LASU Journal of Medical Sciences
Official Publication of the Faculty of Basic Medical Sciences
and Faculty of Basic Clinical Sciences
Lagos State University College of Medicine, Ikeja
www.lasucom.edu.org.
E-mail: lasujms@lasucom.edu.ng

Research Article

Neem-based Poly-herbal Formulation Exerts Anti-Plasmodial and Anti-Haemolytic Activity in *Plasmodium berghei*-Infected Swiss-albino Mice

Wahab Anjola T.[1], Ebuehi Osaretin A.T.[1], Ajibaye Olusola[1], and *Ajagun-Ogunleye Olufemi M.[2]

[1]Department of Biochemistry, College of Medicine, University of Lagos, Nigeria. [2]Department of Medical Biochemistry, Faulty of Basic Medical Sciences, Lagos State University College of Medicine, Ikeja, Lagos State, Nigeria

[*]***Author for Correspondence:***
olufemi.ajagun-ogunleye@lasucom.edu.ng
+2348056008989

Keywords: Malaria, Chemoprophylaxis, Plasmodium berghei, Medicinal plants, Polyherbal formulation, Neem leaves.

ABSTRACT

Objectives: About 216 million cases and 445,000 deaths have been reported due to Malaria in Africa. We hereby evaluated the anti-plasmodial activity of a polyherbal formulation that consisted of *Azadirachta indica* (neem leaves), *Cymbopogon ciratus* (lemon grass), *Morinda lucida*, *Enantia chlorantha* (bark of "awopa" tree), and *Citrus paradisi* (grape fruits) in mice infected with *Plasmodium berghei* Nk65.

Methods: Twenty-five (25) Swiss albino mice (13-19 g) used in this study were grouped as Group A (Sulphadoxine/Pyrimethamine-administered), Group B (distilled water-administered) as the positive and negative controls, respectively. Groups C, D and E were administered the polyherbal formulation at 200, 400 and 800 mg/BW. After five days, they were inoculated intraperitoneally with *Plasmodium berghei* NK65 strain.

Results: Percentage chemoprophylaxis against parasitaemia was observed in a dose dependent manner:10.04%, 19.65% and 62.45% in the treatment groups C, D and E, respectively. Sulphadoxine/Pyrimethamine exerted 100% chemoprophylaxis at 5 mg/kg body weight. Hb (g/dl) concentrations were 14.31 ± 0.39, 12.90 ± 0.44, 13.23 ± 0.36, 13.90 ± 0.55, 13.54 ± 0.55 in Groups A-E, respectively. The WBC and PCV equally showed no heamatological abnormalities ($p < 0.05$). The effects on oxidative stress biomarkers, SOD, GSH and MDA, renal function biomarkers; creatinine and urea, and liver function enzymes; ALT, AST and ALP equally revealed no significant changes in the treated groups compared to the control, ($p < 0.05$).

Conclusion: The neem-based polyherbal formulation is a potent anti-plasmodial therapeutic formula efficient enough to combat drug-resistant malaria endemic without inducing anaemia and oxidative stress.

INTRODUCTION

Malaria has remained a global health challenge for decades and half of the world's population is at risk of the disease.[1] Children under the age of five and pregnant women are the most vulnerable. This infectious disease, caused by *Plasmodium* spp, which is transmitted by the Anopheles mosquito, has caused about 445,000 deaths, with the highest cases occurring in Africa. Previous reports revealed the devastating effects of malaria as reflected in the estimates, which is about one million deaths per year or 3000 deaths per day, most of these deaths occur in African children.[2] Malaria deaths are estimated to be 1.5 to 2.7 million a year, most of which children under five years [3] and pregnant women are the most vulnerable. In other words, 90% of these deaths occur in sub-Saharan Africa.[4] Despite the efforts of the World Health Organization, the prevention and control of the disease is still very challenging.

Plasmodium falciparum is the most virulent and deadliest of all the *Plasmodium* species. The parasite has grown resistance to most available drugs. Modern pharmaceuticals are too expensive in areas where malaria is endemic, therefore, the use of herbal remedies has become popular as an alternative therapy.[5] Resistance to antimalarial drugs has become one of the most significant challenges to human health, and thus has necessitated the quest for new and effective drugs. Consequently, over the years, research into alternative therapy has emerged as an obligation. Up-to-date, neem leaves have been locally used in a variety of ways, one of which is the management of malaria fever.[6]

Due to the development of resistance to earlier drugs, chemotherapeutic management of the disease has remained a global problem.[7] The use of herbs for the treatment and prevention of diseases is as old as humanity.[8] Traditional methods of malaria treatment could be a promising source of new antimalarial compounds.[9] The use of medicinal plants for the treatment of parasitic diseases is well known and documented since ancient times.[10] Anti-malarial compounds have been successfully isolated from plant products.[11] Herbal treatments are the most popular form of traditional medicine, and are highly lucrative in the international marketplace.[12] Chemotherapeutic agents will continue to be in demand for the complete management of malaria.[13] After the resistance of the parasite to chloroquine, the first line treatment gradually changed over time to sulphadoxine–pyrimethamine (SP), and again, most recently, from SP to artemisinin-combination therapy (ACT). Resistance of *Plasmodium falciparum* to ACT has triggered a global concern for malaria prevention and control in Africa.[14]

The malaria species *Plasmodium vivax, Plasmodium ovale, Plasmodium malariae*, and *Plasmodium falciparum* have been implicated in the etiology of the infection. [15] However, the control of these parasites using synthetic antimalarial drugs such as primaquine and chloroquine have been hindered by rapid parasite resistance to these drugs over the last few decades.[16] The drug resistance developed by these parasites has necessitated the hunt for more effectual antimalarial agents from natural products. In malaria endemic countries of the world, natural and traditional products (plants and insects/products) are commonly used to combat malaria.[17] Therefore, there exists a thought that if these natural products used by the traditional herbalists were not helpful, malaria would have shattered Africa a long time ago.[18]

The aim of this study was to prepare a polyherbal formulation from varieties of leaves, bark and fruits of medicinal plants, popularly called "Agbo iba", which is commonly used by the Yoruba tribe (south-western Nigeria), to investigate its effect as a chemoprophylaxis malaria therapy and to evaluate its anti-plasmodial capacity in mice infected with *Plasmodium berghei*.

The polyherbal formulation used in this study consisted of *Azadirachta indica* (neem leaves), *Cymbopogon ciratus* (lemon grass), *Citrus paradisi* (grape fruit), *Enantia chlorantha* bark (awopa), and *Morinda lucida* bark (epo oruwo). *Azadirachta indica* commonly known as Neem, Indian lilac is a tree that belongs to the Mahogany family *Meliaceae*. It is locally known as "Dongoyaro" in Yoruba. The leaves are referred to as the "wonder leaves" due to its medicinal properties. It has been used for ayurvedic remedies for many years.[19]

Cymbopogon ciratus, commonly known as lemon grass is a tropical plant native to Southeast Asia. It belongs to the family *Poaceae*. It is locally called "ewe tea", it has a lot of medicinal and antioxidant activities.[20] *Citrus paradise* also known as grapefruit is one of the tropical evergreen trees of the genus Citrus, it belongs to the family Rutaceae. It is locally called "eso girapufurutu".

Enantia chlorantha bark (locally called "awopa"), is a dense tropical forest tree found along Central Africa and the west coasts of West Africa. Local names include; Awopa, Osu pupa or Dokitaigbo (Yoruba). The bark is very rich in primary and secondary metabolites, the ethanolic extracts from different parts of the plant contains alkaloids, flavonoids, tannins, saponins, phenols, steroids, cardiac glycosides, terpenoids, anthraquinones, proteins, aldehydes/ketones, and carboxylic acid. The tea infusion is locally used in the treatment of Malaria.[21]

Morinda lucida is a tropical rainforest plant that belongs to the family *Rubiaceae*. The bark is used in the treatment of Malaria locally and it is called "epo oruwo". This bark is equally rich in primary and secondary metabolites. The challenge of multiple-drug resistance in eradicating malaria has remained, despite the discovery of Artemisinin-based Combination Therapy (ACT) in the 20th century, whose efficacy has been short-lived. Hence, there is the need for alternative antimalarial therapy that is safe, inexpensive and readily available.[22] Therefore, there is a need for the present study.

MATERIALS AND METHODS

Plant collection and identification

Five freshly harvested medicinal plants were obtained from the botanical garden, University of Lagos, Nigeria and from an herbalist at Yaba herbal market. *Azadirachta indica* leaves (neem) and *Cymbopogon ciratus* (lemon grass) were obtained at the University botanical garden, while *Citrus paradisi* (grape fruit), *Enantia chlorantha* bark (awopa), and *Morinda lucida* bark (epo oruwo) were obtained from the herbalist. The herbs were authenticated by Professor J. O. Olowokudejo, Department of Botany, University of Lagos. The Voucher number is FSC/BOT/HER/21/0018.

Preparation of the polyherbal formulation

Each of the five fresh plant materials were treated separately. They were washed with water and finally rinsed with distilled water after which they were air-dried. The neem leaves, lemon grass, and the two barks were air-dried at room temperature for a week in the laboratory, after which they were powdered separately. 100 g of each plant powder was soaked in 1000 ml of distilled water separately for 24 hrs. with an intermittent stirring. The resulting extracts were filtered with a muslin cloth. Six grape fruits (*Citrus paradisi*), were dry blended all together as a whole, as in the peel, fruit pulp and the seeds, all together. The blended fruit was soaked in distilled water, 100 g of fruit pulp was extracted in 1000 ml of distilled water. The extract was filtered with a muslin cloth. The five filtrates were concentrated with a rotatory evaporator at 40°C. Each of the five extracts (400 mg) was dissolved in 10 ml of water as a cocktail mix. The solutions were mixed together to give 50 ml of the "polyherbal" mixture.

Experiment

Twenty-five male Swiss albino mice weighing 13-19 g aged seven (7) weeks old were obtained from the Nigerian Institute of Medical Research (NIMR), Lagos, Nigeria. They were housed in plastic cages with saw dust as beddings at 25°C with a 12 h dark-light cycle and acclimatized for seven days. Food and water were given *ad libitum.* The mice were handled in accordance with NIH Guide for the care and use of laboratory animals; NIH Publication revised (1985) NIPRD Standard Operation Procedures (SOPs). The animals were distributed into five groups of five mice each as shown below:

- Group A (Positive control): *P. berghei* + 25 mg/kg b.wt of Sulphadoxine/Pyrimethamine (SP) (standard drug).
- Group B (Negative control): Mice infected with *P. berghei* NK65 received distilled water and no treatment.
- Group C (Low dose): *P. berghei* + 200 mg/kg b.wt of polyherbal formulation.
- Group D (Medium dose): *P. berghei* + 400 mg/kg b.wt of polyherbal formulation.
- Group E (High dose): *P. berghei* + 800 mg/kg b.wt of

polyherbal formulation.

The experimental design was approved by the College of Medicine, University of Lagos (CMUL) Ethical Research Committee on the handling and use of laboratory animals. Approval number was CMUL/CHRC/01/002/025. The dosages used were arrived at from preliminary toxicity investigations.

Parasite preparation

The NK65 strain of *P. berghei* used for this study was obtained from Biochemistry Division, Nigerian Institute of Medical Research (NIMR), Yaba, Lagos. An infected donor mouse with the *P. berghei* strain of the rodent malaria *P.* spp was used for parasite inoculum preparation. Each mouse was passaged with 0.1 ml of the infected blood containing about 1×10^6 *P. berghei* parasitized red blood cells intraperitoneally. A sample (1 ml) of blood was taken from donor mice and diluted with 5 ml phosphate buffer; such that 0.1 ml contained standard inoculum of 1×10^7 infected red blood cells.[23]

Chemoprophylaxis Treatment and Parasite Study

The mice in group A were given 25 mg/kg body weight Sulphadoxine/Pyrimethamine (SP). Those in group B were given distilled water. The animals in groups C, D and E were given 200, 400 and 800 mg per kg body weight, respectively, of the polyherbal formulation every day for 5 days using an oral cannula. On the sixth day, the mice were inoculated with 0.1 ml of the infected blood containing about 1×10^6 *P. berghei* parasitized red blood cells intraperitoneally. The mice were allowed to stay for 72 h post inoculation. After the seventy-two hours post inoculation, thick and thin peripheral blood films were prepared from blood collected from the tail of each mouse. The smear was prepared by spreading the blood on a clean slide over an area of 1.5 cm × 2.5 cm, allowed to dry and fixed with methanol, stained with 3% Giemsa stain for 45 min and examined with microscope under the oil immersion objective (100x) to determine the parasite density microscopically (Olympus CX, Japan). This was necessary so as to monitor the level of parasitaemia. For the positive slides, the number of parasites counted per 200 white blood cells was recorded and used to calculate parasite density, assuming 8000 leucocytes/µl of blood. The chemoprophylaxis of parasitaemia in relation to the control was assessed using the recommended formula:

Average (Av) % chemoprophylaxis =

$$\frac{\text{Av\% parasitaemia in control} - \text{Av\% parasitaemia in test} \times 100}{\text{Av\% parasitaemia in control}}$$

Biochemical Parameters

Blood samples were collected from the mice through the ocular vein into heparinized sample bottles at the end of the study (three mice per group on the 3rd day after inoculation). Haematological parameters such as packed cell volume (PCV), white blood cell count (WBC), neutrophils, eosinophils and haemoglobin level (Hb) were evaluated in accordance with standard procedures. Haemoglobin levels were measured using the cyanohaemoglobin method, red blood and white blood cell counts were done using haemocytometer, while PCV was measured by the conventional method of filling capillary tubes with blood and centrifuging using microhaematocrit centrifuge.

Liver function enzymes, AST: Aspartate Aminotransferase, ALT: Alanine Aminotransferase, ALP: Alkaline Phosphatase, renal function biomarkers (urea and creatinine) and total protein were assayed using corresponding Randox kits. Superoxide dismutase (SOD) was assayed utilizing the technique of Kakkar *et al.*[24] A single unit of enzyme was expressed as 50% inhibition of NBT (Nitroblue tetrazolium) reduction/min/mg protein.

Oxidative stress biomarkers, reduced glutathione (GSH) level was determined by the method of Ellman.[25] A measure of 0.1 ml of the supernatant was treated with 0.05 ml of Ellman's reagent (19.8 mg of 5, 5' -dithiobis nitro benzoic acid (DTNB) in 100 ml of 0.1% sodium nitrate) and 3.0 ml of phosphate buffer (0.2 M, pH 8.0). The absorbance was read at 412 nm.

Lipid peroxidation as evidenced by formation of TBARS and HP were measured by the method of Niehaus and Samuelsson.[26] A measure of 0.1ml of serum was treated with 2 ml of (1:1:1 ratio) TBA-TCA-HCL (thiobarbituric acid 0.37%, 0.25N HCl and 15% TCA) and placed in water bath for 15min, cooled and centrifuged at room temperature for 10min at 1,000 rpm. The absorbance of the clear supernatant was measured against reference blank at 535 nm.

Statistical Analysis

The results of the study were expressed as mean ± standard error of mean. Differences in the mean values were analyzed for significance by using Two-way analysis of variance (ANOVA), Dunnett's multiple comparison test using GraphPad Prism version 6.0 statistical software and Statistical Package for the Social Sciences, version 13.0 (SPSS Inc.). The values of $p < 0.05$ were considered statistically significant.

RESULTS

Effect of the polyherbal formulation on average % parasitaemia in mice infected with *P. berghei*

Extract load of the parasite in the blood of the experimental animals revealed the efficiency of the extract in a concentration-dependent manner. Sulphadoxine/Pyrimethamine (SP) standard anti-malaria drug, and distilled water were used as positive and negative controls, respectively. The average % parasitaemia in the groups is presented in Figure 1A. There was an inverse relationship between the dosage and the average percentage parasitaemia.

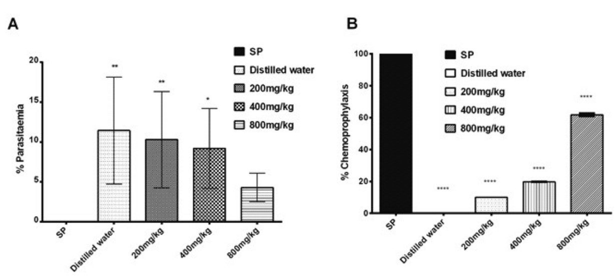

Figure 1: Effect of the polyherbal formulation on (A) percentage parasitaemia and (B) chemoprophylaxis

Group A (25 mg/kg) bw of sulphadoxine/pyrimethamine (SP), group B (2 ml water), groups C, D and E (200 mg, 400 mg and 800 mg/kg bw respectively of the polyherbal formulation; n = 5, (mean ± SEM). * = $p < 0.05$, ** = $p < 0.01$, **** = $p < 0.0001$.

Effect of the polyherbal formulation on chemoprophylaxis in mice infected with *P. berghei*

The percentage (%) chemoprophylaxis effect of the polyherbal formulation and the controls are presented in Figure 1B. As the dosage increased, the percentage chemoprophylaxis increased with the 800 mg dosage having the highest effect at 62.5%. The positive control, Sulphadoxine/Pyrimethamine had 100% chemoprophylaxis, while the negative control had 0% chemoprophylaxis.

Effect of the polyherbal formulation on Haemoglobin, White blood cells and Packed cell volume in mice infected with *P. berghei*

The effect of the polyherbal formulation on haemoglobin concentration, white blood cell concentration, packed cell volume in *P. berghei* infected mice is presented in Table 1. There were no significant differences in the treatment groups compared to the control, the group p-values are 0.9970, 0.999, 0.9979 and 0.9985 respectively.

Effect of the polyherbal formulation on liver function enzymes in mice infected with *P. berghei*

The liver function enzymes measured are AST: Aspartate Aminotransferase, ALT: Alanine Aminotransferase, ALP: Alkaline Phosphatase. There were no significant differences between the liver function parameters between the control group A and treatment groups B ($p = 0.4728$), C ($p = 0.7397$), D ($p = 0.7767$) and E ($p = 0.9834$) (Table 2).

Effect of the polyherbal formulation on kidney function parameters in the *P. berghei* infected mice

The measured biomarkers are Creatinine and Urea. There were no changes in the treatment groups compared to the control. The significant differences between the control group A when compared with the treatment groups are; B ($p = 0.2615$), C ($p = 0.5842$), D ($p = 0.7312$) and E ($p = 0.8841$) respectively (Table 3).

Effect of the polyherbal formulation on oxidative stress parameters of mice infected with *P. berghei*

There were significant differences between the oxidative stress parameters: SOD; Superoxide dismutase, MDA; Malondialdehyde and GSH; Glutathione in the treatment groups compared to the control. There was a significant decrease in the superoxide dismutase enzyme and the oxidative stress biomarker malondialdehyde, there was equally a significant increase in the endogenous antioxidant glutathione compared to the control, $p < 0.0001$, $p < 0.0001$, $p = 0.0122$ and 0.0038 respectively (Table 4).

Table 1. Effect of the polyherbal formulation on Hb, WBC and PCV in *P.berghei* infected mice.

GROUP	A	B	C	D	E
Hb (g/dl)	14.31 ± 0.39	12.90 ± 0.44	13.23 ± 0.36	13.90 ± 0.55	13.54 ± 0.55
WBC (mm3)	^{a}3950 ± 500	^{a,b}5666 ± 2.08	^{b,c}3800 ± 278.4	^{a,c}5500 ± 57.7	^{a,c}5366.67 ± 208.8
PCV (%)	40.33 ± 1.45	38.69 ± 0.2	38.30 ± 0.9	40.0 ± 0.6	39.3 ± 0.3

Group A (25 mg/kg) bw of sulphadoxine/pyrimethamine (SP), group B (2 ml distilled water), groups C, D and E (200, 400 and 800 mg/kg bw. respectively of the polyherbal formulation; n = 5, (mean ± SEM). Hb: Haemoglobin, WBC: White Blood Cell Concentration, PCV: Packed Cell Volume. Differences in superscript letters indicated a significant difference. The group p values are 0.9970, 0.999, 0.9979 and 0.9985 respectively.

Table 2. Effect of the polyherbal formulation on liver function enzymes in mice infected with *P. berghei*.

GROUP	A	B	C	D	E
AST (U/L)	36.20 ± 2.27	40.46 ± 8.43	33.68 ± 5.31	35.73 ± 2.17	42.89 ± 8.74
ALT (U/L)	65.77 ± 1.05	69.32 ± 1.41	69.39 ± 0.76	66.05 ± 0.60	72.42 ± 2.53
ALP (U/L)	690.00 ± 42.74	586.97 ± 69.20	621.00 ± 52.17	754.11 ± 30.90	648.11 ± 45.10

Group A (25 mg/kg) bw of Sulphadoxine/Pyrimethamine (SP), Group B (2 ml distilled water), Groups C, D and E (200, 400 and 800 mg/kg bw. respectively of the polyherbal formulation; n = 5, (mean ± SEM). AST: Aspartate Aminotransferase, ALT: Alanine Aminotransferase, ALP: Alkaline Phosphatase, there are no significant differences between the liver function parameters compared to the control, the group p-values are 0.4728, 0.7397, 0.7767 and 0.9834

Table 3. Effect of the polyherbal formulation on kidney function parameters, Creatinine and Urea in mice infected with *P. berghei*.

GROUP	A	B	C	D	E
Creatinine (mg/dl)	^{a}1.35 ± 0.12	^{a}0.97 ± 0.090	^{a}0.91 ± 0.070	^{a}0.82 ± 0.052	^{a}1.06 ± 0.083
Urea (mg/dl)	79.41 ± 4.93	67.55 ± 7.96	71.47 ± 6.00	^{a}86.82 ± 3.56	74.64 ± 5.26

Group A (25 mg/kg) bw of sulphadoxine/pyrimethamine (SP), Group B (2 ml distilled water), Groups C, D and E (200, 400 and 800 mg/kg bw. respectively of the polyherbal formulation; n = 5, (mean ± SEM). The significant differences between the treatment groups

Table 4. Effect of the polyherbal formulation on Oxidative Stress parameters of mice infected with *P. berghei* in Mice Infected with *P.berghei*.

GROUP	A	B	C	D	E
Total protein (g/L)	29.91 ± 8.1	69.32 ± 1.414	6.82 ± 1.1	47.56 ± 5.6	33.07 ± 0.9
(SOD/min/mg/pro)	40.10 ± 11.02	34.71 ± 1.64	32.16 ± 1.24	35.37 ± 5.07	35.83 ± 1.74
MDA (nmol/ml)	7.23 ± 0.67	5.46 ± 2.52	6.44 ± 2.79	5.18 ± 0.98	6.07 ± 1.61
GSH (µmol/ml)	0.01 ± 0.002	0.11 ± 0.002	0.01 ± 0.002	0.15 ± 0.001	0.15 ± 0.002

Group A (25 mg/kg) bw of sulphadoxine/pyrimethamine (SP), group B (2 ml distilled water), groups C, D and E (200, 400 and 800 mg/kg bw respectively of the polyherbal formulation; n = 5, (mean ± SEM). The significant differences between the treatment and control groups, are $p < 0.0001$, $p < 0.0001$, $p = 0.0122$ and 0.0038 respectively.

DISCUSSION

With respect to a recent ethno-botanical survey carried out on a variety of medicinal plants used in the treatment of malaria, the use of *Azadirachta indica* (neem leaves), *Mangifera indica*, *Carica papaya*, *Cymbopogon citratus* and *Psidium guajava* either singly or preferably in combination for malaria treatment were recommended.[30] In this study, we made use of five medicinal plants (*Azadirachata indica* (neem leaves), *Cymbopogon ciratus* (lemon grass), *Morinda lucida* (bark of "oruwo" tree), *Enantia chlorantha* (bark of "awopa" tree) and *Citrus paradisi* (grape fruit). We hereby present the anti-malarial effect of the combination of these medicinal plants, which are in form of leaves, barks and fruits. The polyherbal formulation was efficient on the titer value of the *P. berghei* in the infected mice. Other studies have also shown the anti-plasmodial activities of *Azadirachata indica*,[31] *Allium sativum* L., *Carica papaya* L., *Vernonia amygdalina* Del., and *Lepidium sativum* L. They are some of the most commonly reported species occasionally used as anti-malarial plants.[32]

There were significant differences between the malaria parasite densities between groups A, B, C, D and E, such that Group A (positive control) which was the group treated with the Sulphadoxine/Pyrimethamine had zero malaria parasite density. Group B (negative control) which was not treated at all, had the highest malaria parasite density. Groups C, D and E which were treated with 200, 400 and 800 mg of the polyherbal formulation respectively had decreasing malaria parasite densities with group E having the least malaria parasite density. This is an indication of a dose-dependent effect of the polyherbal formulation on the growth of the malaria parasite. There was an inverse relationship between the increased doses and the malaria parasite density.

The present results are in line with a previous study which showed the anti-plasmodial effect of another herbal product, *Abaleria*, which displayed a dose-dependent *in vivo* anti-plasmodial effect, biochemical properties as well as improvement of lipid profiles of mice infected with *P. berghei*.[27] It is also in line with the work of Muregi *et al*[28], as they showed the polyherbal effect of extracts of four plants, *Ekebergia capensis*, *Stephania abyssinica*, *Ajuga remota* and *Clerodendrum myricoides* which gave IC_{50} values below 30 µg/ml against both chloroquine sensitive and resistant *P. falciparum* strains. A combination of *E. capensis* and *C. myricoides* with chloroquine showed a synergistic effect against the multi-drug resistant *P. falciparum* isolate.[28] Neem seed-oil showed some antibacterial activity on *Staphylococcus aureus*, however, the Neem seed-oil extract was reported to be acidic and non-edible.[29]

The Neem (Azadirachta indica) plant is valued for it ayurvedic properties[33] it was reported to have shown antimicrobial activities through inhibitory effect on microbial growth and also through the potentiality of cell membrane breakdown. The leaves also have insect repellant property.[19] Alkaloids, flavonoids, phenolics, terpenoids, and glycosides are the most-reported phytoconstituent with effective activity on drug-resistant malaria,[34] the polyherbal formulation used in this study are rich in these phytoconstituents, whose synergistic effect enhances their therapeutic properties.[35]

In a recent study, a combination of the leaves extract of *C. citratus*, *Carica papaya*, *A. indica* and *Ocimum gratissimum* was shown to have anti-plasmodial activities as a result of the synergistic action of their phytochemicals.[36] Our result is in line with this study, as we evaluated the effect of neem leaves, lemon grass, along with three other herbal formulations which the previous study did not examine. Equally, newly synthesized neem-silver nitrate nanoparticles were obtained using the aqueous extract of neem leaves, *A. indica*, especially for malaria therapy, this was shown to possess anti-plasmodial activity as well.[37] Another study showed that the ethanolic extract of *A. indica* has antiplasmodial activity against *P. berghei*.[38] This is also in line with the present study which made use of aqueous extracts of the polyherbal formulation.

Morinda lucida was shown to possess anti-plasmodial activities,[39] the leaves and bark are medicinal agents for malaria treatment hence, could be a potential source of antimalarial templates.[40]The anti-plasmodium effect of the *Morinda lucida* leaves was equally reported.[41] The extract was also shown to possess a potent antioxidant and antitumor activities.[42]

Our work is in agreement with the work of Raymond *et al*, 2021,[42] which revealed the prophylactic and anti-haemolytic effect of lemon grass. Ethanolic leaf extracts of *Cymbopogon citratus*, *A. sativum* and *R. communis* was shown to exhibit larvicidal properties.[20] However, *C. citratus* was most effective followed by *A. sativum* while *R. communis* was the least effective. Lemon grass is a good source of vitamins A and C, folic acid, magnesium, zinc, copper, iron, potassium, calcium and manganese[43], this nutritional components may have contributed to its therapeutic effect, especially the role it plays in malaria treatment.[44]

Haematological abnormalities are considered a hallmark of malaria[45] and this is reported to be most pronounced in *P. falciparum* infections.[46] This study showed that *P. berghei* malaria infections in mice may not necessarily lead to significant changes in hematological parameters. There were no significant differences in the measured parameters, and this is not in line with the work of Kotepui *et al.*,[46] where haematological parameters reduced as an indication of malaria-induced anaemia. The reason for the observed result in this present study could be that the polyherbal formulation prevented the parasites from thriving, hence, preventing the parasite from feeding on haemoglobin which may have led to anaemia.

Renal and hepatic dysfunctions are part of the pathological effects of malaria infection common in children and pregnant women. Creatinine and urea are renal function biomarkers, an increase in these biomarkers in blood is a marker for renal dysfunction, while hepatic dysfunction is also characterized by increase in liver enzyme activities. The polyherbal formulation did not result into an increase in these biomarkers which indicates its mild effect.

The existence of oxidative stress and changes in lipid profiles during acute malaria infection has been demonstrated in some studies. This includes depletion of antioxidants, increased plasma lipid peroxidation and altered fluidity of erythrocyte membrane.[47-48] Although, the oxidative stress appears to be a common phenomenon in acute infection, it may cause a specific consequence in malaria pathogenesis.[48] From the present study, there were no significant differences in the oxidative stress parameters. This could be as a result of the effect of the polyherbal formulation in preventing the parasites from thriving, hence, preventing oxidative stress induced by the parasites. It could equally be as a result of the potent antioxidative activity of the polyherbal mixture, especially due to their synergistic effect. This is in line with a recent study which revealed the fact that neem and its phytoconstituents which are rich antioxidants, play a significant role in scavenging free radical generation and prevention of disease pathogenesis.[49]

CONCLUSION

We hereby suggest that the neem-based polyherbal formulation evaluated in the present study which consisted of *A. indica* (neem leaves), *C. ciratus* (lemon grass), *M. lucida*, *E. chlorantha* (bark of "awopa" tree), and *C. paradisi* (grape fruits), exhibited significant anti-plasmodial activity in mice infected with *P. berghei* NK65 and equally prevented anaemia during malaria treatment. It may be a potent formulation for pharmaceutical drug discovery, in the quest to combat the persistent drug resistance challenge currently faced in the management of malaria.

Competing interest

The authors declare that they have no competing interest.

ACKNOWLEDGEMENT

The authors are grateful for the technical assistance from the laboratory technologists of the Department of Biochemistry, College of Medicine, University of Lagos, Nigeria.

REFERENCES

1. Carlton JM. Malaria parasite evolution in a test tube. Experimental evolution studies reveal drug targets and resistance mechanisms. Science 2018;359(6372): 159–160.
2. Greenwood BM, Fidock Da, Kyle DE, Kappe SHI, Alonso PL, Collins FH, Duffy PE. Malaria: progress, perils, and prospects for eradication. The Journal of Clinical investigation, 2008;118(4):1266-1276.
3. Colborn KL, Giorgi E, Monaghan AJ, Gudo E, Candrinho B, Marrufo TJ, Colborn JM, Spatio-temporal modelling of weekly malaria incidence in children under 5 for early epidemic detection in Mozambique. Scientific Reports, 2018;8(1):9238-9248.
4. Young M, Berti P, FritzGerald S. Insecticide Treated Nets and Vitamin A Supplementation: An integrated approach to control malaria and micronutrient deficiency. Literature review and Malawi case study, The Micronutrient Initiative workshop report. 2002;132.
5. Okochi VI and Okpuzor J. Micronutrients as therapeutic tools in the management of sickle cell disease, malaria and diabetes. African Journal of Biotechnology. 2005;4(13):1568-1579.
6. Oli B, and Gautam D. Medicinal value of *Azadirachta indica*: A review. Modern Phytomorphology, 2022;15(Puri 2005),161–167.
7. Sirima SB, Sawadogo R, Moran AG, Konate A, Diarra A, Yameogo M, Parise ME, Newman RD. Failure of a chloroquine chemoprophylaxis program to adequately prevent malaria during pregnancy in Konpela District, Burkia Faso. Clin Infect Dis., 2003;36:1374–1382.
8. Gafna DJ, Dolos K, Mahiri IO, Mahiri JG, Obando JA. Diversity of medicinal plants and anthropogenic threats in the Samburu Central Sub-County of Kenya. Afr J Tradit Complement Altern Med. 2017;14(5):72–79.
9. Quattara Y, Sanon S, Traore Y, Mahiou V, Azas N, Sawadogo L. Antimalarial activity of *Swartzia madagascariensis* Desv. (Leguminosae), *Combretum glutinosum* Guill. (Combretaceae) and *Tinospora bakis* Miers. (Menispermaceae), Burkina Faso medicinal plants. Afr J Trad CAM. 2006;3(1):75-81.
10. Dua VK, Verma G, Singh B, Rajan A, Bagi U, Agarwal DD, Gupta NC, Kumar S, Rastogi A. Anti-malarial property of steroidal alkaloid conessine isolated from the bark of Holarrhena antidysenterica. Malaria J., 2013;12(194):1-6.
11. Bero J., Frederich M., and Quetin-Leclercq J. Antimalarial compounds isolated from plants used in traditional medicine. Journal of Pharmacy and Pharmacology, 2009;61,1401–1433.
12. El-hassan AM, Shayoub ME, Abdul-kareem AM, Osman HM, Khalifa K. Design, formulation, and evaluation of Senna effervescent tablets. J Food Prod Ind., 2012;1(2):21-25.
13. Mojab F. Antimalarial natural products: a review. A J Phytomed., 2012;2(2): 52-62.
14. Hemming-schroeder E, Umukoro E, Lo E, Fung B, Tom P, Zhou G, Zhong D, Dixit A, Atieli H, Githeko A,

Anne VZ, and Yan G. Impacts of Antimalarial Drugs on Plasmodium falciparum Drug Resistance Markers, Western Kenya, 2003 – 2015. Am. J. Trop. Med. Hyg., 2018;98(3),692–699.
15. Amoa OP, Ntie-Kang F, Lifongo LL, Ndom JC, Sippl W, Mbaze LM. The potential of anti-malarial compounds derived from African medicinal plants, part I: A pharmacological evaluation of alkaloids and terpenoids. Malar J. 2013;12:449.
16. WHO World Malaria Report (2009). Geneva, Switzerland: World Health Organization; 2009.
17. Gupta MP, Correa MD, Solís PN, Jones A, Galdames C, Guionneau-Sinclair F. Medicinal plant inventory of Kuna Indians: Part 1. J Ethnopharmacol., 1993;40:77–109.
18. Elujoba T. Book review traditional medicinal plants and malaria. Afr J Tradit Complement Altern Med., 2005;2:206–207.
19. Kothawade M, Sufiyan M, Daphal G. Active Constituent in Neem (*Azadirachta Indica*) and Their Therapeutic Role. Int. Journal of Pharmaceutical Res. and Appl. 2022;7(5):1233-1245.
20. Ani OC, Ama E, Nnamonu EI. Comparative study on larvicidal potentials of *Cymbopogon citratus stapf*, *Ricinus communis L.* and *Allium sativum L.* on fourth instar larvae of Anopheles mosquitoes. J Basic Appl Zool. 2022;83(1):4-6
21. Davares AKL, Arsene MMJ, Viktorovna PI, Shommya D. *Enantia chlorantha* and its Multiple Therapeutic Virtues: A Mini Review. J Pharm Res Int. 2021 Sep 30;254–259.
22. Abdulkareem A, Babamale A, Anifowoshe A, Opeyemi O, Oloriegbe N, Adamolekun C, *et al.* Extract of *Morinda lucida* leaf rescues hypoglycaemic and dyslipidaemic conditions in *Plasmodium berghei*-infected mice. Bull Nat Sci Res. 2022;12(2):1–6.
23. Maegraith BG, Deegan T, and Jones ES. Suppression of malaria. British Medical Journal, 1952;27,1382–1384.
24. Kakkar PM, Das BBH, Viswanathan PN. A Modified Spectrophotometric Assay of Superoxide. Indian Journal of Biochemistry and Biophysics. 1984;21:130–132.
25. Ellman GL. Tissue sulfhydryl groups. Arch. Biochem. Biophys. 1959;82:70–77.
26. Niehaus Jr WG and Samuelsson B. Formation of malondialdehyde from phospholipids arachidonate during microsomal lipid peroxidation. Eur. J. Biochem. 1968;6:126–130.
27. Adebayo AH, Yakubu OF, Popoola JO, David LC, Okenze G, Agbafor AG, and Okubena O. Evaluation of antimalarial and biochemical profiles of Abaleria in Plasmodium berghei-infected mice. Comparative Clinical Pathology, 2018:27(1):159-1601.
28. Muregi FW, Njagi ENM, Lang'at-Thoruwa CC, Njue WM, Orago ASS, Omar SA, and Ndiege IO. Anti-plasmodial Activity of Some Kenyan Medicinal Plant Extracts Singly and in Combination with Chloroquine. Phytotherapy Research, 2004;18(5):379–384.
29. Assefa H and Dagnaw T. Studies on Antimicrobial Properties of Neem (Azadrichta indica) Seed Oil. Advances in Animal and Veterinary Sciences, 2022;10(2):244–252.
30. Odoh UE, Uzor PF, Eze CL, Akunne TC, Onyegbulam CM, and Osadebe PO. Medicinal plants used by the people of Nsukka Local Government Area, South-Eastern Nigeria for the treatment of Malaria: An ethnobotanical survey. Journal of Ethnopharmacology, 2018;218:1–15.
31. Murugan K, Panneerselvam C, Samidoss CM, Madhiyazhagan P, Suresh U, Roni M, Chandramohan B, Subramaniam J, Dinesh D, Rajaganesh R, Paulpandi M, Wei H, Aziz AT, Alsalhi MS, Devanesan S, Nicoletti M, Pavela R, Canale A, Benelli G. In vivo and in vitro effectiveness of Azadirachta indica-synthesized silver nanocrystals against Plasmodium berghei and Plasmodium falciparum, and their potential against malaria mosquitoes. Res Vet Sci. 2016;106:14-22.https://doi.org/10.1016/j.rvsc.2016.03.001
32. Suleman S, Tufa TB, Kebebe D, Belew S, Mekonnen Y, Gashe F, Musa S, Wynendaele E, Duchateau L, Spiegeller B. De, Wynendaele E., Duchateau L., and Spiegeleer BD. Treatment of Malaria and Related Symptoms Using Traditional Herbal Medicine in Ethiopia. Journal of Ethnopharmacology, 2018;213:262-279.
33. Ahmad S, Maqbool A, Srivastava A, and Gogoi S. Biological Detail and Therapeutic Effect of *Azadirachta indica* (neem tree) Products-A review. J Evid Based Med Health. 2019;6(22)1607–12.
34. Sema YA, Waktola TA. Anti-malarial plants in Ethiopia and their activities on drug-resistant malaria. FEMS Microbes. 2022;3:1-5.
35. Adeleke MT., and Ndah S. Comparative phytochemical analysis of four medicinal plants traditionally used for malaria therapy in Nigeria. World Journal of Biology Pharmacy and Health Sciences, 2022;10(01),80–85.
36. Ghazali SZ, Mohamed Noor NR, and Fadzli Mustaffa KM. Anti-plasmodial activity of aqueous neem leaf extract mediated green synthesis-based silver nitrate nanoparticles. Prep Biochem Biotechnol. 2021;52(1):99-107.
37. Afolabi OJ, Simon-oke AI, and Oluwatobiloba OI. Clinical Pharmacology Antiplasmodial Activity of Ethanolic Extract of Neem Leaf (Azadirachta indica) in Albino Mice Infected with Plasmodium berghei. International Archives of Clinical Pharmacology, 2021;7(1),1–7. https://doi.org/10.23937/2572-3987.1510024
38. Afolabi OJ, Abejide AE. Antiplasmodial activities of *Morinda lucida* (Benth) and Alstonia boonei (De wild) in mice infected with *Plasmodium berghei*. Bull Natl Res Cent. 2020;44(1):85-90.
39. Oladeji OS, Oluyori AP, Dada AO. Antiplasmodial activity of Benth. Leaf and bark extracts against infected mice. Saudi J Biol Sci. 2022;29(4):2475–2482.
40. Calister U, Lydia U, Edith D, Chioma N, Ben A, Andrew E, Ugwu N. Formulation and evaluation of *Morinda lucida* based-phytosome complexes for malaria treatment. PhOL Archives. 2020;3(1):345-357.

41. Bochra G, Ichrak BA, Ezzedine BM, Asma E, Ahmed B. Antioxidant Capacity and Antitumoral Activity of Citrus Paradisi Essential Oil. Biomed J Sci & Tech Res. 2021;40(2):32121-32141.
42. Raymond BB, Germain ST, David DFS, Orelien SMB, Hart Mann AYM, Seraphin Nji OM, *et al*. Prophylactic antimalarial effects of *Cymbopogon citratus* (DC.) Stapf (*Poaceae*) in a mouse model of *Plasmodium berghei* ANKA infection: normalisation of haematological and serum biochemical status. GSC Biol Pharm Sci. 2021;15(1):05–017.
43. Aćimović M, Kiprovski B, Gvozdenac S. Application of Cymbopogon citratus in Agro-Food Industry. J Agron Technol Eng Manag. 2020;3(3):423-436.
44. Chukwuocha UM, Fernández-Rivera O, Legorreta-Herrera M. Exploring the antimalarial potential of whole Cymbopogon citratus plant therapy. J Ethnopharmacol. 2016;193:517–523.
45. Opoka RO, Namazzi R, Bangirana P, John CC., Idro R and Shabani E. Lack of mortality in 22 children with sickle cell anemia and severe malarial anemia. Pediatr Blood Cancer, 2018;65(1):1–4.
46. Kotepui M, Piwkham D, Phunphuech B, and Phiwklam N. Effects of Malaria Parasite Density on Blood cell Parameters. PLoSONE, 2015;10(3):1–11.
47. Das BS, Patnaik Jk, Mohanty S, Mishra D, Mohansy D, Satpathy SK. Plasma antioxidants and lipid peroxidation products in falciparum malaria. Am. J. Trop. Med. Hyg., 1993;49: 720-725.
48. Sibmooh N, Yamanont P, Krudsood S, Leowattana W, Brittenham G, Looareesuwan S, Udomsangpetch R. Increased fluidity and oxidation of malarial lipoproteins: relation with severity and induction of endothelial expression of adhesion molecules. Lipids Health Dis. 2004;25(3):15:1-11.
49. Latif MJ, Hassan SM, Mughal SS, Munir M, Shabbir N, Mushtaq M, *et al*. Therapeutic Potential of Azadirachta indica (Neem) and Their Active Phytoconstituents against Diseases Prevention. Journal of Chemistry and Chemical Science. 2020;10(3):98-110.