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ANTIPLASMODIAL AND B-HEMATIN INHIBITORY POTENTIAL OF THE EXTRACT AND SOLVENT FRACTIONS OF *NEPHROLEPIS UNDULATA*

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Abstract

Background: The beta-hematin-inhibitory potential of the extract and the partitioned fractions of *Nephrolepis undulata* have not been determined, neither the likely constituents responsible for the observed *in vivo* antiplasmodial activities identified, hence this study.
Materials and Methods: The methanol extract of *N. undulata* (MNU), was evaporated *in vacuo* and subsequently partitioned to give HNU (n hexane), DNU (dichloromethane), ENU (ethyl acetate) and ANU (aqueous phase) which were investigated *in vivo* in mice at 0-800mg/kg against chloroquine-sensitive *Plasmodium berghei berghei* using Peter's four-day test. The Beta hematin inhibition assay was carried out on the extract and the fractions as well as Gas Chromatography Mass Spectroscopy for identification of the chemical constituents. Normal saline and chloroquine (10mg/kg) were negative and positive controls, respectively. All the results were subjected to statistical analysis using ANOVA with Student Newman Keul's as *post hoc* test.
Results: The partitioned fractions elicited comparatively lower percentages chemosuppression/inhibition and higher effective doses/inhibitory IC_{50} compared to NU in both experiments respectively suggesting that the constituents were acting synergistically. The GC-MS analysis revealed a total of eleven compounds in MNU partitioned into HNU: Neophytadiene, Hexadecanoic acid methyl ester, N--Hexadecanoic acid, 10 octa decenoic acid methyl ester and Methyl stearate, DNU: 2 ethyl acridine, ENU: Benzoic acid methyl ester and ANU: Benzoic acid methyl ester, 2 ethyl acridine while additional five compounds found in MNU were not detected in any of the fractions.
Conclusion: The constituents of the antimalarial MNU seem to be acting synergistically to inhibit haemozoin formation in the parasite.

Key words: Antimalarial, Beta hematin inhibition; *Nephrolepis undulata*, *Plasmodium berghei berghei,* partitioned fractions.

List of abbreviations used: NU: *Nephrolepis undulata*; **MNU:** methanol extract of *Nephrolepis undulata*; **HNU:** n hexane partition fraction of MNU; **DNU:** Dichloromethane partition fraction of MNU; **ENU**: ethyl acetate partition fraction of MNU; **ANU:** aqueous residue of MNU; **LLINs:** long-lasting insecticide treated nets; **UPRBC:** numbers of unparasitized red blood cells; **PRBC:** numbers of parasitized red blood cells; **PTD:** Average parasitaemia in the test dose; **PNC:** Average parasitaemia in the negative control; **PFTBA:** perfluorotributylamine; **GLC**: Gas Liquid Chromatographic; **GC-MS** : Gas Chromatographic Mass spectrometry; **NIST:** National Institute of Standards and Technology, **PUFA**: Poly unsaturated fatty acids.

Introduction

Malaria is a difficult, life-threatening disease caused by protozoa of the *Plasmodium* genus. The disease which poses a global problem is transmitted to humans through anopheles mosquito bites. The highly adaptable nature of the parasites, vectors involved and the constant movement of people from endemic areas to initially immune

countries (Mooney *et al.,* 2017; Shretta *et al.,* 2017) makes the transmission system to be complex and so a single strategy of control is inadequate. Asides, the physical and biological methods employed in eliminating malaria parasite vectors, increased investment in research and development, long term flexible commitment that includes community involvement, integration with health systems and the development of supporting infrastructure, long-lasting insecticide treated nets (LLINs), rapid diagnostic tests, and Artemisinin-based combination therapies, have resulted in highly effective malaria control over the past few years (Shretta *et al.,* 2017). However, because effective treatment is constantly being threatened by drug resistance, new antimalarial drugs have become a research priority. Although higher plants have furnished antimalarial molecules, search for more seem an unending and inexhaustible venture simply because some plants especially the lower plants are still underexplored. Whereas, the lower plants possess ethno medicinal antimalarial properties, they are scarcely investigated for antimalarial compounds. For example, only a few Pteridophytes such as *Niphidium crassifolium* (Polypodiaceae) and *Drynaria quercifolia* bulb among the non-flowering lower plants, which are traditionally used to treat fever and malaria in the Panama and West Timor region, Indonesia respectively have been discovered to have anti-malarial properties (Angela *et al.,* 2012; Maximus *et al.,* 2018). So also, only *Nephrolepis biserrata* among the single genus family Nephrolepidaceae has been discovered to be used ethno medicinally for the treatment of malaria (Koudouvo *et al.,* 2011). The fresh fronds of *Nephrolepis undulata (*Afzel. Ex Sw.*)* J. Sm. which is commonly called annual sword fern, helecho or ladder fern are used to make decoction for the treatment of fever (Oloyede *et al*., 2014), therefore it should be investigated for antiplasmodial activities and the antimalarial compounds should be isolated or identified.

Haemozoin is a disposal product formed from the digestion of blood by some hematophagous (blood-feeding) organisms such as malaria parasites (*Plasmodium spp.*), *Rhodnius*, and *Schistosoma* (Oliveira *et al.,* 2005). These digest haemoglobin and release high quantities of its non-protein component called free heme which is toxic to cells. The parasites convert free heme into an insoluble crystalline form called hemozoin often called malaria pigment in malaria parasites. The formation of hemozoin is essential to the survival of these parasites, and so it is a very attractive target and an immutable pathway in antimalarial mechanism. Several currently used antimalarial drugs, such as the classical quinolone antimalarials (like quinine, chloroquine, mefloquine, amodiaquine and mepacrine) and artemisinin are all thought to kill malaria parasites by inhibiting hemozoin crystal formation (Weissh and Leiserowitz 2008; Fong and Wright, 2013). Synthetic hemozoin also called β-hematin, is structurally identical to hemozoin and can be produced by chemical synthesis using hemin. This has been adapted in diverse assay formats to measure the hemozoin inhibition of plant extracts, fractions, drugs and new compounds (Thomas *et al*., 2012). The half maximal inhibitory concentration, IC_{50} represents the concentration at which a substance exerts half of its maximal inhibitory effect. It is a value used to characterize the effectiveness of an antagonist in inhibiting a specific biological or biochemical process (Akyul *et al.*, 2016). Heme concentration, incubation time, pH, among other factors that can cause variation in the IC_{50} values of compounds tested for their ability to inhibit hemozoin formation (Nhien *et al*., 2011) should be noted in assays designed to test the activities of extracts, fractions or isolates from plants. Comparative chemosuppressive antimalarial and beta inhibiting activities of the methanolic extracts of *Nephrolepis undulata* and its solvent fractions should confirm it as an antimalarial plant and suggest its mode of action on the parasite, hence this study.

Materials and Methods

Equipment: Grinding machine (Christy and Norris-47362 England), Weighing balance (Mettler Toledo AB204-S), Rotary evaporator (SE-100N, Shimadzu), dissecting set, binocular light microscope (Olympus CH2), macerating flask, Aluminum cages, oral cannula, Pocket digital thermometer (Ultramed), Retort stand, glass slides, feeding and water troughs, filter paper, separating funnel, glass funnel, measuring cylinder (10ml, 20ml, 50ml, 100ml), Sterile disposable syringes, Round bottom flask, sterile disposable syringes, colour pen markers, Paper tape, Test tubes, Spatula, Petri dishes. Cotton wool.

Reagents, chemicals and solvents: Methanol, normal saline, distilled water, heating mantle, heparinized tubes, Distilled water, Giemsa stain (2%), immersion oil, Tween 80, chloroquine phosphate powder.

Solvents: methanol, dichloromethane, butanol, n-hexane and ethyl acetate (GFS Chemicals Inc. Columbus), Animals: Swiss mice,

Collection, preparation and authentication of plant materials

The frond of *N. undulata* was collected at the Road 7, Obafemi Awolowo University within the Oil palm plantation, before the bridge connecting the junior staff quarters of the university. The plant was identified at the Natural History Museum, and authenticated at the Pharmacognosy Department, Faculty of Pharmacy Herbarium, Obafemi Awolowo University. Specimen with Voucher number FPI 2322 was deposited.

Extraction.

Briefly, the frond of *Nephrolepis undulata*, was collected, air dried for 7 days, and powdered using Christy and Norris grinding machine. A 200.0 g portion of the plant material was macerated in 2.0 L of methanol for 72 hours

with intermittent shaking. The resultant extract was filtered, evaporated *in vacuo*, freeze-dried and weighed. The yield was calculated.

Acute toxicity study

The methanolic extracts *of Nephrolepis undulata, was* subjected to acute toxicity testing using Lorke's method. Briefly, the 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 behavioral 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: $LD_{50} = \sqrt{D_0} \times \sqrt{D_{100}}$. (Lorke, 1983)

Rodent Parasite.

The rodent parasite, *Plasmodium berghei berghei* NK 65 passaged into Swiss 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 to about 30% before being used for the experiment.

Preparation of the mice

Seven-week old Swiss 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 randomized into groups of five mice each for the experiment.

Inoculation of the test animals

The parasitized donor mouse with parasitaemia level up to a level of about 30 % was euthanized with dichloromethane. 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 x 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.

Preparation of the test extracts and standard drug

The doses of the extract of *Nephrolepis undulata,* used 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 or fractions in 5.0 ml of normal saline respectively. The doses of normal saline (0.2ml) was administered to the negative control group while chloroquine (10 mg/kg) was administered to the positive control group.

***In vivo* antiplasmodial activity of the extract**

The *in vivo* antiplasmodial activities of the extract, *Nephrolepis undulata,* was determined using the four–day chemosuppressive test in a chloroquine-sensitive *Plasmodium berghei berghei* mouse *in vivo* model alongside *Nephrolepis biserrata, Platycerium angolense Platycerium stemaria* where it was found to be the most active of the four ferns (Odediran *et al.,* 2022)

Partitioning of *N. undulata* extract

The most active fern (*N. undulata*) (2 kg) was extracted with methanol, to obtain 100g methanol extract after being evaporated and dried. A 85.85 g of the dried extract was placed in a beaker and mixed with 100.0 mL of distilled water and poured in a one-litre (1.0 L) separating funnel and then partitioned successively into: *n*-hexane, dichloromethane and ethyl acetate (Fig. 3). The partitioned fractions, HNU, DNU, ENU, including the residual aqueous phase, ANU, were concentrated to dryness *in vacuo* and weighed. The yields were calculated.

***In vivo* antiplasmodial activity of the partitioned fractions**

The *in vivo* antiplasmodial activities of the partitioned fractions HNU, DNU, ENU and ANU were each determined using the four–day chemosuppressive test in a chloroquine-sensitive *Plasmodium berghei berghei* mouse *in vivo* model. Briefly, for the partitioned fraction HNU, (thirty) 30 acclimatized mice were randomized into six groups (**I-VI**) of five animals each (n=5) and inoculated with 0.2 ml of the diluted parasitised blood as in 2.8 above. Two hours after inoculation, Groups II-V of the mice were administered with 100, 200, 400 and 800mg/kg of each of the freeze-dried methanol extracts of *Nephrolepis undulata* 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 by withdrawing blood from the tail of the mouse, using it to make a thin smear, fixing with methanol and staining with Giemsa (3%). The partitioned fractions: DNU, ENU and ANU were similarly tested separately.

Preparation and staining of blood films

The tail of the experimental animals was cut lightly and a small drop of blood was collected from it on a clean slide. A thin smear was made using another slide placed on the blood spot at an angle of 45^0. The thin film of blood was allowed to dry and was fixed using few drops of methanol, and placed in slanting position in order to drain and dry up. The smear was stained with Giemsa stain (3 %) after diluting with distilled water in ratio 1:9 and allowed stand for 30 minutes after which it was drained off and the slide rinsed in water and allowed to air dry (Peters *et al*., 1995).

Estimation of average percentage parasitaemia and percentage chemosuppression

Each of the stained blood film prepared was mounted on the microscope stage, ten fields of view with uniform distribution of red blood cells were viewed using oil immersion (x100) objective. For each of the field selected, the numbers of parasitized (PRBC) as well as unparasitized (UPRBC) red blood cells were counted. 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 (Peters, 1965). From the Average Percentage Parasitaemia, the percentage (%) chemosuppression for each dose extract was 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 (Peters *et al*., 1995).

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.

Beta hematin assay of *N. undulata* and its solvent fractions

The extracts and the partitioned fractions of *N. undulata* (NU, HNU, ANU, ENU and ANU) were subjected to β-hematin assay. This assay is based on the fact that antimalarial drugs act by the inhibition of the formation of haemozoin which is a non-toxic crystal formed by the *Plasmodium* species in response to the toxic ferriprotoporphyrin (IX) haeme within the acidic digestive vacuole. Hence the modified method of Basilco *et al.* (2000) as described by Baelmans *et al.* (1998) was used to determine the ability of NU, HNU, ANU, ENU and ANU to inhibit the β-hematin formation *in vitro* using chloroquine diphosphate as standard drug. Briefly, solutions containing varying concentrations of both the standard drug and NU, HNU, ANU, ENU and ANU were separately prepared. Chloroquine standard was dissolved in distilled water (1.0, 0.5, 0.25, 0.125, 0.0625 mg/mL) and extract and fractions were dissolved in absolute methanol (10, 5, 2.5, 1.25, 0.625 mg/ mL). With distilled water as negative control, each concentration was dispensed in different sterilized Eppendof tubes where 50 µL of 2.5 mg/mL haemin chloride has been added and 250 µL of 0.2M acetate buffer pH 4.4 included to initiate the synthesis of β-hematin. The mixture was incubated at 37 ^oC for 48 h. The tubes were subsequently centrifuged at 4,000 rpm for 15 min to obtain haemozoin pellet while the supernatant was discarded. The pellet obtained was washed twice with 200 µL DMSO. Finally, the washed pellets were dissolved by adding 200 µL of 0.2N NaOH and further diluted with 400 µL 0.1N NaOH. The absorbance was read at 405 nm in a visible spectrophotometer and the read out values of absorption for each was then used to calculate the percentage inhibition of β-hematin synthesis elicited by each of the isolate thus; $I\ (\%) = (A_o - A_i) / A_o * 100$, where, I (%) = Percentage Inhibition, A_o = Absorbance of the control (reaction mixture without extract or standard), A_i = Absorbance of the extract or standard. The IC_{50} was forecasted as a measure of the antimalarial activities of the extract, fractions and standard drug.

Gas Chromatographic–Mass Spectrometric (GC-MS) Analysis

Determination of the levels of phytochemicals in the sample was carried out using GC-MS by operating MSD in Scan mode to ensure all levels of detection of the target constituents. Gas Liquid Chromatographic (GLC) separation was performed on a Gas chromatography (Agilent7820A, USA) hyphenated to an inert mass spectrophotometer (5957C) with electron-impact source and triple axis detector equipped with an auto injector (10 µL syringe) with Helium gas as carrier. Prior to analysis, the MS was auto-tuned to perfluorotributylamine (PFTBA) using already established criteria to check the abundance of *m/z* 69, 219, 502 and other instrument optimal & sensitivity conditions.

All chromatographic separations were performed on a capillary column, specification HP-5 – dimensions: 30 m x 320 µm x 0.25 µm, coated with 5% phenyl methyl siloxan and operated at a constant flow rate of 1.4871 /min of helium gas with other conditions as follows: EI (ion source temperature), 300°C, interface temperature 300°C, initial nominal pressure 1.4902 psi, out time 1.8 mins; 1µL of the samples were injected in splitless mode at an injection temperature of 300 °C. Purge flow to split vent was 15 mL/min at 0.75 min with a total flow of 16.654 mL/min. The oven temperature was initially held for 1.0 min at an initial temperature of 40°C and programmed to increase to 300°C at 12°C/min, held for 10 min and later increased to 250°C at 20°C/min and finally held isothermally for 5 minutes, giving a total run time of 32.667 mins. The mass spectrometer was operated in electron-impact ionization mode at 70eV with ion source temperature of 230 °C, quadrupole temperature of 150 °C and transfer line temperature of 280 °C. Acquisition of ion was via Scan mode (scanning from *m/z* 45 to 550 amu at 2.0s/scan rate). The separated constituents were passed to the detector which recorded the emergence of the constituents as peaks with a retention time. The percentage compositions of the compound in the entire sample were computed from the peak areas automatically generated by the machine. The results were recorded as retention time against percentage composition in the original sample.

Gas Chromatographic–Mass Spectrometric (GC-MS) Analysis of MNU, HNU, DNU ENU and ANU

GC-MS analysis was performed as stated above. Samples were prepared and injected into the GC-MS machine and the result acquired as peaks with respective retention times. Data handling was done using GC-MS solution software. The identities of the components were assigned by comparing their retention times with those of the standard spectra from NIST.

Results

Table 1: Percentage parasitaemia and Percentage chemosuppression (in parenthesis) elicited at different doses (mg/kg) by the partitioned fractions of the methanol extract of the *N. undulata* in an *in vivo* antimalarial activity test

DOSE (mg/kg)	HNU	DNU	ENU	ANU
0	4.15 ± 0.15[a] (0.00 ±0.00[a])	4.15 ± 0.15[a] (0.00 ±0.00[a])	4.15 ± 0.15[a] (0.00 ±0.00[a])	4.15 ± 0.15[a] (0.00 ±0.00[a])
100	3.53 ± 0.26 [b] (14.75 ± 5.35[b])	3.63 ± 0.70 [b] (12.00±2.78 [b])	3.87 ± 8.99 [b] (6.76 ± 8.99 [b])	2.94 ± 0.50 [b] (28.45 ±13.34 [b])
200	3.43 ± 0.55 [b] (17.50 ±12.84 [b])	3.28 ± 0.29 [b] (20.51 ±7.45 [b])	3.35 ± 0.65 [b] (18.68 ±16.82 [b])	3.17± 0.44 [b] (39.61 ±10.50 [b])
400	3.01 ±8.01 [b] (26.85±8.01 [b])	3.24± 0.58 [b] (20.07 ± 15.26 [b])	3.32 ± 0.42 [b] (13.34 ± 11.33 [b])	2.87 ± 0.39 [b] (30.92 ± 9.08 [b])
800	2.95±13.64 [b] (28.15±13.65 [b])	2.21 ± 0.12 [c] (46.53 ± 4.15 [c])	2.27 ± 0.11 [c] (44.98 ± 3.04 [c])	2.54± 0.09[b] (38.37±3.90[b])
CQ	1.59 ± 0.36 [c] (61.51 ± 11.24 [c])	1.59 ± 0.36 [c] (61.51 ± 11.24 [c])	1.59 ± 0.36 [c] (61.51 ± 11.24 [c])	1.59 ± 0.36 [c] (61.51 ± 11.24 [c])

Keys: HNU: N-hexane fraction; DNU: Dichloromethane fraction; ENU: Ethyl acetate fraction; ANU: Aqueous fraction. Data show the mean ± SEM, n = 5, NC (negative control; 0mg/kg): Tween 80 in normal saline; CQ = Chloroquine (10 mg/kg). Only values with different superscripts within columns are significantly different ($p < 0.05$, one-way analysis of variance followed by the Student–Newman–Keuls' post hoc test).

Table 2: Median Effective Doses (ED_{50} and ED_{90}) values of the crude extract and partitioned fractions of *N. undulata* in the *in vivo* 4-day chemosuppressive test model.

DOSES	ED_{50}	ED_{90}
HNU	809.06 ± 253.07[a]	1544.52 ± 646.93 [a]
DNU	692.17 ± 74.44 [a]	1111.68 ± 174.51 [a]
ENU	675.26 ± 98.94 [a]	862.87 ± 398.89 [a]
ANU	625.23 ± 135.12 [a]	1145.97 ± 135.12 [a]
NU	294.27 ± 17.72	

Keys: NU: methanol extract of *N. undulata*; HNU: N- hexane fraction; DNU: Dichloromethane partition; ENU: Ethyl acetate fraction; ANU: Aqueous residue. Data show the mean ± SEM, n = 5+: Significant difference ($p < 0.05$, one-way analysis of variance followed by the Student Newman Keul's post hoc test); from positive control (chloroquine10mg/kg) *: significant difference from negative control.

Table 3: Average survival times and percentage survivor (in parenthesis) of mice in *in vivo* chemosuppressive antimalarial activities test of the partitioned fractions of the methanol extract of *N. undulata* in mice

DOSE	HNU	DNU	ENU	ANU
0	18.6 ±1.9 [b] **(60)**	18.6 ±1.9 [b] **(60**	18.6 ±1.9 [b] **(60**	18.6 ±1.9 [b] **(60**
100	9.8 ±3.3 [a] **(40)**	12.2 ±1.0 [a] **(60)**	19.8± 5.0 [b] **(60)**	16.2±1.4 [a] **(60)**
200	17.8±0.8 [b] **(60)**	16.0 ±4.2 [a] **(40)**	24.0± 1.9 [b] **(40)**	16.2± 3.0 [a] **(40)**
400	16 ±0.6 [b] **(40)**	22.4±2.9 [b] **(80)**	12.4 ± 2.6 [a] **(60)**	12.8 ± 3.2 [a] **(80)**
800	12.7± 1.7 [a] **(20)**	18.8±2.8 [b] **(20)**	18.6 ± 3.9 [b] **(40)**	17.8±2.6 [a] **(20)**
CQ	25.2 ± 2.8 [c] **(80)**	28.0±0.0 [b] **(80)**	28.0±0.0 [b] **(80)**	28.0±0.0 [b] **(80)**

Keys: NU: methanol extract of *N. undulata*; HNU: N- hexane fraction; DNU: Dichloromethane fraction; ENU: Ethyl acetate fraction; ANU: Aqueous residue. Data show the mean ± SEM, n = 5+: Significant difference ($p < 0.05$, one-way analysis of variance followed by the Student Newman Keul's post hoc test); from positive control (chloroquine10mg/kg) *: significant difference from negative control.

Table 4: Absorbance and Percentage inhibition (in parenthesis) for the methanol crude extract and fractions of *Nephrolepis undulata* in a β-hematin experiment

Conc. (mg/mL)	NU	HNU	DNU	ENU	ANU
0.0	0.792±0.00[d] (0.00±0.00)	3.239±0.00[f] (0.00±0.00)	2.46±0.00[c] (0.00±0.00)	2.614±0.00[e] (0.00±0.00)	2.505±0.00[f] (0.00±0.00)
0.625	0.626±0.037[c] (20.95±4.67)	2.7935±0.0235[e] (24.68±1.24)	1.8355±0.0075[b] (25.38±0.63)	2.045±0.017[d] (14.01±1.32)	2.088±0.02617[e] (14.93±0.43)
1.25	0.4575±0.0115[b] (42.23±1.45)	2.7115±0.0025[d] (16.28±0.07)	1.809±0.004[b] (26.40±0.16)	1.8315±0.0075[c] (33.26±0.36)	1.8715±0.02905[d] (23.69±1.69)
2.5	0.3465±0.0085[a] (56.25±1.07)	2.551±0.014[c] (21.24±0.43)	1.76±0.021[b] (28.45±0.85)	1.7445±0.0095[c] (37.18±0.22)	1.74425±0.00407[c] (30.37±0.11)
5.0	0.277±0.0080[a] (65.02±1.01)	2.4415±0.0045[b] (24.62±0.13)	1.661±0.022[a] (32.47±0.89)	1.642±0.006[b] (41.39±1.98)	1.6595±0.01149[b] (33.05±0.47)
10.0	0.2615±0.0085[a] (66.98±1.07)	2.3355±0.0345[a] (27.89±1.06)	1.6165±0.0075[a] (34.28±0.30)	1.532±0.052[a] (14.01±1.32)	1.5525±0.02479[a] (37.20±0.47)
IC_{50} (mg/ml)	**5.53±0.40**	**24.68±1.24**	**25.45±0.63**	**14.01±1.32**	**15.31±0.07**

Keys: Data show the mean±SEM, n = 3. NU = *N. undulata* crude methanol extract, HNU = N-hexane fraction, DNU = Dichloromethane fraction, ENU= Ethyl acetate fraction and ANU = Aqueous phase. Only values with different superscripts (a, b, c or d) within columns are significantly different ($p < 0.05$, one-way analysis of variance followed by the Student-Newman-Keul's post hoc test).

Table 5: Percentage inhibition elicited by Chloroquine in a beta hematin assay

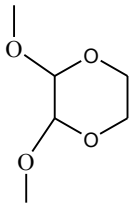
Concentration (mg/mL)	Absorbance	% Inhibition
0.0	0.775±0.000[d]	0.00±0.00[a]
0.0625	0.4135±0.009[c]	46.6452±1.097[b]
0.125	0.336±0.005[b]	56.6452±0.6452[c]
0.25	0.2365±0.018[a]	69.4839±2.258[d]
0.5	0.21±0.002[a]	72.9032±0.2581[d]
1	0.203±0.001[a]	73.8065±0.129[d]

Keys: Data show the mean±SEM, n = 3. Only values with different superscripts (a, b, c or d) within columns are significantly different ($p < 0.05$, one-way analysis of variance followed by the Student-Newman-Keul's post hoc test).

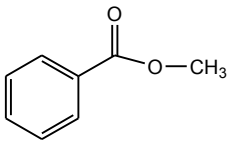
Table 5: Percentage inhibition elicited by Chloroquine in a beta hematin assay

Concentration (mg/mL)	Absorbance	% Inhibition
0.0	0.775 ± 0.000^{d}	0.00 ± 0.00^{a}
0.0625	0.4135 ± 0.009^{c}	46.6452 ± 1.097^{b}
0.125	0.336 ± 0.005^{b}	56.6452 ± 0.645^{c}
0.25	0.2365 ± 0.018^{a}	69.4839 ± 2.258^{d}
0.5	0.21 ± 0.002^{a}	72.9032 ± 0.258^{d}
1	0.203 ± 0.001^{a}	73.8065 ± 0.129^{d}

Keys: Data show the mean±SEM, n = 3, Only values with different superscripts (a, b, c or d) within columns are significantly different ($p < 0.05$, one-way analysis of variance followed by the Student-Newman-Keul's post hoc test).



1,4 dioxane, 2,3 dimethoxy



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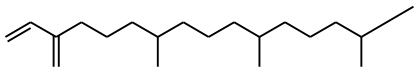

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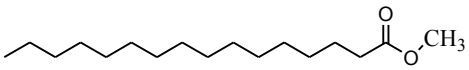
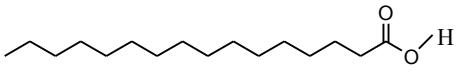
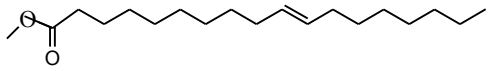


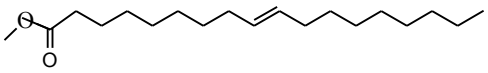
Hexadecanoic acid methyl ester



N-Hexadecanoic acid



10 octa decenoic acid methyl ester



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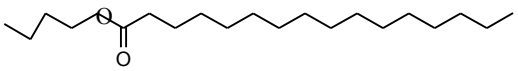

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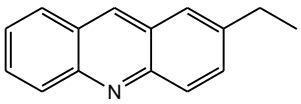
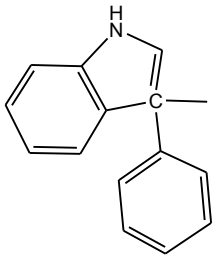
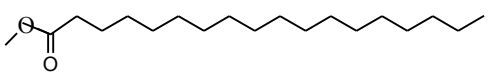


2 ethyl acridine



1H-- indole, 3 methyl -3phenyl



Methyl stearate

STRUCTURES OF SOME COMPOUNDS IDENTIFIED FROM *NEPHROLEPIS UNDULATA*

Discussion

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 (Nasim *et al,* 2022; Chaachouay, and Zidane, 2024). Of late,

research into plants for antimalarial compounds have been geared towards higher plants (Ntie-Kang *et al.,* 2014; Habib *et al.,* 2022) with little investigation into lower or non-flowering plants such as bryophytes and pteridophytes for same. Such lower plants with their diversities seem to have been underexploited in the search for new antimalarial drugs. Since some of them have shown ethno medicinal relevance in the treatment of malaria (Bamigboye *et al.,* 2019), it becomes imperative to venture into them too, hence, a recourse to a lower plant, a pteridophyte in this work.

N. undulata belongs to the order Filicales, family Nephrolepidaceae. Of the members of this genus, only *N. biserrata* have been used ethnomedicinally in the management of malaria, while *N. undulata* have been employed in the management of fever (Bamigboye *et al.,* 2019). A recent work on four Nigerian ethnomedicinal ferns: *Nephrolepis biserrata* (NB), *N. undulata* (NU), *Platycerium stemaria* (PS), and *P. angolense* (PA) and their combination with chloroquine confirms the antimalarial activities of NB and NU (Odediran *et al.,* 2022). Therefore, further testing of the solvent fractions of NU for antimalarial potency and exploring same and the methanol extract for β-hematin inhibitory activities and possible antimalarial constituents is worthwhile.

Antiplasmodial activities of *N. undulata*

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 (Bello *et al*., 2009). Also, the ability of an extract to reduce, suppress the multiplication of, or clear malarial parasites in mice is an indication that it possessed antimalarial activity (Taylor, 1965). The 4-day chemosuppressive test in mice is a classical method for the preliminary *in vivo* screening of drugs with potential antimalarial activity (Peters *et al*., 1995). The comparable ($p>0.05$) percentage chemosuppression values of the tested doses (100, 200, 400 and 800mg/kg) of the crude methanol extract of *N. undulata to* that of *the* positive control (chloroquine 10mg/kg) effective doses, ED_{50} value of 294.27±17.72 and ED_{90} of 569.46±39.5, in a previous experiment showed that it is an active antimalarial drug (Deharo, 2001; Odediran *et al.,* 2022). Also, the chemosuppressive ED_{50} of 279.3 ± 2.9 and 287.1 ± 0.7 obtained in the same laboratory for *Nauclea latifolia* root, *Murraya koenigi* leaf extracts respectively both suggested NU to be active alongside these two extracts (Adebajo *et al.,* 2014). However, the comparable ($p>0.05$) survival times of NU at all doses to that of the negative control and the percentage survivor of 75% at the highest dose of 800mg/kg tested compared to 100% elicited by the positive control drug showed that the NU extract cannot prolong the life of mice beyond the day of drug administration to the mice.

Antiplasmodial activities of the partition fractions

Successive partitioning of the crude extract into solvents of varying polarities is a common method of preliminary purification towards identifying the active constituents of medicinal plants (UNIDO-ICS, 2008). Purifications are usually made activity-guided in order to hasten the process of the identification of the active constituents of plants extracts. The partitioned fractions of NU, the active extract, which are HNU (n hexane), DNU (dichloromethane), ENU (ethyl acetate) and ANU (aqueous phase) were subjected to chemosuppressive antiplasmodial tests at doses 0-800mg/kg. From the experiment, the lowest % parasitaemia and consequently, highest percentage chemosuppression values elicited by DNU and ENU at the highest doses tested (Table 1) and which were comparable to that of chloroquine showed that the two fractions retained the antimalarial activities of NU at the highest dose and so may contain antimalarial compounds while significantly lower values elicited by HNU and ANU at 800mg/kg (Table 1) compared to that of chloroquine indicated their lower contents of constituents with antimalarial actions. Also, the higher chemosuppression elicited by DNU and ENU at 800 mg/kg than HNU and ANU (Tables 1) showed that both DNU and ENU are more likely to contain constituents with greater activities against the malarial parasites. However, the chemosuppression of a little less than 50%, elicited by the ethyl acetate (**ENU**) and dichloromethane (**DNU**) fractions in this experiment can only depict that they were only moderately active (Tarkang *et al*., 2014). The GC MS experiment showed that 2 ethyl acridine was partitioned into DNU while benzoic acid methyl ester was found in ENU. However, ANU which contained both 2 ethyl acridine and benzoic acid methyl ester did not show commensurate synergism of both at the highest dose of 800mg/kg. Also, HNU which contained five constituents gave lower chemosuppressive activities.

Activities of NU and the partitioned fractions

That **NU** extract elicited a higher percentage chemosuppression (58, 63, 66, and 71%) against (12, 21, 20, and 46 %) at 100, 200, 400 and 800 mg/kg respectively for DNU, the seemingly most active partition fraction, and so with the other partition fractions can only suggest that the compounds present were acting synergistically. The overall activities of these fractions were confirmed from their median doses which depicted that they all showed comparable activities to each other. It might also mean that the compounds responsible for the activities in these fractions occur in low and undetectable levels and the dose might not have exerted significant effect on the parasites (Nureye *et al*., 2021). The levels of parasitaemia suppression elicited by the partition fractions (the highest was 45% ENU at 800mg/kg), the lowest 7% (ENU at 100mg/kg) showed weak effects as chemosuppressive agents.

Comparing the percentage chemosuppression of the extract and the partitioned fractions, **NU** elicited higher chemosuppression than any of the partitioned fractions at similar doses. Whereas at the lowest dose, **NU** elicited a percentage chemosuppression of 58, none of the fractions attained up to 30. The same trend could be observed for all the doses tested

including the highest when **NU** gave 71% and **DNU** gave the highest of 47% at similar doses (Table 3). It can be deduced that partitioning did not enhance the activities of the extract and also that the partitioned fractions are poor antimalarial agents compared to the crude extract. Also, it may imply that the compounds present in the partitioned fractions occurred at very low concentrations compared to the extract.

Survival times and Percentage survivor of NU and the partitioned fractions

The extract of NU exhibited a survival time that was comparable ($p>0.05$) to that of the negative control (Odediran *et al.,* 2022), and fractions HNU and ANU elicited survival time values that were lower than that of the negative control and significantly different ($p<0.05$) from that of the positive control at all doses (Table 4) while those of DNU and ENU gave higher values than negative control only at 400 and 200mg/kg respectively and were still statistically comparable ($p>0.05$) to it. This implies that none of the partition fractions gave survival times that were significantly different ($p<0.05$) from that of negative control just like NU. NU gave a PS of 75% at highest dose of 800mg/kg and a similar value of 60% at the other tested doses. However, only DNU and ANU, at 400 mg/kg, of all the partitioned fractions elicited percentage survivor of 80 that were highest and similar to that of the positive control drug. It seems that percentage survivor values of 80 elicited by DNU and ANU identified both as relatively more active especially with DNU eliciting the highest chemosuppression of 47% and higher survival time of all the partition fractions (Table 4). Though Eyasu (2013) has suggested an agreement between percentage chemosuppression and survival time, Adepiti and Iwalewa (2016) opined that chemosuppression readily agrees with percentage survivor rather than survival time as obtained in this experiment. Fraction DNU with relatively high percentage chemosuppression and survival time and similar percentage survivor with the positive control should be the most active partition fraction which may likely contain the most active antimalarial constituents of the plant. It could therefore be explored for the antimalarial constituents of the NU. However, the effective doses depicted comparable ($p<0.05$) activities among the fractions which were lower than that of the mother extract (Table 2).

B-hematin inhibition activities of the partition fractions

B-hematin inhibition assay is a simple, colorimetric assay that is based on the inhibition of hemozoin formation in the food vacuole of malarial parasites as a result of haemoglobin digestion. It is worthwhile to routinely screen plant extracts with the ultimate goal of identifying novel antimalarial agents (Basilco *et al.,* 1998). By targeting this pathway in the parasite a validated target for the discovery of newer antimalarials is assumed to be in place (Baelmans *et al.,* 2000). In the assay, the IC_{50}, (usually forecasted from absorbance and inhibition %) elicited by the plant extract, fractions or isolate is used to identify the potential of an extract or drug to enable this target. Ten (10) antimalarial ethnomedicinal Combretaceae plants were screened using this assay to identify eight plant extracts which demonstrated appreciable activities by inhibiting the formation of hemozoin at 25mg/ml. The best IC_{50} of 2.58 ± 0.447 mg/m was observed in the methanol extract of *Terminalia ivorensis* which was comparable with chloroquine standard drug with IC_{50} 0.55 ± 0.179 mg/mL (Wande and Babatunde, 2017) The absorbance, percentage inhibition and effective inhibitory concentration ($IC_{50,}$ IC_{90}) of **NU, HNU, DNU, ENU** and **ANU** in a β-hematin inhibition assay is as shown in Table 4 while that of chloroquine is shown in Table 5. In the B-hematin inhibiting activities experiment, the concentration dependent increase in % inhibition up to the concentration of 0.25 mg/mL by chloroquine is noteworthy; further increase in the concentration did not increase the % inhibition significantly. Similarly, the methanol crude extract of NU showed a concentration dependent increase in % inhibition up to the concentration of 2.5 mg/mL. The n-hexane, dichloromethane and aqueous fraction of NU gave a comparable % inhibition across all the concentration and did not show any concentration dependent increase in % inhibition. This is an indication that increase in concentration of the fractions did not improve their antimalarial activities. The result also showed that **NU, HNU, ANU, ENU** and **ANU** gave IC_{50} values as follows: 5.53±0.40, 24.68±1.24, 25.45±0.63, 14.01±1.32, 15.31±0.07 respectively while **CQ** has an IC_{50} of 0.05±0.04 mg/ml (Tables 4 and 5). The ability of each of the fractions to inhibit B-hematin vary considerably from that of chloroquine as indicated by the IC_{50}. Comparing the results from this study, it showed that the crude extract of *Nephrolepis undulata* (NU) elicited the strongest inhibitory effect on the formation of hemozoin, while the fractions had low effects. The rank order of crude extract and the fractions based on their inhibitory effect or IC_{50} values are: NU > ENU = ANU > HNU = DNU. The IC_{50} of chloroquine was found to be 0.05±0.04 mg/mL. A lower IC_{50} value indicates a higher potency, meaning that a lower concentration of the test agent is required to achieve a significant inhibitory effect (Berrouet *et al.,* 2020). Based on this, the crude extract of *Nephrolepis undulata* was the most active out of all the agents tested for activity (Table 4). It may also indicate that by the relative values of IC_{50}, NU effects its antimalarial response by inhibiting hemozoin while the fractions could not. This can be due to the synergistic effect NU possesses due to the presence of various compounds as compared to the other fractions which have been purified and contain fewer compounds (Borah *et al*., 2020; Bonincontro *et al*., 2023). The present study also confirmed the synergistic relationships between the NU and its solvent fractions that was obtained from the results of the *in vivo* chemosuppressive effects above. The methanol crude extract of the plant gave the highest chemosuppressive activity of 58 % while none of the partitioned fractions gave an activity that is up to 30 %. Identification of the constituents of the extract NU and its partitioned fractions will be worthwhile in order to confirm the above synergistic relationship and or otherwise enable a conclusion on which of the fractions contain the major constituents of the plant and indirectly give an idea of the possible antimalarial constituents which may further be confirmed by isolation and characterisation. It is for this purpose that the fractions and the methanol extract were all subjected to GC-MS.

GC-MS of methanol extract and partition fractions of *Nephrolepis undulata*

GC in hyphenation with MS has proved to be a valuable tool in natural product research where it can separate and identify chemical compounds in a complex mixture especially volatile oil or organic extracts. It has especially been utilized in separating structurally similar mono and sesquiterpene that are the major constituents of some plants essential oils (Siddiqui *et al*., 2024). In these cases, isolation may not be mandatory if such constituents can be rightly identified and characterised unless it is a new compound. The MS fragmentation pattern is only compared automatically with stored EI spectra data in the computer library; this will suffice in identifying the separated components. For NU and the solvent fractions, the relevant peaks, retention time, the peak area and the quality, which depicted the relative distribution of the compounds in the extract and the fractions were identified as shown in Table 6, where MNU revealed a total of 11 compounds out of which HNU also showed five (5): Neophytadiene, Hexadecanoic acid methyl ester, N--Hexadecanoic acid, 10 octa decenoic acid methyl ester and Methyl stearate; DNU, one: 2 ethyl acridine; ENU, also one: Benzoic acid methyl ester while ANU revealed two: Benzoic acid methyl ester and 2 ethyl acridine. From these, DNU had: 2 ethyl acridine in common with ANU while ENU had Benzoic acid methyl ester in common with ANU. Each of the compounds present in DNU and ENU were both present in ANU. It seems that these various compounds present in the extracts were preferentially partitioned into each of the solvent fractions based on polarity.

It would be recalled that in the *in vivo* experiment as shown above, the order of activity is: NU>DNU= ENU>HNU>ANU while in the *in vitro* it is NU > ENU = ANU > HNU = DNU (Tables 1, 2 and 4) depicting in both cases that NU is more active than any of the partition fractions thus confirming synergism of the constituents in giving activities in the extract. It may also show that the compounds identified in the fractions did not present activities higher than that of the extracts confirming that those compounds were acting synergistically to give a higher activity in the extract. Also, some compounds were retained in the extract that were not detected in the partition fractions. Those components are likely to be the ones which, in combination with those detected in the partition fractions, that were responsible for the higher activities of the extract in both experiments. Neophytadiene, which is one of the constituents identified in MNU and HNU was detected along with others such as palmitic acid, myristic acid, fucosterol, phthalic acid, di (2-methylbutyl) ester, loliolide, has been identified as one of the main constituents in the active fractions of Antarctic Alga *Desmarestia antarctica* in a GC-MS analysis against parasitic infections including multi-drug-resistant *Plasmodium falciparum* (dos Santos, *et al.,* 2020). Neophytadiene is also one of the terpenoids that correlated with anti *P. falciparum* NF54 in a correlation experiment between abundance of plant metabolites and anti-*Plasmodium falciparum* activity in response to different light conditions (Sankhuan *et al.,* 2022). The antipyretic, analgesic, anticonvulsant, anxiolytic, sedative, with the probable participation of the GABAergic system, antimicrobial, anti-inflammatory, antifungal, anti-oxidant activities of the compound has been reported in addition to its antiplasmodial properties earlier mentioned (Gonzalez-Rivera *et al.,* 2023). Hexadecanoic acid methyl ester, also identified in MNU and HNU was also found along with 2-hydroxy-1, 3-propanediyl ester; octadecanoic acid; 9, 12-octadecadienoicacid (Z, Z) - methyl ester; 7, 10-octadecadienoic acid methyl ester; and squalene was isolated from the leaves *Pistia stratiotes L*. It is also a potent hepatoprotective compound and also found to have the following properties: antioxidant; nematicide; pesticide; antibacterial; antifungal; antiarthritic; antitumor; anticancer; anticoronary; anti-inflammatory; hypocholesterolemic; (Gupta *et al.,* 2023). It has also been identified as one of the antimalarial constituents of *Trichilia heudelotti* leaf (Sulaiman *et al.,* 2023). Hexadecanoic acid methyl ester along with N--Hexadecanoic acid was found as one of the constituents of methanol extract of *D. arborescens* roots that is most likely responsible for its antiplasmodial properties (Enenebeaku *et al.,* 2021). These two compounds were identified in MNU and HNU. Acridine is a popular antimalarials moiety, therefore, the identification of 2 ethyl acridine in MNU, DNU and ANU shows promises as an antimalarial. Recent structure-activity relationship studies have revealed new promising antimalarials based on acridine and acridinone core. For example, Haloalcoxyacridinones which are acridines, gave extremely potent antiplasmodial activity *in vitro* (Winter *et al.,* 2006), novel acridinedione with potent inhibitory activity of mitochondrial bc1 Complex (Biagini *et al.,* 2008), and 10-N-substituted acridinones showing strong chemosensitizing properties and heme polymerisation inhibition (Kelly *et al.,* 2009) are the most significant structures. Polyunsaturated fatty acids such as hexadecanoic acid, methyl ester, 9,12-octadecadienoic acid methyl ester (linoleic acid), 9,12,15-octadecatrienoic acid, methyl ester (linoleic acid), 9-octadecenoic acid (Z)-2-hydroxyethyl ester and eicosanoic acid, 2-(acetyloxy)-1-[(acetyloxy) methyl]ethyl ester have been found in the active antiplasmodial fraction. These PUFAs above have been implicated in antiplasmodial activity and this activity has been reported to increase with the degree of unsaturation (Okokon *et al.,* 2017). The above evidences of antiplasmodial activities of the various compounds identified in MNU and its solvent fractions seem to reinforce their possible antiplasmodial activities and so, are the antiplasmodial constituents of the plant.

Summary and Conclusion: The methanolic extract of NU elicited significantly higher antiplasmodial and beta inhibitory activities than the solvent fractions. Its constituents which were partitioned into the solvent fractions seem to be acting synergistically in the extract to inhibit haemozoin formation in the parasite.

Acknowledgments section

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Authors' contribution

The research was conceived by SAO and RAB. SAO supervised ORA and PAA for Postgraduate and undergraduate thesis respectively. DAA worked and reviewed the literature at every stage. SAO wrote the preliminary draft with RAB. All authors read and approved the final draft.

Declaration of conflicting interest:

'The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article'.

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Ethical approval and informed consent statements

The protocol for this work was approved. All animals were cared for in strict accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996), and the experimental design was approved by the by the Board of Postgraduate College, OAU for student with Registration Number PHP/18/19 /H/0282 on April 22, 2021.

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