Prophylactic, Suppressive and Curative Potentials of a Polyherbal Antimalaria Mixture in Plasmodium Berghei Infected Mice

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Abstract:
Objective
There is an increasing interest in the development of combination therapies used in the treatment of malaria and other ailments in folk medicine. This study evaluates the antimalarial potential and constituents of a Polyherbal extract used in the western part of Nigeria in treating malaria.

Materials and methods
Polyherbal extract (PE) was prepared from a decoction of the dried leaves of Morinda lucida (ML) and Azadiracta indica (AI) and stem barks of Alstonia boonei (AB), Harungana madagascariensis (HM) and Enatia chlorantha (EC) in ratio 1.0:0.5:1.5:1.0:1.5 respectively. Acute toxicity and anti-malarial assay were evaluated in vivo at different doses of 200, 400 and 800mg/kg with Chloroquine and distilled water as controls using Chloroquine sensitive Plasmodium berghei infected mice. Phytochemical constituents, thin layer chromatographic conditions and Fourier Transform Infrared Spectroscopy (FTIR) spectrum of the polyherbal were assessed.

Results
Safety of the PE at 5000mg/kg was recorded. Prophylactic activity of the extract exhibited significant (p>0.05) low percentage parasitaemia (0.55%) at 800mg/kg compared to chloroquine (1.56%). Chemo-suppressive was significantly effective at a lower dose (200mg/kg) in comparison with negative control. The extract exhibited insignificant curative effect in the Plasmodium infected mice. Alkaloids, saponins, triterpenoids, flavonoids, cardiac glycosides were found to be present in the extract. FTIR spectrum showed the fingerprint identity of the extract.

Conclusion
These findings could therefore substantiate the use of the herbal mixture in folk medicine for preventing and treating malaria infection.

Keywords: Anti-malaria, Harungana madagascariensis, Ethnomedicine, Fingerprint

1. INTRODUCTION
Malaria is one of the most prevalent and debilitating diseases afflicting humans. It is regarded as a tropical disease. Malaria is a life-threatening disease caused by parasites that are transmitted to people through the bites of infected female anopheles mosquitoes [1]. Plasmodia parasites have complex life cycles involving vertebrate and insect hosts. Five species of Plasmodium are known to infect humans and these include P. Ovale, P. vivax, P. malariae, P. knowlesi, P. falciparum. In 2017, there were an estimated 219 million cases of malaria in 87 countries. The estimated number of malaria deaths stood at 435,000 in 2017. The WHO African Region carries a disproportionately high share of the global malaria burden. In 2017, P. falciparum accounted for 99.7% of estimated malaria cases in the WHO African Region, as well as in the majority of cases in the WHO regions of South-East Asia (62.8%), the Eastern Mediterranean (69%) and the Western Pacific (71.9%). The diagnosis of malaria usually
consists of microscopic examination of the parasites in stained thick and thin blood smears of peripheral blood. Malaria is managed by targeting the parasite in one or more of the stages of the life cycle prominently during the schizogonic phase to give rise to either clinical cure or radical cure. In clinical cure, the blood schizonts and/or the tissue schizonts are partly eliminated bringing about the elimination of the malaria symptoms because the parasite population left will not be enough to give rise to malaria symptoms. In radical cure, the entire Plasmodium population is eliminated including the hypnozoites that occur with the *P. vivax* and *P. malariae*. This is done with tissue hypnozoonticites such as primaquine \(^2\). However, the emergence of resistance in the treatment and advent of artemisinin combination therapy has given credence to investigating plant mixtures and decoctions from ethnomedicine. A wide variety of plants belonging to several families have been previously identified through ethnobotanical and ethnopharmacological studies as anti-malarial medicinal plants \(^3\). Plant combination therapy has been the model of treatment in most African traditional medicine.

*Alstonia boonei* De wild belong to the family Apocynaceae. It is also known as “God’s tree” in West African origin. It’s a tall forest tree that can reach 45min height and 3m in girth, with leaves oblongoconate in shape, and airborne whorls at the end. The flowers are yellowish-white and fruits pendulous paired, with follicles up to 16cm long containing seeds. It is used in the management of painful menstruations, painful urethritis in men and diabetes. The latex of the bark is applied to swellings of the kidney, mastitis, otitis and abscess in the ear. It is also been reported to have gastroprotective, hepatoprotective, antimicrobial, antiprotozoal, antisickling, anti-inflammatory, anticonvulsant and analgesic and pyretic activity \(^4\). Pharmacological activities including Antimalaria, antifertility, anti-inflammatory, antibacterial, antiplasmodial, antiadibetic, antiadriarhoea and antipsychotic of *A. boonei* has previously been reported \(^5\). *Azadirachta indica* A. Juss. belongs to the family Meliaceae and commonly known as "Neem tree" in English is a perennial tree that can reach a height of 15–20 metres (49–66 ft), and rarely 35–40 metres (115–131 ft). Its medicinal use includes its use in poultry, as the bark is used to treat wounds, diarrhoea, ticks and lice. They are also used in skin disorders including ringworm, alopecia, eczema, urticarial, scabies, ticks and lice. Other minor indications include metritis, orchitis and tetanus, rinderpest, rheumatism, stoppage of urination, swelling of the kidney, mastitis, otitis and abscess in the ear in veterinary use \(^6\). Neem has been implicated for having anti-malarial, hepatoprotective, antimicrobial, anti-malarial, antiadibetic, anticancer and wound healing activity \(^7\). *Morinda lucida* known as Oruwo in the Southwestern part of Nigeria is a medium-sized tree at maturity. Stem bark infusion is used as an anti-malarial and antiadibetic \(^8\). Anti-malarial, antimicrobial, gastroprotective, antioxidant, antiviral, antiadibetic, anti-inflammatory, anticonvulsant and analgesic and pyretic activity of *Morinda lucida* extracts have all been reported \(^9\). *Harungana madagascariensis* belongs to the family Hypericaceae and is native to tropical Africa. It has been reported to have antioxidant, antimicrobial, antiprotozoal, antisickling, anti-inflammatory and antioxidant \(^10\). *Enantia chlorantha* is an ornamental tree that belongs to the family annonaceae. It may grow up to 30 m high with dense foliage and a spreading crown. It has also been reported to have gastroprotective, hepatoprotective, antiviral, anticonvulsant, anti-inflammatory, fertility booster and anti-malarial activity \(^11\). The present study was aimed at evaluating the combined effect of the five agents of malaria in plasmodium infected mice.

2. **MATERIALS AND METHODS**

2.1. **Plant Collection**

The stem bark of HM, AB and the leaves of AI used for this investigation were collected from the botanical garden of the Faculty of Pharmaceutical Sciences, University of Port-Harcourt, Rivers state while ML leaves were collected from Rumualogu, Rivers State. EC was purchased from mile 3 market, Rivers State. The plants were further identified and authenticated in the Herbarium of the Department of Pharmacognosy and Phytotherapy, Faculty of Pharmaceutical Sciences, University of Port Harcourt. Voucher numbers were obtained for the plants viz: UPHH462, UPHA466, UPHM463, UPHR464, UPHA465 respectively.

2.2. **Preparation of the PE**

The dried and powdered leaves of AI and ML and the dried and powdered stem barks of AB, HM, and EC were mixed in the ratio 0.5:1.0:1.5:1.0:1.5 respectively to obtain a total of 386.5g of Combined powdered plant material. The mixed plant materials were boiled with 2L of distilled water at 100°C for one hour, allowed to cool and then filtered. The marc was re-extracted in the same manner twice. The extract was combined and evaporated to dryness on a water bath.
2.3. Experimental Mice
Both male and female mice *Mus musculus* (Muridae), 4-6 weeks of age, weighing between 19g and 31g were used for all experiments. The mice were obtained from the animal house of the Department of Pharmacology and Toxicology of the Faculty of Pharmaceutical Sciences, University of Port Harcourt. They were kept at 25 ± 2°C with a 12 h photoperiod per day and provided with standard feed and water *ad libitum*.

2.4. Acute Toxicity Studies (LD$_{50}$)
The aqueous plant extract was given to animals which were divided into six groups of three animals each, according to their body weights (150-160g). The acute toxicity study was carried out on experimental healthy albino rats using Lorke's method [12]. In the first phase of the study, nine mice were randomized into three groups of three mice each and were given 10,100, and 1000mg/kg body weight of the PE orally. They were observed for 24 hours for death and any sign of toxicity. In the second phase of the study, another fresh set of nine albino rats were randomized into three groups of three mice each and were given 1600, 2900 and 5000mg/kg body weight of the PE. They were observed for 24 hours for signs of toxicity. The oral median lethal dose was calculated using the formula:
\[
\text{LD}_{50} = \sqrt{\text{Minimum toxic dose} \times \text{maximum tolerated dose}}.
\]

2.5. Plasmodium Berghei
Chloroquine-sensitive *Plasmodium berghei* strain was used for the induction of malaria in the experimental mice. The parasite was obtained from the Malaria Research and Reference Reagent Resource Center (M-RAB) of the University of Port-Harcourt. Three mice infected with *Plasmodium berghei* were used as the donor source. The parasitaemia levels of the three mice were determined after which equal quantities of blood were obtained from the tails of the respective mice and diluted with 0.9% normal saline solution as the stock and used to infect both the suppressive and prophylactic test mice by injecting 0.5ml of the diluted blood via intraperitoneal injection to each of the mice.

2.6. Evaluation of the Prophylactic Anti-Malarial Activity of the PE
The chemoprophylactic activity of the PE was performed using a modified method described by Voravuth *et al.*, (2018) [13]. There were five groups of *P. berghei* NK 65 infected mice in which each group contains six mice. The mice in groups 1, 2 and 3 were administered 200, 400 and 800mg/kg of the PE orally respectively. The mice in group 4 were administered 10mg/kg of chloroquine orally. The mice in group 5 were administered 0.2ml of distilled water and the dosage administration continued once daily for the next three days respectively. At 24 hours after dosage administration, the mice were inoculated with 32.3% parasitized erythrocytes by intraperitoneal injection. At 72 hours after the inoculation of the mice with the *P. berghei*, both thick and thin smears of the blood of each of the mice in all the groups were obtained through the tail. The smears were fixed with ethanol and stained with Giemsa for microscopic identification and quantification of parasitized and total red blood cells.

2.7. Evaluation of the Suppressive Anti-Malarial Activity of the PE
Evaluation of the chemosuppressive anti-malarial activity of the polyherbal using a modified standard four-day suppressive test [13] involves five groups of mice in which each group contains six mice each. Each of the groups was inoculated with 32.3% parasitized erythrocytes by intraperitoneal injection. Three hours after inoculation, the mice in groups 1, 2 and 3 were administered 200mg/kg, 400mg/kg and 800mg/kg of the PE respectively, the mice in group 4 were administered 10mg/kg of chloroquine orally and the mice in group 5 were administered 0.2ml of distilled water and the dosage administration continued once daily for the next three days respectively. At 24 hours after day 3 of the dosage administration, both thick and thin smears of the blood of each of the mice in all the groups were obtained through the tail and prepared as stated in prophylactic evaluation.

2.8. Evaluation of the Curative Anti-Malarial Activity of the PE
The curative potential of the polyherbal was evaluated as described by Ryley and Peters (1970) [14]. After three days post-induction of the *P. berghei* NK 65 parasite, the mice were grouped in four different cages containing six mice each. Group 1, 2, and 3 received 200, 400 and 600mg/kg of the PE respectively. The mice in group 4 were administered 10mg/kg of chloroquine. Mice in group 5 were...
not inoculated but administered 0.2ml of distilled water and the dosage administration continued once daily for the next seven days respectively. Thin blood smears were obtained and prepared as stated in the chemo-suppressive test on days 0, 3, 7 and 14 to monitor the level of parasitaemia by microscopic assessment of parasitized and total red blood cells.

2.9. Determination of Percentage Survival

Mortality was monitored daily throughout the study, from the time of infection up to the end of the 7th day in all the groups. The percentage of survival time was calculated for each group by using the formula:

\[ \% \text{ survival} = \frac{\text{number of animals (Day 0)} - \text{number of animals (Day 7)}}{\text{Number of animals (Day 0)}} \times 100 \]

2.10. Phytochemical Screening

Phytochemical screening was carried out on the PE to detect the presence of secondary metabolites using described standard methods\(^{[15, 16]}\).

2.11. Thin-Layer Chromatography (TLC)

The PE was predisposed to chromatographic profiling using TLC based method. A pre-coated silica gel GF\(_{254}\) plate of 10 x 4 cm marked 1cm away from both ends. Glass capillary tubes were used to spot on the plate and developments were done using varied ratios of mobile phases containing butanol, acetic acid, water. The plates were visualized in both daylight and ultraviolet light (UV). The chromatograms were detected with 60% sulphuric acid spray in which the plate was activated at 100°C\(^{[17]}\).

2.12. Fourier Transform Infrared Spectroscopy (FTIR) Assay

Buck scientific M530 USA FTIR was used for the analysis. This instrument was equipped with a detector of deuterated triglycerine sulphate and beam splitter of potassium bromide. The software of the Gram A1 was used to obtain the spectra and to manipulate them. An approximately 1.0g of sample was properly placed on the salt pellet. During measurement, FTIR spectrum was obtained at frequency regions of 4,000 – 600 cm\(^{-1}\) and co-added at 32 scans in 4 cm\(^{-1}\) resolution. FTIR spectra were displayed as transmitter values.

2.13. Data and Statistical Analysis

The average parasitaemia was determined by obtaining the percentage of the ratio of parasitized to the total number of RBC. The average percentage of chemo-suppression (or parasite clearance) was calculated as 100x \((A-B)/A\), where A is the average parasitaemia of the negative control group and B is the average parasitaemia of the test group. The effective doses (ED\(_{50}\)) were determined online (www.aatbio.com) while the one-way analysis of variance between groups and negative control was done with GraphPad Prism 9.0.2. A P value of <0.05 was considered statistically significant.

3. RESULTS

3.1. Percentage Yield

The air-dried and powdered stem bark of \(AB\) (200g), \(HM\) was (135g), \(EC\) (200g) and leaves of \(ML\) (135g) and \(AI\) (67g) and were combined. The 737g of the powdered plant materials yielded 150g after decoction with a percentage yield of 20.32%.

3.2. Acute Toxicity

The animals survived the doses of the PE given to them at a single dose at different phases of the study. Signs of toxicity were not exhibited by the animals. The LD\(_{50}\) was therefore estimated to be 5000mg/kg.

3.3. Effect of PE on Prophylactic Evaluation

In Table 1, the treatments showed significant (p>0.05) percentage reduction of parasitaemia level in the \(P. berghei\) infected mice in a dose-dependent manner when compared to distilled water, control
groups. Consequently all the treatments have a lower percentage of parasitaemia than the chloroquine treated group.

**Table 1.** Effect of PE on percentage parasitaemia level in *P. berghei*-infected mice in the prophylactic evaluation

| Treatment           | Mean parasitaemia level = Standard deviation | ED50  |
|---------------------|---------------------------------------------|-------|
| PE 200 mg/kg        | 1.17±0.1528%*                               | 456.7 |
| PE 400 mg/kg        | 1.04±0.4928%*                               |       |
| PE 800 mg/kg        | 0.55±0.5119%*                               |       |
| Chloroquine 10 mg/kg| 1.56±0.2182%*                               |       |
| Distilled water     | 2.22±0.4481%                                |       |

Values represent Mean±Standard Deviation of 6 mice, PE: Median Effective dose, *Significantly different (p>0.05) when compared to the negative control.

**Table 2.** Percentage survival of infected treated mice after 14 days

| Days                  | PE200 | PE400 | PE800 |
|-----------------------|-------|-------|-------|
| Number of mice at Day 0| 6/6   | 6/6   | 6/6   |
| Number of mice at Day 14| 6/6   | 5/6   | 5/6   |
| Percentage survival   | 100%  | 83.3% | 63.3% |

The numerator represents the number of mice that are active while the denominator represents the total number of mice.

3.4. Effect of PE on Chemo-Suppressive Evaluation

It was observed that the group treated with 200 and 400mg/kg showed a significant (p>0.05) decrease in percentage parasitaemia when compared to the negative control (distil water) in Fig 1. Only the 200mg/kg treatment group showed a significant percentage of parasitaemia when compared with the positive control (chloroquine 10mg/kg)

**Fig 1. Suppressive effect of the Polyherbal on the mice**

![Graph showing suppressive effect of the Polyherbal](image)

Values represent Mean±Standard Deviation of 6 mice, PE: Med Effect dose.

3.5. Effect of PE on Curative Evaluation

None of the concentrations of the extract in fig. 2 exhibited a reduction in parasitaemia level of the *P. berghei* infected mice. The percentage parasitaemia in the extract treatment groups was similar to the negative control while chloroquine exhibited 100% clearance of the parasitaemia.
Values represent Mean±Standard Deviation of 6 mice. PE: PE

3.6. Percentage Survival after 14 Days

Mortality was monitored from the time of infection to the end of the 14th day in all the treatment groups in the curative evaluation. In the group treated with chloroquine (10mg/kg) and 200mg/kg of the polyherbal, the percentage survival was 100%. In 400 and 800mg/kg treated groups, the percentage survival was 83.3 and 66.7% respectively.

3.7. Phyto-Constituents of the PE

The phyto-constituents detected in the PE were alkaloid, saponins, tannins, flavonoids, cardiac glycosides, triterpenoids and carbohydrates as presented in Table 3. Anthraquinone was not detected.

Table 3. Phytochemicals of the Ployherbal extract

| Phytochemicals | Remark |
|----------------|--------|
| Alkaloids      | +      |
| Anthraquinones | -      |
| Flavonoids     | +      |
| Saponins       | +      |
| Tannins        | +      |
| Triterpenoids  | +      |
| Cardenolides   | +      |
| Carbohydrates  | +      |

+: Present, -: Absent

3.8. Chromatographic Identification of the PE

The PE showed a better separation of its characteristic constituents on a thin layer chromatography on a pre-coated Silica Gel GF254 in a mobile phase of butanol: acetic acid: water (4:1.5) and visualized at 365nm. Notable Rf were 0.73, 0.63, 0.53, and 0.33 with characteristic light blue, brown, yellow and brown colours respectively.

3.9. FTIR Spectrum of the PE

The fingerprint obtained from the infrared spectrum the PE in Fig 3 was to specifically identify the quality of the PE. The fingerprint region showed a unique pattern that describes the PE. The frequency region also showed the presence of OH (3287-3438) stretching, alkyl group, CH2 and CH3 frequencies at 2133, 2902, and 2973 respectively.
One major constraint of anti-malarial monotherapy is the growth of resistance by the malarial parasites [18, 19]. With the discovery of artemisinin from *Artemisia annua* and the adoption of artemisinin combination therapy (ACT) as the current anti-malarial treatment policy, it is logical that anti-malarial plant drug discovery efforts be tilted towards exploring decoctions of medicinal plant materials used for anti-malarial in traditional medicine [20]. Decoctions in herbal medicine are multicomponent and could therefore be principally considered as 'natural' combination therapies [21]. Hence, resistance to the malarial parasite is not known in multicomponent herbal mixtures which often consist of two or more morphological parts of plant(s) to provide synergistic antiplasmodial actions or to perform other formulation roles as pharmaceutical excipients [22].

Each of the plants in the mixture has earlier been reported to have anti-malarial potential: *Alstonia boonei* bark [23]; *Azadirachta indica* and *Morinda lucida* leaves [24]; *Enantia chlorantha* [25]; *Harungana madagascariensis* stem bark [26].

The primary aim of this study was to ascertain the efficacy and safety of the claimed use of the PE in treating malaria. In this study, acute behavioural signs of toxicity such as paw licking, salivation, stretching, and reduced activity, were observed at dose 5000mg/kg however there was no mortality at all the dose levels. The oral median lethal dose (LD<sub>50</sub>) was estimated to be > 5000mg/kg body weight. This high safety profile may have been responsible for its widespread use in ethno-therapeutic interventions. *Plasmodium berghei* parasite is used in treatment outcomes of any suspected antimalarial agent due to its high sensitivity to Chloroquine making it the appropriate parasite for this study [27].
In the suppressive study, although 200mg/kg and 400mg/kg showed a significant decrease in parasitaemia level (P < 0.05), the decrease in parasitaemia was regressing with an increase in the concentration of the PE. It was also observed that the mice in the groups of the higher doses were dying before the end of the experiment. This, therefore, suggests that an increase in the dose of the extract become toxic to the animals and does not hinder the progression of the parasitaemia level in the mice and thereby leading to the death of the animals in the group given the higher doses. It is as well suggests that lower doses below 200mg/kg could be a better range to be administered to obtain a better anti-malarial suppressive effect as observed that the fixed smear of the blood of the animals treated with a lower concentration of the PE showed lesser bleaching of the red blood cells of the animals. With an increase in dose, it could as well be suggested that the active sites of the PE were being blocked which resulted in the diminishing anti-malarial suppressive effect with an increase in dose.

In the curative study where malaria has already been established in fig 3, the PE did not show a significant (p<0.05) decrease in parasitaemia level even at a dose of 800mg/kg up to the fourteenth day. This, therefore, suggests that the PE has no curative effect on already established malaria infection. It was also observed that the group treated with a higher concentration of the extract had weight loss, shivering, and seizure which progressively led to the death of some of the animals before the end of the experiment. This could be as a result of a progression of the malaria infection and possible antagonistic effect of the scaffold constitutes of the Polyherbal.

In the prophylactic study, the polyherbal treated group exhibited a dose-dependent and significant decrease in P. berghei when compared with the untreated group. The study, therefore, indicated that the doses of 400mg/kg and 800mg/kg have a better prophylactic treatment effect of P. berghei (P < 0.05) than the standard chloroquine drug.

The absence of anthraquinones in the extract could suggest a possible reason for its ineffectiveness in early and established malaria infection stages since it was present in the individual plants that were claimed to have anti-malarial activity. The FTIR spectrum corroborated the phytochemical result with the absence of carbonyl functional group which could signal the presence of anthraquinone.

The thin layer chromatogram of the polyherbal which is also its identity showed the best separation in using a mobile phase of Methanol, Acetic Acid, Ethylacetate (5:0.5:4) and Butanol, Acetic Acid, Water (4:1:5)

In conclusion, the polyherbal was found to be safe at 5000mg/kg. It is effective in treating the early onset of malaria at low doses and prophylactic stage of P. berghei infection and ineffective in treating the early and established P. berghei infection which means that it should not be taken. The identity of the polyherbal is recorded for its thin layer chromatography and FTIR fingerprint spectrum.

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AUTHORS CONTRIBUTION

Kio Anthony Abo conceived the experiment. Suleiman Mikailu and Afieroho Ozadheoghene Eriarie designed the experiment and wrote the manuscript. Vigara Sophia Ledumbari, Nzereogu Chibuike Stephen and Doris Nnenna Ajibo conducted the research.

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Prophylactic, Suppressive and Curative Potentials of a Polyherbal Antimalaria Mixture in Plasmodium Berghei Infected Mice

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