extract.

2.7.2 SMEAR PREPARATIONS

Using a 1ml syringe, blood was collected from each of the mice's lateral tail veins on a clean microscope slide. When the blood was is placed on the clean slide, another clean slide was used as a spreader for making smears. The spreader is moved in such a way as to contact the blood (the spreader slide was positioned at an angle of 45^0 to the other slide where the blood was dropped). Once the contact is made, the blood is allowed to spread on the entire surface via capillary action. The spreader was then pushed forward while holding firmly. The smear was then allowed to dry.

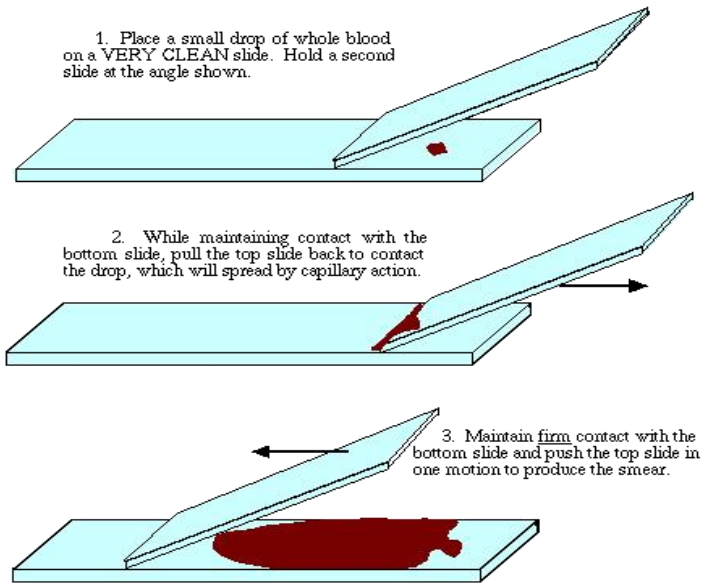

Figure 5: Procedure for blood smear preparation

2.7.3 FIXATION

The smears were left to dry for about 2-3 minutes. After drying they were allowed to sit in a bottle containing methanol of 99% for about a minute and allowed to air dry after removal from the bottle. This fixation is done to ensure that the smear remains bound to the microscopic slide.

2.7.4 STAINING

After fixing the slides were stained using Giemsa stain to allow for examination using the microscope. Gustav Giemsa is a German born scientist that was mostly employed as a chemist until his death in 1948. His name is attached to a staining technique that was developed mainly to show parasites in malaria, but was also used in histology due to its excellent nuclear membrane and chromatin staining, the ability to metachromasia some cellular components, and the variable qualities of cytoplasmic staining based on the type of cell (Barsia, 2007).

CHAPTER THREE

3.0 RESULTS

On day 9 of study, the following data (Figure 6) was obtained following the calculation of % parasitemia. Groups treated with BHE4 were observed to show a significant decrease in parasitemia as compared with the negative control group.

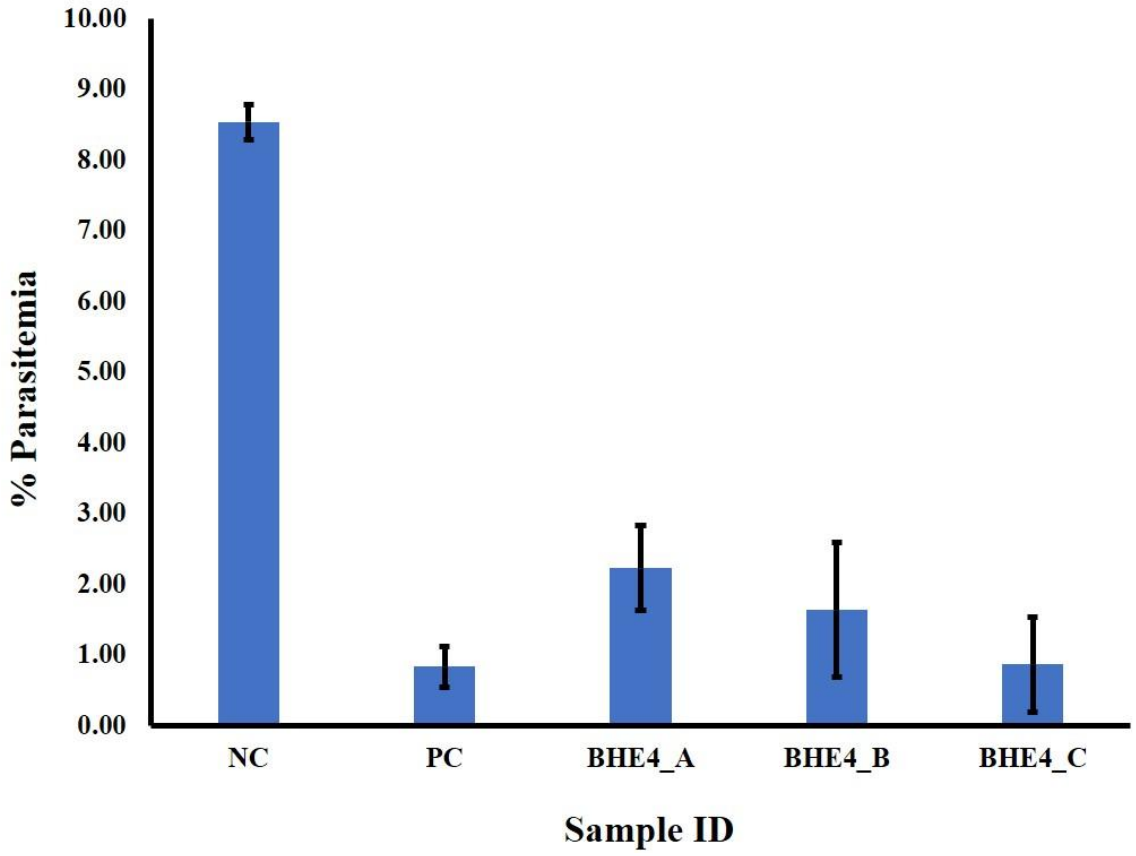

Figure 6: Percentage parasitemia of mice infected with *P. berghei* and treated with increasing doses of BHE batch 4. Results is presented as mean ± SD.

Key: NC: Negative control group, PC: positive control group, BHE4_A: Treatment with 50 mg/kg body weight of BHE batch 4, BHE4_B: Treatment with 100 mg/kg bogy weight of BHE batch 4, BHE4_C: Treatment with 250 mg/kg body weight of BHE batch 4

3.2 MEAN SURVIVAL TIME AND STANDARD DEVIATION

						MEAN SURVIVAL TIME	STANDARD DEVIATION
BHE_4A	**3**	**3**	**30**	**30**	**30**	**19.2**	**14.78851**
BHE_4B	**21**	**24**	**30**	**30**	**30**	**27**	**4.242641**
BHE_4C	**3**	**22**	**23**	**30**	**30**	**21.6**	**11.05893**
PC	**30**	**30**	**30**	**30**		**30**	**0**

Table showing mean survival time and standard deviation

CHAPTER FOUR

DISCUSSION AND CONCLUSION

4.1 DISCUSSION

From the results obtained (chapter 3), the negative control group had the highest percentage parasitemia because mice in this group were only administered normal saline that acted as a vehicle in this study. Mice in the positive control group (PC) were administered chloroquine which is a standard antimalarial drug. Consequently, they had a very low % of parasitemia. Mice in BHE4_A group received 50 mg/kg b.wt. of the extract decreased parasitemia but not as much those in BHE4_B (administered 100 mg/kg b.wt.). Mice in BHE4_C (administered 100 mg/kg b.wt.) group when had the lowest % parasitemia when compared with the lower doses. It was able to significantly reduce the % parasitemia in mice as compared to the negative control group.

In conclusion, this study which was aimed at assessing the antimalarial potential of a bi-herbal combination of *Enantia chlorantha and Nauclea latifolia* (BHE Batch 4) in mice infected with chloroquine sensitive strain of *Plasmodium berghei* has proved to be progressive as traditional medicines could be a significant and long-lasting source of treatment given the issues with rising drug resistance and access to affordable, effective antimalarial medications in underdeveloped areas. The data from this study together support initiatives to record the local application of native medicinal plants with possible antimalarial properties and offer foundational information for upcoming toxicological, pharmacological, and conservation research.

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APPENDIX

SAMPLE PREPARATION

Preparation of bi-herbal extract for administration

Samples were prepared on a daily basis, thus administered samples were prepared fresh. Treatment was made using 50 mg,100 mg and 250 mg per kg body weight of samples A, B and C (i.e. BHE 4_A, BHE4_B and BHE4_C).

FOR SAMPLE A

50 mg = 1000 g

X = 22 g

$$x = \frac{50\ mg\ x\ 22\ g}{1000\ g} = 1.1\ mg\ per\ mouse$$

1.1 mg x 6 (mice in each group; 6 was used for calculation incase an incident occurs and the sample for 5 is not enough) = 6.6 mg (i.e. 0.007 g of sample in 1.2 ml of normal saline to achieve 50 mg per kg body weight

FOR SAMPLE B

100 mg=1 kg=1000 g

X= 22 g

$$x = \frac{100\ mg\ x\ 22\ g}{1000\ g} = 2.2\ mg\ per\ mouse$$

2.2 mg x 6 (mice in each group; 6 was used for calculation incase an incident occurs and the sample for 5 is not enough) = 13.2 mg (i.e. 0.013 g of sample in 1.2 ml of normal saline to achieve 100 mg per kg body weight.

FOR SAMPLE C

250 mg =1 kg = 1000 g

X = 22 g

$$x = \frac{250\ mg\ x\ 22\ g}{1000\ g} = 5.5\ mg\ per\ mouse$$

5.5 mg x 6(mice in each group; 6 was used for calculation incase an incident occurs and the sample for 5 is not enough) = 33 mg (i.e. 0.033 g of sample in 1.2 ml of normal saline to achieve 100 mg per kg body weight.

PREPARATION OF CHLOROQUINE FOR POSITIVE CONTROL MICE

For Chloroquine;

25 mg = 100 g

X = 22 g

$$x = \frac{25\ mg\ x\ 22\ g}{1000\ g} = 0.55\ mg\ per\ mouse$$

0.55 mg x 6 (mice in each group; 6 was used for calculation in case of an accidental spill and the sample for 5 is not enough) = 3.3 mg (i.e. 0.033 g of sample in 1.2 ml of normal saline to achieve 100 mg per kg body weight.

0.033 g of Chloroquine was weighed and placed on a mortar. It was then dissolved with normal saline. After dissolving, it was made up to mark (1.2 ml) in an Eppendorf tube.

Each mouse in the positive control group (PC) received 0.2 ml of chloroquine.

For negative control group each mouse received 0.2 ml of normal saline which served as the vehicle.