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In vivo chemosuppressive activities of combinations of four Nigerian ethnomedicinal antimalarial ferns

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Abstract

Background to the study: The *in vivo* antimalarial activities and the effects of combining the methanol extracts of four ferns namely, *Nephrolepis biserrata* (NB), *N. undulata* (NU), *Platycerium stemaria* (PS), and *P. angolense* (PA) randomly and with chloroquine, was determined.

Methods: The four ferns were collected, separately air-dried, powdered and the extracts was obtained by cold maceration with methanol and evaporation *in vacuo.* They were subsequently investigated (0-800mg/kg) for antimalarial potency against chloroquine-sensitive *Plasmodium berghei berghei* in mice using the Peters' four-day test after preliminary toxicity studies, using Lorke's method. They were further tested separately, combined with chloroquine and randomly combined with each other at their respective median effective doses.

Result: NB and PS elicited comparable ED_{50} and lower activities than NU while PA was the least active. The percentage chemosuppression elicited by all the individual extracts were comparable [p>0.05] to that of chloroquine when combined, with chloroquine and each other, except for NU and PS. Also, NB+PA, NB+PS, NU+PA+PS, NU+NB+PS and NU+NB+PA gave comparable [p>0.05] chemosuppression to CQ. However, NU+PA, NU+CQ and PS+CQ elicited similar survival times and % survivor with chloroquine. The activities of the lower - acting PA and PS was only improved by combination with either NU or NB and did not give better effects than the most active individual drug.

Conclusion: The study confirms the ethnomedicinal use of NU and NB for malaria and indicate that combining the ferns did not give any significant increase in activity better than the most active individual or the standard drug.

Keywords: Ferns; Antimalarial; Chemosuppressive; Combination; *Nephrolepis biserrata; N. undulata; Platycerium stemaria; P. angolense*

1. Introduction

Combination therapy, which is a common concept in medical discipline where two or more drugs are combined to improve efficacy and delay development of resistance to individual component of a drug is not entirely new to herbal management of malaria [1]. Many herbal remedies which are usually prepared as combinations of many medicinal plants, have been used in treating febrile illness based on the fact that the component plants perform complementary functions in the body [2]. Several combinations of extracts of higher plants have also been reported in the management of several diseases [3] [4]. In a similar manner, lower plants that are active can also be combined to improve efficacy. Some lower plants combined in a random manner can show synergistic effects of the individual component which can yield standardized herbal remedies.

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Most of the standard drugs used in the treatment of various diseases including malaria, were obtained through rigorous research on some traditional herbal remedies [5]. These remedies, included a combination of ethnomedicinal plants for which parasites are rarely known to develop resistance. Recently, parasites are developing resistance to standard drugs against which they were formerly effective. Artemisinin Combination therapy (ACT) became developed to combat resistance of the parasite against single antimalarial drugs. It is therefore reasonable to approach the old remedies or develop standardized remedies with optimum or high activities against the parasite for development of effective drugs. A major innovation in developing effective standardized remedies is to scientifically combine the component plants to obtain verified activity profiles [6, 7]. In the treatment of malaria and search for effective antimalarial molecules from plants, higher plants are much in vogue [8, 9] while the lower plants such as bryophytes and pteridophytes seem to be scarcely investigated, though they also suggest ethno medicinal antimalarial properties. Among the non-flowering plants, only a few Pteridophytes such as *Niphidium crassifolium* (L.) Lellinger and *Drynaria quercifolia* (bulb*)* under the family Polypodiaceae commonly called Calaguala and Manliras respectively which are traditionally used to treat fever and malaria in the Panama region and by the Tetun ethnic people in Malaka, West Timor region, Indonesia respectively have been discovered to have anti-malarial properties [10, 11]. Also, among the single genus family Nephrolepidaceae, only *Nephrolepis biserrata* has been shown to be used ethno medicinally for the treatment of malaria [12]. Some pteridophytes particularly from the families Polypodiaceae and Nephrolepidaceae namely: *Platycerium stamaria, P. angolense* and *Nephrolepis biserrata, N. undulata* respectively are commonly found epiphytic ferns in southwestern Nigeria and so investigation of their antiplasmodial activities may be necessary either as single plant or in combination.

Nephrolepis undulata whose fresh fronds are commonly used to make decoction for the treatment of fever [13], grows in terrestrial habitat and sometimes as epiphytes on palm trees [14] is commonly called annual sword fern, helecho or ladder fern. *N. biserrata* which grows in a similar habitat possess larger leaves and without rhizomes [15]. It is used in the treatment of malaria in traditional medicine, as well as for cough, boils, abscesses and blisters [12, 16]. *N. undulata's* fresh frond also finds use in traditional medicine as cough and skin diseases remedy in India [17], the rhizomes for rheumatism, chest congestion and anorexia while the leaves are used in the treatment of wounds, stomach ache, jaundice and as pregnancy booster [14, 18].

P. angolenses also called *P. elephantotis* which is used to treat pulmonary troubles, as genital stimulants/depressants and as anti-abortifacients during pregnancy has its plant ash used in the treatment of heart diseases [19]. The sterile frond is used as ecbolic (to induce uterine contractions in order to facilitate delivery). It is an epiphytic large fern native to dry forests of tropical Africa and grows well in warm and low temperatures and produces large, un-branched, dark green foliar fronds. The triangle staghorn fern, *Platycerium stemaria* is native to tropical Africa and consists of semi-erect, large foliar fronds with a silvery cast when young. Its drooping inverted Y-like fertile leaves form the shape of long triangle. It produces pulps readily but more difficult to grow from spores [20]. Methanol and petroleum ether extract of *N. undulata* has been discovered to have anti-microbial activities while aqueous extract of *Nephrolepis biserrata* has antihypertensive potential [21].

The antiplasmodial activities of *Nephrolepis biserrata, N. undulata, Platycerium stemaria, P. angolense,* which are pteridophytes from the families Nephrolepidaceae and Polypodiaceae need to be explored. Therefore they were evaluated singly and in combination in order to assess the effects of combining them either with each other or with standard drug on their antimalarial properties as single drugs.

2. Material and methods

2.1. Collection, preparation and authentication of plant materials

The leaves (frond) of *Nephrolepis biserrata* and *N. undulata* were collected at the Oil Palm plantation, before Opa dam, Road 7, Obafemi Awolowo University, Ile-Ife, Nigeria, while those of *Platycerium stemaria* and *P. angolenses* were collected behind Oduduwa Hall lecture theatre, Obafemi Awolowo University, Ile-Ife, Nigeria. The plants were identified by Dr (Mrs.) R. A. Bamigboye and authenticated at the Faculty of Pharmacy herbarium by Mr. I. I Ogunlowo of the Pharmacognosy Department, Obafemi Awolowo University, Ile-Ife, Nigeria. Specimen with Voucher numbers FPI 2320, FPI 2322, FPI 2319 and FPI 2321 for *Nephrolepis biserrata*, *N. undulata, Platycerium stemaria* and *P. angolenses* respectively were deposited at the Faculty of Pharmacy herbarium, Obafemi Awolowo University, Ile-Ife, Nigeria.

2.2. Extraction

Briefly, the leaves of each of the four plant species: *N. biserrata, N. undulata*, *P. stemaria* and *P. angolenses* were separately air dried for 7 days, and powdered using Christy and Norris grinding machine. A 200.0 g of each plant

material was separately macerated in 2.0 L of methanol for 72 hours with intermittent shaking. The resultant extracts were filtered, evaporated *in vacuo*, freeze-dried and weighed. The yields were noted.

2.3. Acute toxicity study

Each of the methanolic extracts of *N. undulata, N. biserrata, P. angolense,* and *P. stemaria*, was subjected to acute toxicity testing using Lorke's method. Briefly, each extract was administered at 10, 100, 1000 mg/kg doses respectively to 3 groups of three mice per group in a phase I evaluation. The mice were observed for 24 hours for behavioural changes and mortality. In the second phase, three mice grouped into three groups of one mouse each and administered with higher doses of 1600, 2900 and 5000mg/kg of the extracts respectively. They were also observed for 24 hours for behavior changes as well as mortality. For each of the phases, highest dose that gave no mortality (D_0) and lowest dose that produced mortality (D_{100}) were determined. These were used to calculate the LD_{50} of the extracts respectively using the formula: $LD_{50} = \sqrt{D_0} \times \sqrt{D_{100}}$ [22].

2.4. Rodent Parasite

The rodent parasite, *Plasmodium berghei berghei* NK 65 passaged into Swiss albino mouse was obtained from the Institute of Advanced Medical Research and Training (IMRAT), University College Hospital, Ibadan. It was maintained by serial passaging into other mice and was monitored keenly for rise in the parasitaemia level before being used for the experiment.

2.5. Preparation of the mice

Seven-week old Swiss albino mice of either sex weighing between 18 to 24 g (male and female, not pregnant) were obtained from the Animal House, Obafemi Awolowo University, Ile-Ife, where they were housed in aluminum cages with wood shavings used as beddings and allowed free access to water and food (Growers' mash) under 12 hours day/night cycle. They were acclimatized for at least seven days before use. The mice were handled in accordance with NIH Guide for the care and use of laboratory animals; (NIH Publication, No. 83-123 (revised), 1985). The animals were randomly divided into groups of five mice each for the experiments.

2.6. Inoculation of the test animals

The parasitized donor mouse with parasitaemia level up to a level of about 30 % was euthanized with chloroform. Blood was obtained through cardiac puncture using sterile needle and syringe into a heparinized bottle. The blood was diluted with normal saline such that 0.2 mL of the resultant solution will contain standard inoculum of 1×10^7 infected red blood cells (RBC). The inoculum of 0.2 mL was given to each of the test mouse intraperitoneally in order to commence the antiplasmodial test.

2.7. Preparation of the test extracts and standard drug

The doses of each of the extract of *N. biserrata*, *N. undulata*, *P. stemaria* and *P. angolenses*, to be utilized for the experiment (100, 200, 400 and 800 mg/kg) were prepared by dissolving 50, 100, 200 and 400 mg of each of the extract in 5.0ml of Normal saline respectively. The doses of each of the extracts and Normal saline were administered to the test groups and the negative control group respectively while chloroquine (10 mg/kg) was administered to the positive control group. For the combination experiments, 1401.82 mg, 882.81 mg, 1512.8, 1402.8 mg of NB, NU, PA & PS respectively were solubilized in 30.0 ml of normal saline to give doses of 467.27, 294.27, 504.31, 467.65 mg/kg being the respective ED_{50} (median effective dose) for the individual drugs (Fig. 1). These were administered as appropriate to the mice accordingly in the combination experiments.

2.8. *In vivo* antiplasmodial dose- response studies on the fern individual extracts.

The *in vivo* antiplasmodial activities of each of the extracts of NB, NU, PA & PS was determined using the four – day chemosuppressive test. Briefly, (thirty) 30 acclimatized mice were randomly divided into six groups (I-VI) of five animals each and inoculated with 0.2 ml of the diluted parasitised blood as above. Two hours after inoculation (D_0), Groups' II-V of the mice were administered with 100, 200, 400 and 800mg/kg of the freeze-dried methanol extracts of *Nephrolepis biserrata*, dissolved in normal saline respectively, while Group I received normal saline to serve as negative control and Group VI received chloroquine (10 mg/kg) to serve as positive control. This was repeated daily for three consecutive days (D_1-D_3) after measuring their rectal temperature. The level of parasitaemia was determined on the fifth day (D_4) for each mouse, from which the percentage chemosuppression and the median effective doses were calculated. This whole procedure was also repeated each for *N. undulata, P. angolense, P. stemaria* respectively.

2.9. Estimation of average percentage parasitaemia, percentage chemosuppression and median effective doses

Ten fields of view with uniform distribution of red blood cells were selected from a thin and stained smear of blood obtained from the experimental mouse and viewed using oil immersion (x100) objective of the microscope. The numbers of parasitized (PRBC) as well as unparasitized (UPRBC) red blood cells were counted for each field. The percentage parasitaemia for each field of view was then calculated from the formula: 100 x (total number of parasitized red blood cell (PRBC) /total number of parasitized (PRBC) and unparasitised (UPRBC) red blood cell). The averages of these percentage parasitaemia for the 10 fields per mouse were calculated while the average of these results for five mice gave the average percentage parasitaemia per dose with their respective ±SEM values [23].

From the Average Percentage Parasitaemia, the percentage (%) chemosuppression for each dose of each of the extracts were afterwards calculated using this formula: 100 x (Average parasitaemia in the negative control (PNC) - Average parasitaemia in the test dose (PTD) / Average parasitaemia in the negative control (PNC)). The values were recorded as percentage (%) chemosuppression ±SEM.

The effective doses (ED_{50} and ED_{90}), for each fern extract was forecast from a plot of dose against percentage chemosuppression using Microsoft Excel programme package.

2.10. Survival times and percentage survivor of mice

The treated mice were observed for mortality for 28 days from the day of drug administration in order to determine the survival times and percentage survivors elicited by the extracts in the mice. The survival time for each mouse was recorded, in days and the average for each group determined as days ± SEM. The percentage survivor for each group was estimated from the average survival time as the percentage number of mice eliciting survival time that falls within the average for the whole group.

2.11. *In vivo* antiplasmodial combination studies on the fern individual extracts.

The extracts were tested individually in mice at their respective median effective doses (ED_{50}) in mg/kg obtained from the dose-response experiment above (Fig. 1). Briefly, six groups of 5 mice each were administered with appropriate volumes of the diluted extracts, NB, NU, PA and PS, inclusive of normal saline and chloroquine (CQ), 10mg/kg as negative (NC) and positive controls respectively equivalent to the ED_{50} values (467, 294, 504 & 467) in mg/kg according to their weights and subjected to *in vivo* chemosuppressive tests as above. The doses were administered after 2 hours of inoculation and subsequently daily for 3 days. The level of parasitaemia was determined on the fifth day (D_4) from a blood smear made from each mouse. Percentage chemosuppression was afterwards calculated from the percentage parasitaemia as the parameter for the activity of the extracts.

2.12. *In vivo* antiplasmodial studies on the combination of fern extracts and the standard drug

Appropriate volumes of the diluted extracts of NB, NU, PA and PS, equivalent to the respective median effective doses (ED_{50}) in mg/kg obtained from the dose-response experiment above (Fig. 1) were administered successively with 10mg/kg of CQ to six groups of 5 mice each. Normal saline and chloroquine (CQ), 10mg/kg were negative (NC) and positive controls respectively. The doses were administered after 2 hours of inoculation and subsequently daily for 3 days. The level of parasitaemia was determined on the fifth day (D_4) from which the percentage chemosuppression was calculated.

2.13. *In vivo* antiplasmodial studies on the 2- and 3-plant combinations of fern extracts.

For the combination of the different extracts, thirteen (13) groups of five mice each were prepared and administered respectively with the combinations of extracts at their respective median effective doses in a successive manner and based on the weight of each mouse as follows: NU+PA, NB+PS, NU+NB, NU+PS, NB+PA, PA+PS, NU+ NB+ PS, NU +PA +PS, NU+NB+ PA, NB+ PA+ PS, NU+ PA+ PS+ NB. Normal saline and chloroquine (CQ) were negative (NC) and positive controls respectively. The extracts were administered in combination, two hours after inoculation with parasite on the first day of the experiment and daily for three consecutive days as above. The level of parasitaemia was determined on the fifth day (D_4) as above to obtain the percentage chemosuppression for each combination.

2.14. Statistical analysis

Values of percentage parasitaemia, percentage chemosuppression and the median effective doses for the various extracts and combinations as the case may be, were expressed as mean±SEM and analyzed statistically using One-way

Analysis of Variance (ANOVA) followed by Student Newman Keul's post-hoc test for comparisons to determine the source of significant difference for all values. Values of $p < 0.05$ were considered to be of statistical significance.

3. Results and discussion

Traditional herbal remedies which are prepared by using the different parts of same or different medicinal plants have been employed in the treatment of malaria for over a number of centuries across several parts of the world [24]. That the first antimalarial drug, quinine, and the latest one, artemisinin were isolated from *Cinchona succirubra* bark and *Artemisia annua* herb respectively is an encouragement to test more plants for antimalarial activities [25, 26, 27].

Generally, plants have been a huge reservoir of chemicals, the development of which has yielded many drugs for the amelioration of various diseases, malaria inclusive [28].

Globally, there has been a rise in the use of plant remedies as well as the search for new phytochemicals that can be potential sources of new and more potent drugs for the treatment of malaria and other infectious diseases [29]. In obtaining plant remedies, several plants are combined [2] while for the purpose of obtaining potent drugs, plants were randomly selected and screened for antimalarial activity in a process which was unnecessarily laborious until a better approach which identifies potential plants based on their ethnomedicinal uses against fever and/or malaria was recently adopted by researchers [30]. Several higher plants has been confirmed to have antimalarial activity using this procedure, having been screened using various *in-vivo* and *in vitro* antimalarial activity test models. For example, a diversity of higher plants including, *Morinda lucida* [31] *Tithonia diversifolia* [32, 33], *Khaya grandifoliola* ([34, 35], *Guiera senegalensis* [36], *Quassia amara* [37, 38], *Vernonia amygdalina* [39, 40], *Crossopteryx febrifuga* ([33, 41] and *Spathodea campanulata* [31] have all been proven to exhibit *in-vivo* activity against the malaria parasite, *Plasmodium berghei berghei*.

Of late, research into plants for antimalarial compounds have been geared towards higher plants with little venture into lower plants. Though lower plants also suggest ethnomedicinal antimalarial properties, there has not been a pronounced search for antimalarial agents from them. Therefore, it becomes imperative to venture into lower plants with their varied diversities [42] for remedies and potent antimalarial compounds. Also, the fact that some lower plants have shown ethno medicinal relevance in the treatment of malaria suggest that investigations into their potentials as antimalarial agents or remedies is worthwhile [10]. Therefore, a recourse to these categories of living organisms in this work is necessary [43].

Two families of Polypodiaceae and Nephrolepidaceae were focused in this work with the major aim of identifying the antimalarial potential of each of the plants and their various combinations using the *in vivo* four-day test. This would naturally add to the remedies that can be used in treating or managing malaria and those exempted from consumption. The plants: *Nephrolepis biserrata, N. undulata, Platycerium stemaria, P. angolense*, were collected in their natural habitats in mid July 2019, during the rainy season, dried, powdered and extracted with methanol, concentrated i*n vacuo* and tested.

3.1. Acute toxicity testing

The acute toxicity testing was to establish the safety, or otherwise of the extracts and identify the range of doses that may be administered to mice. The LD_{50} up to 5000mg/kg elicited by leaf of *N. undulata* and *P. stemaria* showed that they were safe [44]. Also neither *of the mice* showed evidence of skin changes, aggressiveness, diarrhoea, restiveness, seizures, dizziness, weakness nor withdrawal from food. *The* LD_{50} values of *t*hose of *Nephrolepis biserrata* and *P. angolense* were up to 2900 mg/kg and 3807.89 mg/kg respectively. LD_{50} values should guide in the choice of dosage for testing the activity and the potential for human and animal use. Non-toxic extracts can be tested at minimum doses 20 times lower than the LD_{50} value. For *N. undulata* and *P. stemaria*, 250 mg/kg is a reasonable minimum dose to use. However, for *N. biseratta* and *P. angolense*, 145 and 190 mg are minimum doses respectively. They were therefore tested at doses 100, 200, 400 and 800 mg/kg [45].

This discovery confirms the earlier report that *N.biserrata* is safely used in the ethnomedicinal management of malaria *and N. undulata* in the treatment of fever [13, 16].

3.2. Antiplasmodial activities of the individual extracts

The validity of rodent *in vivo* models in antimalarial activities screening for most of the tested compounds has been established through its value in the identification of several conventional antimalarial agents such as chloroquine, halofantrine, mefloquine, and artemisinin derivatives [46]. Also, the chemosuppressive test is an approved model by

WHO for assessing the antimalarial activities of new antimalarial drugs through plant studies [47]. It has the added advantage of being able to simulate the Africa's malarial endemic status hence the application of this model in this experiment. The parasite chemosuppression, median effective doses, survival times and percentage survivors are reasonable parameters that may capture the antimalarial potential of each of the extracts [48]. *In vivo* antiplasmodial activities determinations of chemosuppressive, prophylactic and curative have been variously used to ascertain antimalarial potencies of medicinal plants in their various plant parts [49].

Table 1 Average percentage parasitaemia elicited by methanol extracts of *N. undulata, N. biserrata, P. stemaria,* and *P. angolense*

Dose (mg/kg)	NU	NB	PS	PA
0	4.94 ± 0.3 ^{b}	3.91 ± 0.1 ^{b}	4.94 ± 0.3 ^{b}	3.91 ± 0.1 ^{b}
100	2.05 ± 0.3 ^{a}	3.43 ± 0.4 ^{b}	4.31 ± 0.3 ^{a}	3.09±0.1 ^{b}
200	1.80 ± 0.1 ^{a}	2.81± 0.5 ^{a}	3.55 ± 0.7 ^{a}	2.48±0.3 ^{a}
400	1.66 ± 0.1 ^{a}	1.81± 0.7 ^{a}	3.51 ± 0.4 ^{a}	2.18±0. 0^{a}
800	1.40 ± 0.1 ^{a}	1.58 ± 0.3 ^{a}	2.18 ± 0.2 ^{a}	1.85±0.2 ^{a}
CQ	1.92 ± 0.2 ^{a}	1.61 ± 0.3 ^{a}	1.92 ± 0.2 ^{a}	1.61±0.3 ^{a}

Keys: Data show the mean ± SEM, *n* = 5. NU- *Nephrolepis undulata,* NB- *N. biserrata,* PS- *Platycerium stemaria,* PA- *P. angolense,* PC-*positive control,* NC (negative control): Tween 80 in normal saline; Chloroquine (10 mg/kg) = positive controls. Only values with different superscripts within columns are significantly different ($p < 0.05$, one-way analysis of variance followed by the Student–Newman–Keul's post hoc test).

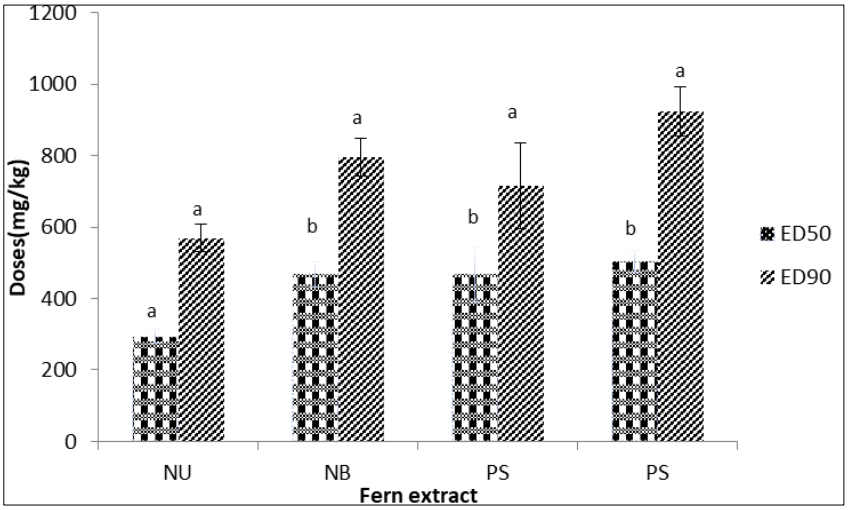
Table 2 Percentage chemosuppression elicited by methanol extracts of *N. undulata, N. biserrata, P. stemaria* and *P. angolense*

Dose (mg/kg)	NU	NB	PS	PA
0	0.00 ± 0.0 ^{a}	00.00± 0.0 ^{a}	0.00 ± 0.0 ^{a}	0.00 ± 0.0 ^{a}
100	57.74 ± 7.2 ^{b}	13.09 ± 4.1 ^{a}	31.60 ± 4.7 ^{a}	20.97 ± 3.3 ^{a}
200	62.69 ± 4.4 ^{b}	27.75 ± 11. 2 ^{a}	31.62 ± 12.0 ^{a}	36.27 ± 9.3 ^{b}
400	66.41 ± 0.8 ^{b}	50.05 ± 13.6 ^{b}	31.62 ± 8.9 ^{a}	44.07 ± 5.4 ^{b}
800	71.20 ± 3.2 ^{b}	59.28 ± 6.5 ^{b}	35.60 ± 0.2 ^{a}	52.82 ± 4.1 ^{b}
CQ	67.40 ± 14.0 ^{b}	58.82 ± 12.1 ^{a,b}	67.40 ± 14.0 ^{b}	58.82 ± 12.0 ^{b}

Keys: Data show the mean ± SEM, *n* = 5. *Nephrolepis undulata,* NB- *N. biserrata,* PS- *Platycerium stemaria,* PA- *P. angolense,* PC-*positive control,* NC (negative control): Tween 80 in normal saline; Chloroquine (10 mg/kg) = positive controls. Only values with different superscripts within columns are significantly different ($p < 0.05$, one-way analysis of variance followed by the Student–Newman–Keul's post hoc test).

In this study, at all the doses tested, each of the extract of the ferns gave a dose-dependent reductions in parasitaemia (Tables 1 and 2). *N. undulata* produced the lowest percentage parasitaemia at the lowest and the highest dose tested. It also produced percentage chemo suppressions that were highest at the lowest and the highest doses. At all the doses tested, the extracts of *N. undulata* and *P. stemaria* gave percentage parasitaemia levels that were significantly lower ($p<0.05$) than that of the negative control while those of NB and PA were comparable ($p>0.05$) to that of the negative control at 100 mg/kg only but were comparable to the positive control from 200-800 mg/kg (Table 1). Also, all doses of NU and PS gave values that were comparable to each other and the positive control (Table 1). The use of NB and NU for malaria and fever in ethnomedicine is respectively justified [12]. PS with a % suppression of 36 at the maximum tested dose should not be listed among the antimalarial active plants while PA which gave 53% chemosuppression at 800mg/kg like NB (59 %) have only moderate antimalarial activities (Table 2). A 53 % chemosuppression elicited by *Polyalthia longifolia* at 800 mg/kg dose was considered to be a moderate activity [50]. The median effective doses ED_{50} value of 294.27±17.7 and ED_{90} of 569.46±39.5 mg/kg elicited by *N. undulata* showed that it was the most active of the four extracts while *P. stemaria, P. angolense*, and *N. biserrata* elicited comparable ED_{50} to all rank next to *N. undulata* (Fig. 1). This was consistent with the lowest average % parasitaemia of 1.4 and highest percentage chemosuppression of 71.2 % elicited by *N. undulata* (Table 1 and 2). However, *N. undulata, P. stemaria* and *N. biserrata* elicited comparable ED_{90} values to rank better than only *P. angolense* (Fig. 1). The order of activity is *N. undulata* > *N. biserrata =P. stemaria >P. angolense*. The rank ordering of the extracts usually indicate their relative antiplasmodial activities that may guide

further work on the plants. Although there is no previous report on the activity of *P. stemaria* and *P. angolense* against malaria ethnomedicinally, the result from the study gave a profile of their activities for the first time with that of P. angolense being relatively inactive. The employment of *P. berghei berghei parasite* in the prediction of plant antiplasmodial activities was because of its higher accessibility and sensitivity. Chloroquine was used as the standard drug because of the parasite's sensitivity and its significant suppression of this parasite [51].



Keys: Data show the mean ± SEM, *n* = 5. **NU**-*Nephrolepis undulata,* **NB**-*N. biserrata,* **PS**-*Platycerium stemaria,* **PA**-*P. angolense.* **PC**-*positive control,* **NC** (negative control): Tween 80 in normal saline; Chloroquine (10 mg/kg) = positive controls. Only values with different superscripts within columns are significantly different ($p < 0.05$, one-way analysis of variance followed by the Student–Newman–Keul's post hoc test).

Figure 1 Effective Doses (ED_{50} and ED_{90}) of the methanol extracts of *N. biserrata*, *N. undulata*, *P. stemaria* and *P. angolense*

3.3. Survival times and percentage survivors of the individual extracts in the dose response experiment

Table 3 Survival times and percentage survivors (in parenthesis) elicited by the methanol extracts of *N. biserrata, N. undulata, P. stemaria and P. angolense*

Dose(mg/kg)	NU	NB	PS	PA
NC	16.60 ± 0.9 [a] (40)	16.60 ± 0.9 [a] (40)	16.60 ± 0.9 [a] (40)	16.60 ± 0.9 [a] (40)
100	15.80 ± 1.2 [a] (60)	14.60 ± 2.6 [a] (60)	14.80 ± 4.0 [a] (75)	9.40 ± 4.0 [a] (40)
200	15.20 ± 1.0 [a] (60)	13.80 ± 2.1 [a] (40)	9.60 ± 3.3 [a] (60)	14.80 ± 9.3 [a] (80)
400	17.40 ± 1.6 [a] (60)	13.00 ± 2.0 [a] (40)	16.00 ± 1.8 [a] (60)	10.80 ± 1.6 [a] (60)
800	12.80 ± 3.5 [a] (75)	14.60 ± 2.5 [a] (80)	15.60 ± 1.4 [a] (60)	16.40 ± 1.6 [a] (80)
CQ	28.00 ± 0.0 [b] (100)	28.00 ± 0.0 [b] (100)	28.00 ± 0.0 [b] (100)	28.00 ± 0.0 [b] (100)

Keys: Data show the mean ± SEM, *n* = 5. *Nephrolepis undulata,* NB- *N. biserrata,* PS- *Platycerium stemaria,* PA- *P. angolense,* CQ-*positive control*-Chloroquine (10 mg/kg), NC-Negative control. Tween 80 in normal saline. Only values with different superscripts within columns are significantly different ($p < 0.05$, One-way analysis of variance followed by the Student–Newman–Keul's post hoc test).

The survival times elicited by all the extracts at all doses tested were comparable ($p>0.01$) to that of the negative control but significantly different ($p<0.01$) from that of the positive control. The mice could not survive beyond the day of drug administration.

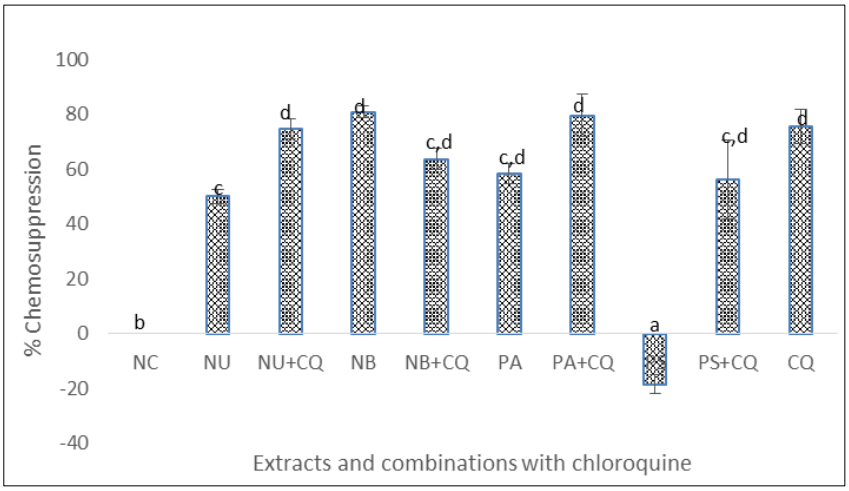
N. biserrata and P. angolense which gave lower % chemosuppression however gave higher percentage survivor profile of 80% at the highest dose compared to 75% given by *N. undulata* which elicited higher antiplasmodial activities. All the ferns elicited survival times that were comparable to the negative control (Tables 2 and 4). It has been opined in similar studies that percentage parasitaemia reduction is better correlated with % survivor rather than with survival times [52, 53]. Effective antimalarial drugs should also increase survival times or percentage survivors significantly in addition to better antiplasmodial activities. Exceptions may indicate toxicities of such extracts, thus suggesting further toxicity evaluation of the extracts before employing them as antimalarial agents in practice. It should be noted that *N. biserrata* and *P. angolense* were safe only up to 2900 and 3802 mg/kg in Lorke's evaluation of acute toxicity (section 3.1 above). Based on the relative antiplasmodial activities the survival time and percentage survivor profile of the four extracts, NU was selected as the most active fern extract.

3.4. Antiplasmodial activities of the combination of the fern extracts with standard drugs

Conventional antimalarial drugs were isolated from plants among a variety of other associated compounds in combination *in situ.* When combined in the plants, they face the parasites together and so less prone to resistance. In single isolated states, they are more prone and so may need other chemicals to achieve the initial combined state. Combination of chemicals have been shown to exhibit better activities against the parasite and so increase the life span of antimalarial drugs. ACT have been advocated and adopted by the WHO for this reason.

Also, most of the time, patrons and patients combine plants extracts or products with orthodox drugs prescribed in the hospital especially in cases like cancer and hypertension. This usually affect the outcome of the treatment either positively or negatively [54]. It may therefore be desirable to test the effects of plant extracts on the orthodox antimalarial drugs and thus predict the effects of this unexpected combination on the activities' of the prescribed drugs in practice. Similar experiments have considered such where it was observed that plant extracts lowered the activities of chloroquine [48, 55]. Inadvertent combinations of standard drugs and plant extracts may therefore becloud interventions in a diseased state.

The fern extracts under test were each combined at their median effective doses with 10mg/kg, chloroquine, which is the standard antimalarial drug in this experiment. All the combinations with the standard drug, gave percentage parasitaemia that were not significantly different ($p>0.05$) from that elicited by the standard drug (Fig 2). This means that the combination or co administration of each of the fern extracts with chloroquine did not affect the activity of chloroquine significantly; also imply that inadvertent co administration in practice may not significantly affect the response of chloroquine in malarial treatment. The incorporation of standard drugs like sedatives into some herbal preparation was to increase activity of same for competitive advantage [48]. This now calls for sundry investigations into the effect some herbal drugs may have on some pharmaceutical drugs used in the treatment of some diseases.



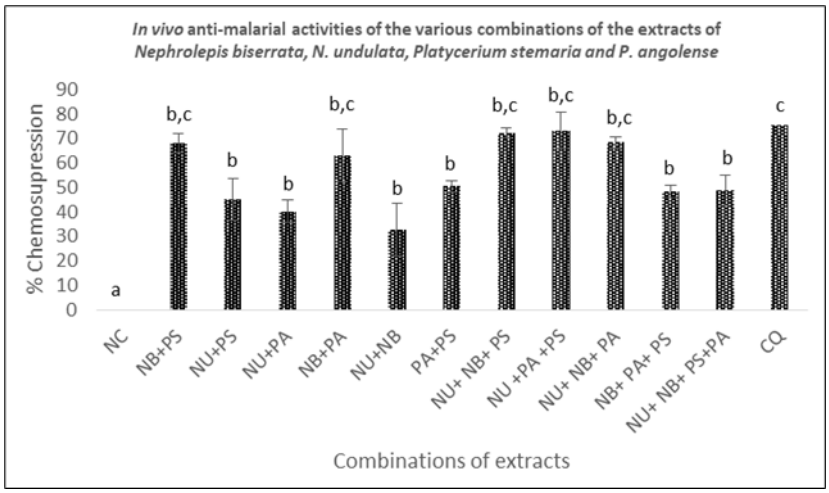
Keys: Data show the mean ± SEM, $n = 5$. *Nephrolepis undulata,* NB- *N. biserrata,* PS- *Platycerium stemaria,* PA- *P. angolense,* PC-*positive control*-Chloroquine (10 mg/kg), NC-Negative control. Tween 80 in normal saline. Only values with different superscripts on the bar are significantly different ($p < 0.05$, One-way analysis of variance followed by the Student–Newman–Keul's post hoc test).

Figure 2 *In vivo* anti-malarial activities of combinations of standard drug with extracts of *N. biserrata, N. undulata, P. stemaria and P. angolense*

3.5. Antiplasmodial activities of the combination of the plants extracts

In ethnomedicine, plants are combined for many reasons but principally to complement the activities of each other, though such combination may give varied effects. Therefore, the random combination of the ferns in twos, threes, and the four at their median effective doses were to identify useful and the not so useful combinations. The higher percentage chemosuppression elicited by NB than NU and PA similarly tested showed NB to be the most active here, its activities being comparable to that of CQ, the standard antimalarial drug. But PS with a % chemosuppression of -11.5 was relatively inactive. The order of activity is NB>NU=PA>PS. The fern extracts have no effect on the activities of CQ.

The comparable activities ($p>0.05$) elicited by the combination of NU with any of the other extracts, and the significantly ($p<0.05$) lower activities given by a combination of PA and PS than that of NB or CQ (Figs 2 and 3) is noteworthy. So also is the significantly ($p<0.05$) reduced antiplasmodial activities in the combination of each of NB or NB+NU together with PA+PS respectively. A combination of NB with PA or PS, which significantly lowered the activity of NB; and the combination of NU with PA+PS, NB+PS or NB+PA with similar trend of lowered activities only confirmed the obvious effect that combination of medicinal plants can give higher or lower antimalarial activities. For example, combinations of the leaf of *Citrus aurantifolia* and its fruit gave enhanced curative antimalarial activities when compared with the individual plant extracts while the same combination gave reduced prophylactic antimalarial activities in mice [48]. It became obvious that combining the ferns did not show any significant increase in activity better than the most active individual drug. This also stands to affirm the necessity of scientific investigation before combining medicinal plants in traditional medicine. Some combinations are beneficial while others are not. For instance, combinations of *Nauclea latifolia* root extract (34.4%) plus *Murayya koenigi* leaf extract (48.5%) and *Enantia chlorantha* (56.4%) plus *Murayya koenigi* (48.5%) at their respective ED_{50} values gave better chemosuppression values (78.9% and 76.9%) than those given by the individual plants while that of *Nauclea latifolia* (34.4%) *plus Enantia chlorantha* (56.4%) *combined* in a similar way gave lower chemosuppression value of 42.4% [55].



Keys: Data show the mean ± SEM, *n* = 5. *Nephrolepis undulata,* NB- *N. biserrata,* PS- *Platycerium stemaria,* PA- *P. angolense,* PC-*positive control*-Chloroquine (10 mg/kg), NC-Negative control. Tween 80 in normal saline. Only values with different superscripts on the bar are significantly different ($p < 0.05$, One-way analysis of variance followed by the Student–Newman–Keul's post hoc test).

Figure 3 *In vivo* anti-malarial activities of the various combinations of the extracts of *N. biserrata, N. undulata, P. stemaria and P. angolense.*

3.6. Survival times and percentage survivors of the combination of the fern extracts with standard drugs

An effective antimalarial drug should prolong the life of the patient and not give toxic reactions or unmanageable side effects. For rodents and other experimental animals, survival time is used to assess the ability of an extract to prolong life for a specified period of time after the administration of the drug. In antimalarial experiments, the mice is usually observed for 28 days to see whether it would survive or not and for how many days. The percentage of the participating subjects' that will attain the survival time of the group usually termed survival rate [56] or percentage survivor is also determined. The percentage of people in a study or treatment group still alive for a given period of time after diagnosis is a part of survival analysis. It is used to describe prognosis in certain disease conditions and also can be used as yardstick for the assessment of standards of therapy [56]. Adapted to *in vivo* experiments in mice, it may be used as a complementary parameter to assess the activities of extracts.

The standard drug attained the highest survival time (ST) of 28 days while all the single drugs elicited comparable ST with the negative control (Table 4). This indicates that none of these extracts could prolong the life of mice beyond the day of drug administration. However, when combined with CQ, the extracts of NU and PS did not reduce the ST of CQ as NU +CQ and PS+Q gave comparable ST with the standard drug. It implied that other extracts apart from NU and PS which gave significantly lower ST, will reduce the effect of chloroquine in treating malaria. On the other hand, PS which attained the least antiplasmodial activity of the four (Fig. 3), in combination with chloroquine improved its activity and also the survival time. This is an 'incentive' to the bad practice of adulterating plant remedies with standard drugs practiced by some unscrupulous practitioners which should be outrightly discouraged.

Also, the highest percentage survivor of 80 given by PS despite being the least antiplasmodial - active shows that other factors apart from parasitaemia reduction may modulate malarial disease. When combined with CQ, it attained a 100% survivor like CQ implying that the extract did not reduce the survivor of chloroquine like NB and PA which gave 60 and 80% respectively in combination with CQ. It further shows the involvement of extra plasmodial reduction factors in the amelioration of the malaria disease. NU, like PS, did not reduce the survivor of CQ just as none of the extracts reduced the antiplasmodial activities of CQ.

Table 4 Survival times and percentage survivors (in parenthesis) elicited by of combinations of standard drug with extracts of *Nephrolepis biserrata, N. undulata, Platycerium stemaria and P. angolense*

DRUG	Survival times	% survivor
NC	12.2±2.0[a]	80
NU	21.2± 4.7[a]	60
NU+CQ	28±0.0[b]	100
NB	18.4±4.5[a]	40
NB+CQ	19.4±5.3[a]	60
PA	16.2±5.4[a]	40
PA+CQ	27.2±0.8[a]	80
PS	23.8±4.2[a]	80
PS+CQ	28±0.0[b]	100
CQ	28±0.0[b]	100

Keys: Data show the mean ± SEM, *n* = 5. *Nephrolepis undulata,* NB- *N. biserrata,* PS- *Platycerium stemaria,* PA- *P. angolense,* PC-*positive control*-Chloroquine (10 mg/kg), NC-Negative control. Tween 80 in normal saline. Only values with different superscripts in the columns are significantly different ($p < 0.05$, One-way analysis of variance followed by the Student–Newman–Keul's post hoc test).

3.7. Survival times and percentage survivors of the plant-plant combination of the fern extracts

Just as combination of each of the extracts can have effect on the antiplasmodial activities, ST and survivor of CQ if combined together so also can each extract have effects on each other when combined. All the individual extracts gave comparable survival times to each other and to the negative control implying that none was able to prolong life beyond the day of drug administration.

The inactive PS significantly reduced the chemosuppresive activity of PA in PA+PS, but increased survival times and % survivor while PA on the other hand, could be said to have increased the activities of PS (Figs. 2 and 3). Its survival time was increased, however, without affecting the % survivor (Tables 4 and 5).

The combination, NU+PA (with PA+PS) gave significantly different ST of all the 2-plant combination and its value was comparable to that of CQ (Table 5). Also, with % survivors of 60 and 40% for NU and PA respectively, only the combination elicited a survivor of 100 like CQ. PA+PS elicited a survivor of 80%. The other 2- plant combinations gave ST that was comparable ($p>0.05$) to those of the individual drugs implying that their combinations did not improve ST (Tables 4 and 5).

Generally, PA combined with any of the individual extracts improves its survivor, PS combined with any stabilizes the survivor while NU improves all except with NB when in combination.

Table 5 *In vivo* survival times and percentage survivors of the various combinations of the extracts of *N. biserrata, N. undulata, P. stemaria and P. angolense*

DRUG	Survival times days	% Survivor
NB+PS	25.8±1.7 [a]	80
NU+PS	25.6±2.4 [a]	80
NU+PA	28±0.0 [b]	100
NB+PA	24.4±3.1 [a]	80
NU+NB	20.4±3.1 [a]	40
PA+PS	27.6±0.4 [b]	80
NU+ NB+ PS	25.6±2.4 [a]	80
NU +PA +PS	27.6±0.2 [a]	60
NU+ NB+ PA	13.8±3.5 [a]	40
NB+ PA+ PS	23.8 ±4.2 [a]	80
NU+ NB+ PS+PA	21.8±3.8 [a]	60
CQ	28±0.0 [b]	100

Keys: Data show the mean ± SEM, *n* = 5. *Nephrolepis undulata,* NB- *N. biserrata,* PS- *Platycerium stemaria,* PA- *P. angolense,* PC-*positive control*-Chloroquine (10 mg/kg), NC-Negative control. Tween 80 in normal saline. One-way analysis of variance followed by the Student–Newman–Keul's post hoc test).

Unlike the 2-plant combination, 3-plant combination did not strictly improve survivor, for example combination of NU+PA with 100% survivor to PS and NB reduced to 60 and 40 respectively while NB+PA (80) combined with NU reduced to 40 %, PA +PS (80) combined with NU gave 60. Generally instead of improving survivor like in the antiplasmodial testing, the 3- plant combination reduced survivor, although some others like NB+PS (80) combined with either PA or NU, NB +PA with PS, NU+PS with NB, PA+PS with NB did not affect survivor. It all boiled down to strict experimentation in order to predict the effect of combining medicinal plants in malarial therapy.

4. Conclusion

Ferns can also elicit antimalarial properties as higher plants. They should not be combined arbitrarily neither should they be administered with standard antimalarial drugs inadvertently.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The authors hereby declare that there is no conflict of interest with regard to the preparation of this article.

Statement of ethical approval

The protocol for the work was approved by the Board of Postgraduate College, OAU for student with the Registration Number PHP/18/19/H/0282**.** Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable.

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