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Antimalarial potential of herbal combination containing *Harungana madagascariensis, Costus afer* and *Citrus aurantifolia*

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Abstract
There has been continuous research for herbal remedies to combat malaria which is gradually becoming resistant to the available orthodox drugs. These drugs pose a risk of toxicity to humans and are becoming more expensive in Nigeria. This research is aimed at investigating the antimalarial potential of the herbal combinations containing *Harungana madasgariensis, Costus afer*, and *Citrus auritifolia*. The Herb was prepared by the maceration of the stem bark of by *H. madagascariensis* at g/ml of distilled water. The stem of *Costus afer* was debarked by peeling and then pounded in a wooden mortar to extract its juice and filtered. The juice of *Citrus aurantifolia* was obtained by squeezing the fruit and filtering. The three plants *H.madagascariensis, C.afer*, and *C.aurantifolia* were combined in a ratio of 100ml:100ml:10ml respectively in a bottle and preserved at 4ºC. Acute toxicity of the herbal combination was assessed using Lorke method. The antimalarial assay was evaluated in vivo using Chloroquine sensitive *Plasmodium berghei* induced mice. The herbal preparation was screened for Phytochemical constituents, Thin layer chromatography and Fourier transform Infrared analysis were carried out for characterization. The acute toxicity showed LD50 of 1300mg. The curative antimalarial study showed that the highest dose (260mg/kg) exhibited a significant reduction of the parasitemia level against the control group. The Phytochemical screening of the herbal combination showed the presence of tannins, cardenolides, deoxy sugars and triterpenes. The FTIR showed the presence of free OH, aliphatic CH, and C=C bonds. Thus, it could be concluded that the Herbal combination of *H. madagascarensis, C. afer*, and *C. auritifolia* is safe at 1300mg/kg and has a significant curative effect at the highest dose (260mg/kg) and its effect could be attributed to the functional groups present in the combination.

Keywords: *Harungana madagascarensis, Costus afer, Citrus auritifolia, Plasmodium berghei*

1. Introduction
Malaria is an infectious and life-threatening disease which is caused by a single-celled protozoan parasite of the genus *Plasmodium spp*; these parasites cause disease via several pathways which depend on the type of *Plasmodium* spp. There are five species known to infect humans which include *Plasmodium falciparum, P. Ovale, P. vivax, P.malariae, P. knowlesi.* There are currently over 100 countries and territories where there is a risk of malaria transmission. The world health organization estimates that 438,000 people died of malaria in 2015; the vast majority are young children in sub-Saharan Africa.
The malaria parasite is transmitted to people by the female anopheles' mosquito and through blood transfusion, organ transplant, or the shared use of needles or syringes contaminated with blood [1]. The use of medicinal plants in the treatment of diseases and ailments has a long history worldwide. The use of herbal mixtures in the treatment of malaria in Africa is still highly acceptable despite the emergence of effective combination therapies such as Artemisinin-based combination therapy (ACT) although the emerging potent drugs originated from plants. The first antimalarial drug was extracted from the bark of the *Cinchona* species. Infusions of the plant bark were used to treat human malaria as early as 1632 [2]. Years later, quinine was isolated and characterized [3]. Another ancient plant that has been employed in the treatment of malaria is *Artemisua annua*, discovered in China in the seventies and it's an important source of Artemisinins [4].

Harungana madagascariensis (Family: Hypericaceae) is commonly known as Dragon Blood Tree. It is a shrub or tree that has broad ovate leaves and produces an exudate of a bright orange juice. It is widespread and locally abundant in areas where annual rainfall exceeds 1300mm. [5, 6]. The stem, root and leaf extracts have been used in the treatment of various diseases including dysentery, bleeding piles, trypanosomiasis, fever, cough, black tongue, scabies, cold, jaundice, boils [7]. The efficacy of the medicinal plant is majorly due to the presence of phytochemicals and the successful extraction of these compounds from the plant material which largely depends on the type of solvent used in the extraction procedure [8]. The Aqueous leaf extract has been shown to have anti-microbial activity on different strains of bacteria (*Bacillus subtilis*, *Staphylococcus aureus*, *Eschericia coli* and *Salmonella typhi*), thus substantiating its use for gastro-intestinal disorders [9]. The stem bark extract inhibited *Entamoeba hystolytica* growth at a concentration of less than 10µg/ml [10]. The most recent work of Ndjakou *et. al*., (2007) [11] reported six anti-plasmodial compounds isolated from the root bark of *H. madagascariensis*.
Costus afer belongs to the family Zingiberaceae. It is commonly called bush sugar cane or monkey sugar cane [12]. It is commonly found in the moist or shady forest of West and Tropical Africa and highly valued for its anti-diabetic, anti-inflammatory and anti-arthritic properties in South-East and South-West Nigeria [13]. Several literatures have confirmed the antidiabetic, anti-inflamatory, analgesic and liverprotective effect of *Costus afer* [14].

Citrus aurantifolia belongs to the Rutaceae family. It is a polyembryonic plant cultivated in many countries all over the world and grows in hot subtropical or tropical regions such as Southern Florida, India, Mexico, Egypt, and the West Indies [15]. Some of the major classes of compounds in citrus include flavonoids, limonoids, coumarins and phytosterols [16]. Studies have reported that *C. aurantifolia* posses antibacterial activity. The extracts of the root of *C. aurantifolia* have been found effective in inhibiting the growth of *Staphylococcus aureus, Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas aeruginosa, Beta-haemolytic streptococci, Escherichia coli* and *Neisseria gonorrhoeae* [17]. However, this study investigates the *in vivo* safety, Curative effect, and phytochemicals of the herbal combination of *H.madagascariensis, Costus.afer* and *Citrus auritifolia* in *Plasmodium berghei* infected mice.

2. Materials and Methods

Plant collection

The stem bark of *Harungana madagascariensensis* and stem of *Costus afer* (monkey sugar cane) used for this investigation were obtained from the botanical garden of the Faculty of Pharmaceutical Sciences, University of Port Harcourt. *Citrus aurantifolia* (lime orange) was purchased from the Choba market, Rivers state. The plants were further identified and authenticated in the faculty Herbarium

Extraction of plant material

The fresh stem bark of *Harungana madagascariensis* (100g) was scrapped off of its outermost layer leaving the fleshy part alone. A 100g of the bark was macerated with 100ml of distilled water by constant shaking for one hour and filtered. The stem of *Costus afer* was debarked by peeling and then pounded in a wooden mortar to extract its juice and filtered. The juice of *Citrus aurantifolia* was obtained by squeezing and filtering. The three plants *H. madagascariensis, C.afer*, and *C.aurantifolia* were combined in a ratio of 100ml:100ml:10ml respectively, freeze dried and preserved in the refrigerator at 4^0 C before its use.

Experimental Animals

Plasmodium berghei infected albino mice (12-20g) were obtained from the National Institute of Medical Research (NIMR), Yaba Lagos and housed in five cages containing four animals each. The animals were maintained at room temperature of 25 ^{o} C in the animal house and fed with standard diet and water *ad libitum*.

Acute toxicity study

The aqueous plant extract was given to animals which were divided into six groups (1, 2, 3, 4, 5 and 6) of three animals each, according to their body weights (20-30g). The acute toxicity study was carried out on experimental healthy mice using the Lorke's method [18] to estimate the level of toxicity of the plant extract in other to determine the safety margin. In the first phase, three groups (1, 2, and 3) were treated orally with doses of 10,100, and 1000mg /kg body weight respectively. They were observed for 24 hours for death and any sign of toxicity. In the second phase, three groups (4, 5, and 6) were treated with doses of 1600, 2900 and 5000mg/kg body weight respectively. They were observed for 24 hours for signs of toxicity.

Plasmodium induction

A chloroquine-sensitive strain of *Plasmodium berghei* (NK 65) was used for the induction of malaria in the experimental mice. A mouse that was previously infected was used as a donor and subsequently maintained in the laboratory by the serial passage of blood from the donor infected mouse to the uninfected mice through the intraperitoneal route. This was done by preparation of a standard inoculum of 10^7 parasitized erythrocyte from the blood harvested from the donor mouse (>30% parasitized) with normal saline and administered (200uL) through the intraperitoneal route to each of the experimental mouse.

Treatment (Seven Day Curative test)

Seventy-two hours after induction of the *P. berghei* parasite according to Ryley and Peters (1970) [19] with slight modification, the mice were grouped in five different cages containing five mice each. The groupings are as follows:

Group 1: contains Normal untreated mice

Group 2: contains infected mice treated with 10mg/kg bodyweight of chloroquine.

Group 3: contains infected mice treated with 65mg/kg bodyweight of plant extract.

Group 4: contains infected mice treated with 130mg/kg bodyweight of plant extract.

Group 5: contains infected mice treated with 260mg/kg bodyweight of plant extract.

The plant extract and chloroquine used in this study were administered orally. Each mouse was treated once daily for seven days and the level of parasitemia were examined on Day 0, Day 3 and Day 7.

Peripheral blood-smear preparation and determination of parasitemia

Thin and thick smears of blood were made from the tail of each mouse. The smears were applied on microscopic slides and the blood was drawn evenly across a second slide to make a thin blood film and allowed to dry at room temperature. The slides were fixed with 10% methanol and stained with Giemsa's stain for 30minutes. The parasitemia level was determined by counting the number of parasitized erythrocytes in random fields of the microscope. The percentage parasitemia level was calculated by using the formula [20]:

$$\% \text{ Parasitemia} = \frac{\text{Number of infected RBC's}}{\text{Total number of RBC}} \times 100$$

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:

% survival = number of animals (Day 0) – number of animals (Day 7) X 100 Number of animals (Day 0)

Statistical Analysis
Data were expressed as mean ± standard error and analyzed using the students' T-test for test of significance.

Phytochemical screening
Phytochemical screening was carried out on the mixture for the presence of secondary metabolites using standard methods [7, 21].

Thin-Layer chromatography (TLC)
The combined plants extract was subjected to chromatographic profiling using TLC based method. A pre-coated silica gel GF_{254} plate of 10 x 4 cm was marked 1cm away from both end. Glass capillary tube was used to spot on the plate and development was done using mobile phase of butanol: acetic acid: water (40: 10: 50). The plate was visualized in both day light and ultraviolet light (UV). It was also detected with 60% sulphuric acid spray in which the plate was activated at 100˚C.

Fourier transformer infrared (FTIR) of the extract
Fourier transform infrared absorption signals of the studied extracts was measured at room temperature (20˚C) in the wavelength range 4000 - 400 cm^{-1} using a computerized recording FTIR spectrometer (Mattson 5000, USA). The dried extract was mixed with KBr in the ratio 1:100 for quantitative analysis and the weighed mixtures were subjected to a load of 5 t/cm^2 evocable discs. The IR absorption spectrum was immediately measured after preparing the discs to avoid moisture attack.

3. Results

Acute toxicity
In the first phase of the study when dose of 10 – 100mg/kg was given all the animals survived. In the second phase, from the first dose of 1600mg/kg group, one animal died and others were sluggish and inactive. In the groups of other doses no one survived. The median lethal dose was therefore calculated to be 1300mg/kg of the rats.

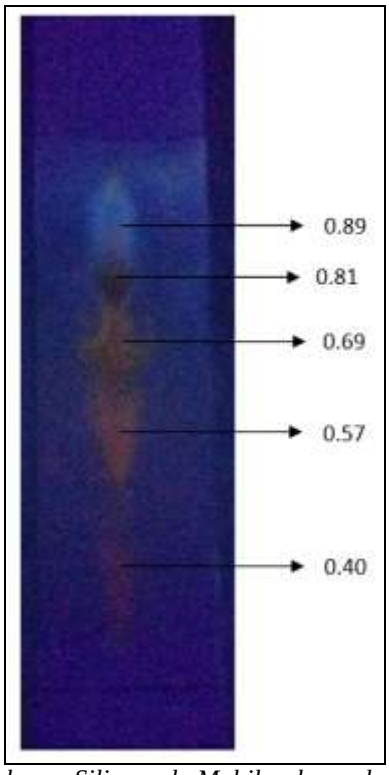
Seven-day curative test
In this experiment the reduction in percentage parasitemia in Table 1 was observed in the treated groups on day 3 while the untreated group showed increase in percentage parasitemia when compared to day 0. On day 7 it was obvious that the highest dose (260mg/kg) showed the highest percentage reduction in parasitemia even though there was increase when compared to day 0.

Percentage survival of animals
It was observed that both the untreated and treated survived from day 0 to day 3 but by day 7 two death was recorded (Table 2) in which one was from untreated group and the other was from 65mg/kg treated group.

Phytochemical screening
The screening revealed the presence of flavonoids, tannins, triterpenoids, and cardiac glycosides in the herbal combination extract, Table 3.

Thin layer chromatography (TLC)
This experiment shows that the herbal combination extract can be identified on a TLC using a mobile phase of butanol: acetic acid : water (40 : 10 : 50) to separate it to five distinct spots with their Rf on Fig 1. The spots showed characteristic colours under the Ultraviolet light (UV_{365nm}) viz; 0.40 (pink), 0.57 (pink), 0.69 (light brown), 0.81 (deep brown) and 0.89 (light blue).

FTIR
The region of 1600nm to 600nm in Fig 2 shows the unchangeable spectrum of the extract at all time while the upper region indicates the presence of functional groups present in the extract which include OH-group and =CH or $=CH_2$ groups at 3633nm and 3028nm respectively.



Stationary phase: Silica gel; Mobile phase: butanol:acetic acid: water (4:1:5); Visualization: UV_{365} ; Values are Rf.

Fig 1: The chromatogram of the herbal combination extract on Thin-layer Chromatography (TLC)

Table 1: Effect of herbal combination extract on Percentage parasitemia level of *Plasmodium berghei* infected mice.

GROUPS	DAY 0	DAY 3	DAY 7
Untreated (Negative Control)	6.83 ± 0.39	8.94 ± 3.14	19.89 ± 3.43
Treated with the chloroquine 10mg/kg	-----	12.35 ± 2.32	*14.33 ± 1.33
Treated with extract 65mg/kg	20.48 ± 11.70	9.29 ± 3.80	16.14 ± 3.21
Treated with extract 130mg/kg	9.00 ± 3.16	6.68 ± 2.41	16.52 ± 4.19
Treated with extract 260mg/kg	11.03 ± 1.32	8.39 ± 4.18	*11.95 ± 1.24

Values represent Mean ± SEM (standard error of the mean) of percentage parasitaemia of 5 mice; * Significantly different when compared to the negative control on day 7 ($p<0.05$).

Table 2: Percentage survival of the animals.

Number of mice at Day 0	25/25
Number of mice at Day 7	23/25
Percentage of survival	88%

Numerator signifies the number of mice that are alive while the denominator represents the total number of mice.

Table 3: Phytochemical screening result

Phytoconstituents	Inference
Alkaloids	-
Anthraquinones	-
Flavonoids	+
Saponins	-
Tannins	+
Triterpenoids	+
Cardenolides	+
De-oxysugar	+

- Means negative; + means present

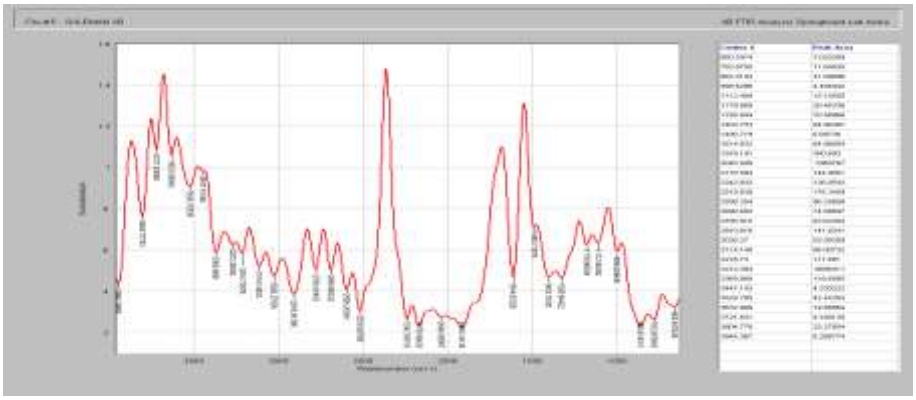


Fig 2: The FTIR spectrum of the herbal combination extract

4. Discussion

The combination of the three plants (*Harungana madagascariensis*, *Costus afer* and *Citrus aurantifolia*) extracts in the ratio of 10:10:1 is a recipe taken once malaria symptoms are observed by a patient in the folk. However, there has been a report of the antimalarial effect of *Harungana madagascariensis* [22]. *Costus afer* has also been reported to have the same effect [23]. It was claimed that a glass shot of the combination is equivalent to 40 ml is the daily dosage. The 40ml of the herbal mixture is equivalent to 760mg after freeze-drying. However, the study was aimed at evaluating the safety, curative effect and phytochemicals contained in the herbal combination.

The Acute toxicity result showed an LD_{50} of 1300mg/kg. The acute toxicity showed that the 40ml of the herbal combination is safe since it is equivalent to 760mg which is below the LD_{50} as tested in the mice.

The herbal combination showed a significant ($p<0.05$) percentage reduction of parasitemia to 11.9% level at the highest dose (260mg/kg) on Day 7 of treatment while the control showed a parasitemia level of 19.83%. It is also obvious that the herbal extract showed a lower parasitemia level when compared with the standard drug, Chloroquine (10mg/kg) which had 14.33% parasitaemia level on the Day 7 of treatment. During the period of the experiment, the untreated group exhibited elevated body temperature and shivering while the extract treated and chloroquine treated mice were active without elevated temperature which is indicative of being cured of the plasmodium infection.

The Phytochemical screening showed that the herbal combination contained Tannin, flavonoids and cardiac glycosides. The presence of Tannins and flavonoids were further confirmed through the results obtained from FTIR but not specific on TLC. Tannins and flavonoids have been implicated in several pharmacological activities including antimlarial [24].

The FTIR in Fig 2 showed the presence of free OH-groups stretching at 3633nm and bonded OH groups at 3216 – 3447nm which could be attributed to the flavonoids and tannins that were detected in the phytochemical screening.

Stretching at 3028nm indicates =CH or $=CH_2$ groups of possibly the aromatic rings of the flavonoids present in the extract. The fingerprint region of the FTIR represents the signatory identity of the combined herbal extract. The TLC results of the extract also serve as another quality control measures in identifying the extract against adulteration.

Percentage survival, Table 4 is a parameter that is used in the evaluation of the antimalarial prospect of an extract. A plant extract that can prolong the survival time of infected experimental animals when compared to the untreated group is considered a promising agent for malaria treatment [25]. In this study, the control group and group been administered with the low dose (65mg/kg) experienced the death of one animal each while the median dose and the highest dose showed no death of the animals. This however shows that ineffective dose made the parasite to kill the mouse since death was not recorded in the higher dose. With the 88% survival, there is indication that the extract was able to prolong the life of the infected animals hence suggestive of good antimalarial.

5. Conclusion

From this study, it could be concluded that the herbal preparation is safe for human consumption at the dose it was taken in folk (40ml daily equivalent to 760mg) since it is far below the LD50. It also contains various phytochemicals which include free OH, aliphatic C-H, and bonded OH which is responsible for its curative activity in the *Plasmodium berghei* infected mice.

6. Conflict of Interest

The authors declare no conflict of interest.

7. Acknowledgments

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