Original Research Article

IN VIVO CHEMOSUPPRESSIVE EFFECTS OF DIFFERENT RATIO COMBINATIONS OF THREE NIGERIAN ANTIMALARIAL PLANTS ON PLASMODIUM BERGHEI BERGHEI PARASITE IN MICE

Abstract:

Aims: Individual medicinal plants that are usually combined in arbitrary ratios or by intuition as herbal recipes for malaria in African ethnomedicine should be evaluated scientifically to obtain appropriate ratios. This would serve the purpose of designing a model for investigation of known or formulating new effective remedies from existing arrays of active antimalarial plants.

Place and duration of the study: The study was conducted at the Department of Pharmacognosy, Obafemi Awolowo University, Ile-Ife Nigeria.

Study Design: The relative antimalarial activities of three Nigerian ethnomedicinal antimalarial plants, E. uniflora leaf (E), G. latifolium root and stem (G) and A. altilis stem bark (A) in their dried powdered forms, singly and in different ratio combinations of two's (E:G, G:A, E:A) and three's (E:G:A) were determined.

Methodology: The infusions were separately concentrated in vacuo, freeze-dried and each evaluated against Plasmodium berghei berghei parasite in mice at a single dose of

400mg/kg using the Peter's four-day chemosuppressive model. Normal saline and chloroquine (10mg/kg) were negative and positive controls respectively.

Results: The binary ratio combinations E: G and G: A of 1:1, 1:3, 2:3 elicited percentage chemo suppressions: 66, 69, 78 and 31, 42, 31 respectively which were comparable (P=.28; P=.07) to those of the single drugs. However, all the tested combination ratios in the E: A group, gave relatively low percentage chemosuppression, barely above 20% with only the ratios 2:3 and 3:2 giving percentage suppression which were significantly (P=.00) higher than negative control. Only the E: G ratios gave activities that were comparable (P=.28) to the positive control. The trinary combination E: G: A 2:1:2 and 3:3:2 gave suppressions that were significantly (P=.00) higher than the negative control with others but comparable (P=.33) activities to those of the individual drugs: E, G and A which gave 49, 51 and 38% respectively. The other ratios with low suppression values were relatively inactive. But three ratios, E: G: A 3:1:2, 2:1:1, 1:1:1 elicited survival times doubled (204, 242 and 202 %) that of the negative control without commensurately high antiplasmodial activities.

Conclusion: Ethnomedicinal antimalarial plants should not be combined without a data of previous scientific evaluations.

Introduction

The antiplasmodial and the antimalarial activities with the safety of *E. uniflora* leaf, *G. latifolium* root and stem and *A. altilis* stem bark, have been established in various studies [1, 2, 3, 4, 5, 6, 7, 8]. However, such studies have not taken into consideration the possibility of preparing a combination of these plants as a standard antimalarial recipe, which may be used in African ethnomedicine [9]. Taking into consideration that most remedies are prepared as a combination of many plants for improved effects [10, 11, 12, 13], it is expedient that antimalarial plants are tested in their combination forms particularly with the ones with which they are usually combined in ethnomedicine or those with similar ethnomedicinal claims. This will enable investigators to explore the potential of combining such medicinal plants in therapy and further establish the values of or otherwise of the combination of these plants in the treatment of prevalent disease conditions. Plants have multiple chemical components [14, 15] and so can exhibit different pharmacological profiles known as polypharmacological effects when combined together especially in various ways [16]. In this study, the various combination ratios of *E. uniflora* leaf, *G. latifolium* root and stem and *A. altilis* stem bark in twos and threes were prepared as infusions, concentrated to small volume, freeze dried and tested for chemosuppressive antimalarial activities in a *Plasmodium berghei berghei* – infected mouse model. This was with a view to justifying the possible combination of these plants in ethnomedicine including identification of the best combination ratio of the chosen medicinal plants for treatment of malaria.

Materials and Methods

Equipment: Grinding machine (Christy Norris), Digital Balance, Hot plate, Microscope, Rotary evaporator, Dissecting set, Thermometer (Kwest digitherm). **Materials:** Glass jar, Tap water, Round bottom flask (1L), Glass rod, Funnel, Cotton wool, Aluminum cages, Mice, Animal feed (Grower pellets), Needle & syringe (1ml & 5ml), Test-tubes, Metal cannula, Spatula, Normal saline, Chloroquine tablets, Giemsa stain, Microscope slides, Immersion oil.

Plant collection and authentication

The leaf of *Eugenia uniflora*, the stem & root of *Gongronema latifolium* and stem bark of *Artocarpus altilis* were collected from the Medicinal plant garden and the Teaching and Research Farm area, Obafemi Awolowo University, Ile-Ife, Osun state on 8th June, 2018. The plant parts were identified and authenticated by Mr. I. I. Ogunlowo, the plant curator, Department of Pharmacognosy, Faculty of Pharmacy, Obafemi Awolowo University, Ile-Ife, voucher specimen with numbers FPI 2208, FPI 2209 and FPI 2207 were deposited in the herbarium, Department of Pharmacognosy, Faculty of Pharmacy. The plants were separately dried, powdered using the Christy Norris grinding machine and kept in a cool dry place until it was used for the experiment.

Extraction

Powdered samples (40.0g) each of *Eugenia uniflora* leaf, *Gongronema latifolium* root & stem and of *A. altilis* stem bark were weighed into different jars. Also, separate powdered samples of each plant were mixed together as follows: *E uniflora*: *G. latifolium* **(E:G)** and *G. latifolium: A. altilis* **(G:A)** and *E uniflora*: *A. altilis* **(E:A)** with each combination in the ratios 1:1, 1:2, 1:3, 2:1, 2:3, 3:1, 3:2, while *E. uniflora*: *G. latifolium: A. altilis* **(E:G:A)** were constituted in the ratios 1:1:1, 1:1:2:, 1:2:2, , 2:1:1, 2:1:2, 2:2:1, 3:1:2, 3:2:2, 3:3:2, respectively. These were all weighed into different glass jars to make a 40.0g mixture each. A 500ml of boiling water was added to each individual powders and mixtures, stirred and left to stand for 30 minutes before it was filtered. The filtrates were concentrated to small volume *in vacuo* at 70°C using the rotary evaporator, freeze dried, weighed and kept in a desiccator. The yields were calculated.

Preparation of Mice

Swiss albino mice weighing between 18.0g to 22.0g (male and female, not pregnant) were purchased from the Animal house of the Faculty of Basic Medical Sciences, College of Health Sciences, Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria. They were housed in aluminum cages under a 12-hour day/night cycle and fed with Grower pellets and clean water. They were made to acclimatize for at least 10 days before use. The acclimatized mice were divided into 10 groups of five mice each for **(E: G)**, 10 groups for **(G: A),** 10 groups of five mice each for **(E: A)** and 12 groups of five mice each **(E: G: A)** including positive and negative control and a single plant respectively, of five mice per group.

Parasite and passaging of the test animals

The rodent parasite, chloroquine-sensitive *Plasmodium berghei berghei* NK 65 was obtained from Professor O. G. Ademowo, of the Institute of Advanced Medical Research and Training (IMRAT), University College Hospital, Ibadan. Sufficient volume of blood was obtained through cardiac puncture from a euthanized donor mouse with about 30% parasitaemia. The blood was diluted with normal saline so that 0.2ml will contain $1x10^7$ infected erythrocytes.

Preparation of Test Drug and Control Drug

The test drugs were each prepared by dissolving 0.2g of the dried extract in 2.0 ml of normal saline in a test tube and shaken to form a paste; normal saline was then added to make up to 5.0 ml for all animals in a group.

The control drug was prepared by dissolving 8.33mg of chloroquine tablet powder in 5ml of normal saline for each animal in a group.

Fifty (50) acclimatized and inoculated mice were grouped into 10 groups (I – X) of five mice each. They were each administered with 400.0mg/kg/day of **E: G** in ratio 1:1, 1:2, 1:3, 2:1, 2:3, 3:1, 3:2, *Eugenia uniflora* leaf extract and 10mg/kg chloroquine and distilled water as negative control. In the same way, **G: A** was also administered to another set of 50 mice in the ratio 1:1, 1:2, 1:3, 2:1, 2:3, 3:1, 3:2, *Gongronema latifolium* stem and root extract, 10mg/kg chloroquine and distilled water as negative control. **E: A** was also combined in the same ratio of 1:1, 1:2, 1:3, 2:1, 2:3, 3:1, 3:2, and similarly tested. Also, **E:G:A** was administered to another set of 60 mice in 12 groups of five mice each in the ratio 1:1:1, 1:1:2:, 1:2:2, , 2:1:1, 2:1:2, 2:2:1, 3:1:2, 3:2:2, 3:3:2, *A. altilis* stem bark extract, 10mg/kg chloroquine and distilled water as negative control. These were administered orally two (2) hours after inoculation and for three consecutive days (D_0 - D_3). On the fifth day (D_4), blood smear was taken. The percentage parasitaemia was determined by recording the number of parasitized cells out of total red blood cells counted in 10 random fields of blood smear views under light microscope using the oil immersion objective. Average Percentage parasitaemia was calculated using the formula: % Parasitaemia= 100* Average number of parasitized red blood cells)/ Total number of parasitised and unparasitised red blood cells. Also, the average percentage chemosuppression was calculated from the Average Percentage parasitaemia using the formula: 100{(A - B) / A}, where A is the average percentage parasitaemia in negative control group and B is the percentage parasitaemia in the test group.

Laboratory determination of percentage parasitaemia in mice

2.3.1 Blood film preparation

Clean slides were arranged and masking tapes were placed at the base of the slides to identify the mice from which smear was collected and on which surface of the slide the smear was made. Drop of blood from each mouse tail were put on each slide, another clean slide held at an angle of 45° to the end of the first slide and pushed carefully along the length of the first slide to produce a thin smear of blood. The blood film was allowed to dry and then fixed by dipping in methanol. The methanol was allowed to dry.

2.3.2 Staining of the blood film

Giemsa stain solution (100ml) was prepared by diluting 10ml of Giemsa stain (3%) to 100ml with tap water, mixed thoroughly and poured in a staining tank. The fixed slides were arranged in the tank and left for 30 minutes. It was then removed, rinsed gently in tap water, air-dried, packed neatly in tissue paper and labeled appropriately.

2.3.3 Microscopical examination of stained film

The stained slides were viewed using oil immersion objective from which ten random fields were estimated for the number of parasitised and unparasitised red blood cells for each slide. The percentage parasitaemia for each mouse was calculated as a percentage ratio of the number of parasitized and total number of parasitized and un-parasitized red blood cells. The average percentage parasitaemia ± standard error of the mean (SEM) were recorded for each group of five mice treated.

2.4 Survival time, percentage survival time and percentage survivor

The survival time for each mouse was estimated by counting the number of days each mouse survived, post-treatment after observing for 28 days. The mean survival time ± SEM for each group was calculated and recorded. Percentage survival time relative to that of negative control (% of NC) was obtained by expressing the raw survival time for each group as a percentage of the value for the negative control group of mice. The percentage survivor is the percentage of the number of mice that elicited survival time that fall within the mean survival time of the group.

2.5 Statistical analysis

One-way analysis of variance with Student Newman Keuls as *post hoc* was used for comparison to determine the source of significant differences for all values. Values of $p < 0.05$ were considered to be of statistical significance. The data analysis was performed using Vinstat Instant Demo Software.

Results

The antiplasmodial activities of the extract obtained from the infusions of the different ratio combinations of *Eugenia uniflora* leaf and *Gongronema latifolium* stem and root, *Gongronema latifolium* stem and root with *Artocarpus altilis*, *Eugenia uniflora* leaf with *Artocarpus altilis*, and the various combinations of *Eugenia uniflora* leaf, *Gongronema latifolium* stem and root plus *Artocarpus altilis* stem bark were evaluated using the chemosuppressive test model performed on *Plasmodium* NK 65 parasite in Swiss albino infected-mice at a dose of 400.0 mg/kg. The percentage yield relating to the plant extract was also calculated.

The percentage parasitaemia obtained for the extracts of the individual plants, their different ratio combinations and the percentage chemo-suppression obtained from the average percentage parasitaemia will enable the determination of the ratio with highest chemosuppressive activity of all the combinations tested. Survival time and percentage survivor was calculated from the number of days the animal survived after treatment. The antipyretic activity was determined against malaria-induced fever using the daily record of temperature of mice obtained before drug administration. The results of the above determinations are presented as follows:

Table 1: Chemosuppressive antiplasmodial effects (Percentage chemosuppression) elicited by the different binary ratio combinations of *Eugenia uniflora* leaf, *Gongronema latifolium* stem root and *Artocarpus altilis* extracts

RATIOS	**E: G**	**G: A**	**E: A**
NC	0.00± 0.0[a]	0.00± 0.0[a]	0.00± 0.0[a]
1:1	65.97 ± 8.2[c]	30.53 ± 3.5 [c]	15.90 ± 3.6[b]
1:2	14.24 ± 2.8[a]	9.85 ± 7.5[a, b]	17.19 ± 2.4[b]
1:3	68.81 ± 7.0[c]	42.20 ± 1.1 [c]	16.80 ± 2.9[b]
2:1	25.87 ± 6.7[b]	20.94± 4.6 [a,b]	14.87 ± 3.4 [b]
2:3	78.82 ± 3.4[c]	31.07 ± 4.2[c]	17.70 ± 3.9 [b]
3:1	65.84 ± 8.1[c]	20.06± 4.0[a, b,c]	19.68 ± 1.8[b]
3:2	69.49 ± 7.5[c]	28.4 ± 4.0 [b,c]	21.90 ± 2.0[b]
G	51.63± 7.7[c]	50.07± 3.0 [b]	-
E	49.28±13.8 [c]	-	49.28±13.8[b]
A	-	37.87±5.5 [b]	34.09±8.3[b]
CQ	73.68 ± 7.0[c]	79.78± 1.7[d]	79.78± 1.7[c]

Keys: Data show the mean ± SEM, *n* = 5: **NC** (negative control Tween 80 in normal saline): **G**= *Gongronema latifolium*, **E**= *Eugenia uniflora;* **A** = *Artocarpus altilis*; **CQ** = Chloroquine (10 mg/kg). Only values with different superscripts of alphabets within columns are significantly different ($p < 0.05$, one-way analysis of variance followed by the Student Newman Keul's post hoc test).

Table 2: Chemosuppressive antiplasmodial effects elicited by the different trinary ratio combinations of *Eugenia uniflora* leaf, *Gongronema latifolium* stem root and *Artocarpus altilis* extracts

RATIOS	E: G: A
NC	0.00 ± 0.0[a]
1:1:1	11.98 ± 8.7[b]
1:2:1	3.88 ± 1.2[b]
1:2:2	36.64± 8.2 [b]
2:1:1	6.65 ± 1.9[b]
2:1:2	57.76 ± 3.6[c]
2:2:1	2.22 ± 0.8[b]
3:1:2	21.43± 10.5[b]
3:2:2	1.59 ± 0.9[b]
3:3:2	45.40 ± 10.1[c]
CQ	79.83±1.41[d]

Keys: Data show the mean ± SEM, *n* = 5: **NC** (negative control Tween 80 in normal saline): **G**= *Gongronema latifolium*, **E**= *Eugenia uniflora;* **A** = *Artocarpus altilis*; **CQ** = Chloroquine (10 mg/kg). Only values with different superscripts of alphabets within columns are significantly different ($p < 0.05$, one-way analysis of variance followed by the Student Newman Keul's post hoc test).

Table 3: Percentages of survival time and percentage survivors of mice in the chemosuppressive antiplasmodial studies of *Eugenia uniflora* leaf and *Gongronema latifolium* stem and root and Artocarpus altilis extracts and their different binary combinations ratio.

RATIOS	E: G	G: A	E: A
NC	100.00± 0.[a]	100.00± 0.0[a]	100.00± 0.0[a]
1:1	163.12± 34.0 [a]	190.40± 45.2 [a]	110.83± 23.1[a]
1:2	174.71± 41.0 [a]	173.35± 32.6 [a]	77.01± 11.1[a]
1:3	205.13± 54.7 [a]	254.63± 72.5 [b]	163.65± 64.8[a]
2:1	185.54± 64.5 [a]	224.05± 92.8 [a]	153.13± 54.7 [a]
2:3	126.12± 18.7 [a]	187.35± 71.2 [a]	213.46± 63.4[a]

3:1	174.49± 32.9 [a]	207.85± 50.9 [a]	139.69± 41.4[a]
3:2	218.97± 54.5 [a]	206.60± 36.3 [a]	215.63± 57.0[a]
GL	148.12±20.0 [a]	148.12±20.0 [a]	148.12±20.0 [a]
EU	220.51±55.2 [a]	220.51±55.2 [a]	220.51±55.2 [a]
AR	147.60±22.0 [a]	147.60±22.0 [a]	147.60±22.0 [a]
CQ	276.74± 71.5 [a]	276.74± 71.5 [b]	276.74± 71.5 [a]

Keys: Data show the mean ± SEM, $n = 5$: **NC** (negative control Tween 80 in normal saline): **G**= *Gongronema latifolium*, **E**= *Eugenia uniflora;* **A** = *Artocarpus altilis*; **CQ** = Chloroquine (10 mg/kg). Only values with different superscripts of alphabets within columns are significantly different ($p < 0.05$, one-way analysis of variance followed by the Student Newman Keul's post hoc test).

Table 4: Percentages of survival time and percentage survivors of mice in the chemosuppressive antiplasmodial studies of *Eugenia uniflora* leaf and *Gongronema latifolium* stem and root and Artocarpus altilis extracts and their different triple combinations ratio.

RATIO	AVERAGE SURVIVAL TIME (DAYS)	PERCENTAGE SURVIVOR (%)
0	100.00 ± 0.0[a]	20
1:1:1	204.18 ± 44.1 [a]	40
1:2:1	197.93± 47.3 [a]	40
1:2:2	152.69± 38.4 [a]	40
2:1:1	242.03± 77.8 [a]	40
2:1:2	177.32± 49.4 [a]	60
2:2:1	128.03± 26.3 [a]	60
3:1:2	201.57± 81.6 [a]	60
3:2:2	149.66± 57.0 [a]	60
3:3:2	169.58±42.7 [a]	40

E	220.51± 55.2 [a]	60
G	148.12± 20.0 [a]	60
A	147.60± 22.0 [a]	80
PC	276.74 ± 71.5 [a]	100

Keys: Data show the mean ± SEM, n = 5: **NC** (negative control Tween 80 in normal saline): **G**= *Gongronema latifolium*, **E**= *Eugenia uniflora;* **A**= *Artocarpus altilis*; **CQ** = Chloroquine (10 mg/kg). Only values with different superscripts of alphabets 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: Comparative activities of the most active combination ratios of *Eugenia uniflora* leaf and *Gongronema latifolium* stem and root and *Artocarpus altilis* in chemosuppressive antiplasmodial studies in mice.

DRUG	% CHM	ST (% of NC)	PS
NC	**0.00±0.0[a]**	**100.00±0.0 [a]**	**20**
E:G 1:1	65.97 ± 8.2[c]	163.12± 34.0 [a]	60
E:G 2:3	78.82 ± 3.4[c]	126.12± 18.7 [a]	80
E:G 1:3	68.81 ± 7.0[c]	205.13± 54.7 [a]	60
G:A 1:3	42.20 ± 1.1 [b]	254.63± 72.5 [b]	20
G:A 1:1	30.53 ± 3.5 [b]	190.40± 45.2 [a]	80
G:A 2:3	31.07 ± 4.2[b]	187.35± 71.2 [a]	80
E:A 2:3	17.70 ± 3.9 [a]	213.46± 63.4[a]	60
E:A 3:2	21.90 ± 2.0[a]	215.63± 57.0[a]	60
E:G:A 1:2:2	36.64± 8.2 [b]	152.69± 38.4 [a]	40

E:G:A 2:1:2	57.76 ± 3.6[b]	177.32± 49.4 [a]	60
E:G:A 3:3:2	45.40 ± 10.1[b]	169.58±42.7 [a]	40
CQ	79.83±1.41[c]	276.74 ± 71.5 [b]	80

Keys: Data show the mean ± SEM, n = 5: **NC** (negative control Tween 80 in normal saline): **G**= *Gongronema latifolium*, **E**= *Eugenia uniflora;* **A**= *Artocarpus altilis*; **CQ** = Chloroquine (10 mg/kg); ST: survival times expressed as percentage of values in days obtained for the negative control; **CHM:** chemosuppression; **PS**: percentage survivors. Only values with different superscripts of alphabets within columns are significantly different ($p < 0.05$, one-way analysis of variance followed by the Student Newman Keul's post hoc test).

DISCUSSION AND CONCLUSION

Since creation, medicinal plants have occurred in nature, to serve medicinal and other purposes [17, 18]. Most, if not all the drugs employed in the treatment of diseases have their origin directly or indirectly in natural products including plants [19, 20]. Malaria is a deadly disease for which breakthrough in control or treatments have not been found [21]. The drugs, quinine and artemisinin that have been used the treatment of malaria were obtained from plants [22]. One major challenge of orthodox drugs used in the treatment of malaria is resistance of the parasite to the drugs, whereas multicomponent medicinal plants used in ethnomedicine rarely give resistance to the disease but rather give complimentary therapeutic actions [23]. Therefore, the adoption of combination therapy in malaria and other disease conditions was to avoid parasite resistance to treatment [24]. Moreso, multicomponent nature of medicinal plants should offer a distinct advantage in the management of malaria. Therefore, further combination of different plants may further increase the options of a multicomponent recipe for the treatment of malaria. Also, most of the herbal recipe for malarial in ethnomedicine are a cocktail of different medicinal plants, the basis of which must be investigated. Earlier investigation of combination of medicinal plants [10,5,25] have focused on the three modes of malarial but the endemic nature of malarial in Africa may suggest concentration on recipe that that have chemosuppressive effects. Also, the recent outbursts in the investigations on the combination of medicinal plants in the treatment of malaria should guide the selection of medicinal plants and the various doses to be used in the management of malaria. This could also be extended to other diseases managed by the Traditional medical practitioners contrary to the common arbitrary combination in quantity and quality being practiced for self-medication by the populace and treatment by supposed professional herbalists [25]. Herbal drug development in Africa could be a way to prepare for the eventual co recognition or integration of herbal medicine [26] into the health care system in terms of the availability of recipes to be prescribed for the various ailments. The employment of a combination of plants in this work may therefore lead to a recipe or recipes that will avert the problem of resistance. The current work is to explore the possibility of three investigated antimalarial plants, when combined together in twos or threes to elicit better activity than the single plants. It could at the same time give information as to the compatibility of some plants being combined together in ethnomedicine. It was also thought to prepare the extracts of these combinations as infusions so that the chosen combination can be easily used as a dosage form by patrons. Therefore, extracts obtained from the infusions of a mixture of *E. uniflora* leaf + *A. altilis* stem bark, *E. uniflora* leaf +*G. latifolium* root and stem and *G. latifolium* root and stem +*A. altilis* stem bark were each prepared in different ratios of 1:1, 1:2, 1:3, 2:1, 2:3, 3:1 and 3:2 while the three combinations *E. uniflora* leaf+ *G. latifolium* root &stem +*A. altilis* stem bark were in the ratios 3:1:2 , 2:1:1, 2:1:2, 2:2:1, 3:2:2, 3:3:2, 1:1:1, 1:2:2, 1:2:1 with a view to optimizing the combination. The ratios were tested alongside the individual drugs and chloroquine and normal saline as positive and negative controls respectively using the chemosuppressive model of antiplasmodial testing in *Plasmodium berghei berghei*–infected mice [27].

After the preparation of the infusion and the eventual *in vacuo* evaporation and freeze-drying, the percentage yields were calculated in order to find the efficiency of the extraction from the combination ratios relative to the individual drugs (Table 4). The percentage parasitaemia with percentage chemosuppression, survival times and percentage survivors were used as parameters for assessment of the activities of the different combination ratios. The effect on malarial –

induced pyrexia was also evaluated after recording the rectal temperature before the daily drug administration [28].

***In vivo* antimalarial activity of individual plant extracts**

The extracts of the individual plants were tested at 400mg/kg, the dose which was obtained by a comparison of the percentage chemosuppression elicited by the same plants in earlier experiments in the same laboratory [5, 6, 29]. The suggested combinations tested were based on the median effective doses elicited by the plants in the previous experiments and so were all tested at 400mg/kg. The percentages chemosuppression obtained from the infusions of individual plants as follows: *Eugenia* 49.28±13.80, *Gongronema* 51.63±7.72, and *Artocarpus* 37.87±5.48 portrays their relative antiplasmodial activities and so justify the use of the parameter of percentage chemosuppression for evaluation of antiplasmodial activities of the combination in this work. The parameters of % chemosuppression or reduction of parasite population have been used in the evaluation of *in vivo* and *in vitro* antimalarial activities of medicinal plants and their combinations in previous experiments [1, 5, 6]. The extracts of the three plants gave comparable (P=.57) antiplasmodial activities to each other (Table 1).

In vivo* antimalarial activity of the combination *Eugenia* and *Gongronema

Among the other ratios tested in this combination, only E**: G** 1:2, gave comparable (P=.55) % chemo suppression (14%) to the negative control (Table 1). This implies that *Eugenia* and *Gongronema* should not be combined together in ethnomedicine in this ratio. This was closely followed by the ratio combination 2:1 (26%). The ratio combinations **E: G** 1:1, 1:3, 2:3, 3:1 and 3:2 with relatively high percentage chemo suppressions: 66, 68.8, 78.8, 65.8, 69.5%) respectively were significantly (P=.001) different in activity from the negative control and so possessing significant activities against the malaria parasite (Table 1). Various ratio combinations of medicinal plants have elicited various levels of activities when tested against parasites in mice for example, the ratio combinations of *Mangifera indica, Alstonia boonei*, *Morinda lucida* and *Azadirachta indica* in **MAMA** (1:1:1:1), **MAMA-1** (1:2:2:2), **MAMA-2** (2:1:2:2), **MAMA-3** (2:2:2:1), **MAMA-4** (1:1:2:2) as antimalarial combinations, elicited varying activities as indicated by their respective median effective doses [30]. These active ratio combinations were also comparable (*P =.55)* in activity to each other and the positive control with the % chemosuppression of 74 (Table 1). These combination ratios were also found to elicit relatively higher % chemo suppressions than each of the individual plant extracts in their uncombined state. Lower doses of combinations are usually required to elicit similar responses given by individual extracts of medicinal plants. For example, 170.0 mg /kg of MAMA-1 (1:2:2:2) was required to elicit a similar response of 68.2% chemosuppression given by 200.0mg/kg of *Alstonia boonei* while 400.0 mg of *M. lucida* elicited a similar suppression of 63% similar to 85.0 mg of MAMA (1:1:1:1) [31] The ratio E: G 2:3 is the optimum for this combination and could be chosen for further investigation among others that are equally active. This gives credence to the need for careful selection and precise combination of medicinal plants in the amelioration of diseases; for instance, in bio prospecting new plant drugs for antimicrobial activities, it was discovered that direct ethnopharmacological method was better than random, and indirect ethnopharmacological approach [32]. This work goes further to suggest an assessment of their efficacies when combined in different proportions after obtaining their relative activities.

In vivo* antimalarial activity of the combination *Gongronema* and *Artocarpus

In the **G: A** group, combination ratios 1:3, 1:1 and 2:3 with % chemosuppressions: 42, 30 and 31 elicited the lowest % chemo suppressions which were comparable (*P*=.07) to each other. They also gave comparable (*P*=.07) % chemo suppressions to the single drugs and the positive control. The combinations here do not possess better activities than the individual drugs. This implies that either of the components could have been used to treat malaria in mice instead of attempting to combine them. Other tested combination ratios: 1:2, 3:2, 2:1, 3:2 gave comparatively lower % chemosuppression, hence, high parasitaemia level easily comparable to the negative control. This confirms the inappropriateness of combining these two plants in the treatment of malaria. Such combination could do more harm than good. The order of activity is 1:3= 1:1 = 2:3=Eu=GL=PC>3:2= Aa >3:1>2:1=1:2> NC. Only G: A 1:3 gave survival time that was significantly different (*P<.01*) from that of the negative control and was also comparable (*P=.58*) to that of the positive control but the percentage survivor is 20%. A departure from the assertion that the % parasitaemia reduction are better correlated with % survivor rather than with survival time [34].

In vivo* antimalarial activity of the combination *Eugenia* and *Artocarpus

In the **E: A** group, all the tested combination ratios gave relatively low **percentage** chemosuppression barely above 20%. The individual drugs gave 49 and 38% respectively. The the ratios 2:3 and 3:2 which gave relatively better % chemo suppressions of 17.7 and 22.2 respectively were comparable (*P=.66*). Both possess comparable (*P=.35*) activities to the individual drugs tested at 400mg/kg. Other ratios also elicited comparable (*P=.35*) activities to the negative control and so were inactive. Either plants must have been inhibiting each other at the ratios employed in the combination. This was not the case when *Artocarpus* was replaced with *Gongronema* (**E: G 2:3 or 3:2**). An arbitrary combination of these two plants for the management of malaria which though may be suggestive in ethnomedicine could not have given a good result.

***In vivo* antimalarial activity of the combination *Eugenia, Gongronema* and Artocarpus**

More than two plants may be combined in ethnomedicine for malaria [10,22], but not all the combinations may be effective. Of all the combination ratios of **E: G: A** tested, three ratios, 1:2:2, 2:1:2 and 3:3:2 gave % chemo suppressions that were significantly (*P* =.00) higher than the negative control. These were also comparable in value to those of the individual drugs at 400mg/kg tested (*P*=.20) and the positive control (*P*=.20). Other ratios which elicited comparable activities were relatively inactive. Also, three ratios, E:G:A 3:1:2, 2:1:1, 1:1:1 elicited survival times doubled (204, 242 and 202 %) that of the negative control without commensurately high antiplasmodial activities (Table 5). Doubled survival times is a criteria for curative activity in antimalarial testing [33] Mukherjee, 2019). It has also been suggested that a similar activity found in prophylactic and chemosuppressive tests could be used as a criteria for activity [25]. Also, of all the individual drugs tested in this work, only *E. uniflora* elicited doubled survival time but not with the highest chemosuppression (Tables 2 and 5). Of the antiplasmodial-active ratios, only 2: 1: 2 with survival time 177% gave percentage survivor of 60% while of the survival-time active only ratios, 3:1:2 with 202 % survival time also gave a percentage survivor of 60%. Also, of the individual drugs only *A. altilis* gave a percentage survivor of 80% without a correspondingly high survival time. Some previous studies have correlated % parasitaemia reduction with % survivors rather than with survival times [34,35].

Comparative antiplasmodial activities

In order to obtain the most active combination of all the tested ratios, the percentage chemosuppression, percentage survival times and percentage survivors of the most active from each group were compared (Table 5). Thus **E: G** 1:1, 1:3, 2:3, **G: A** 1:3, 1:1, 2:3, **E: A** 2:3, 3:2 and **E: G: A** 1:2:2, 2:1:2, 3:3:2 were statistically compared. Though all exhibited chemosuppression levels higher than that of the negative control, **E: G 2:3, E: G 2:1, E: G 1:1** elicited comparable *(P=.55)* activities to chloroquine (10mg/kg), the positive control drug. Whereas **E:G 1:3, G:A 1:3, E: A 2:3, E:A 3:2** and CQ elicited double survival times, only CQ of the group gave the highest PS of 80. Others (**E: G 2:3, G: A 2:3, G: A 1:1** and **G: A 2:3**) however gave PS of 80% similar to CQ without corresponding doubled survival times, thus confirming the non-correlation of survival time and PS with antiplasmodial chemosuppressive activities [34,35].

Ratio **E: G 2:3** with PS of 80% and relatively high % chemosuppression similar to chloroquine may be selected as the most active while **E: G 1:3** with doubled survival times can be next in activity. In general, the individual drugs displayed better activities than the 2- or the 3- combination drugs tested. Most of the active ratios were comparable in activity to the positive control. The most active combination was **E: G 2:3** while the others were comparable in activities (*P=.55*) to each other.

Conclusion

In conclusion, while it seems good to combine plants in the treatment of malaria in ethnomedicine, consideration should be given to their individual relative activities and in combination including their compatibility. This study vividly confirms *Eugenia* and *Gongronema* as the most active individual drugs tested and their ratio 2:3, the most active combination ratio. The inclusion of *Artocarpus* as a third plant in the combination nor its inclusion with either of the other two did not enhance activities.

Ethical Approval

Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee

REFERENCES.

1. Agbedahunsi JM, and Aladesanmi AJ. Effect of *Eugenia uniflora* on early malaria infection, *Fitoterapia.* 1993; 62(2):174-175.
2. Boonphong S, Baramee A, Kittakoop P, Puangsombat P,. Antitubercular and antiplasmodial prenylated flavones from the roots of *Artocarpus altilis*. *Chiang Mai Journal of Science*. 2007; 34: 339–344.
3. Etim EN, Useh MF, Okokon JE. Pharmacological Screening and Evaluation of Antiplasmodial Activity of *Gongronema latifolium* (utazi) Against *Plasmodium berghei berghei* infection in Mice *Nigerian Journal of Health and Biomedical Sciences* 2008; 7 (2): 51-55.
4. Akuodor CG, Idris-Usman M, Ugwu TC, Akpan JL, Ghasi SI, Osunkwo UA. In vivo Schizonticidal Activity of Ethanolic Leaf Extract of *Gongronema latifolium* on *Plasmodium berghei berghei* in Mice *Ibnosina Journal of Medicine and Biomedical Sciences* 2010; 2(3): 118-124 10.4103/1947-489X.210981.
5. Adebajo AC, Odediran SA, Nneji CM, Iwalewa EO, Rukunga GM, Aladesanmi AJ, Gathirwa JW, Ademowo OG, Olugbade TA, Schmidt TJ, Verspohl EJ. Evaluation of ethnomedical claims II: Antimalarial activities of *Gongronema latifolium* root and stem. *Journal of Herbs, Spices & Medicinal Plants.* 2013;19 (2): 97 – 118.
6. Adebajo CA, Odediran SA, Aliyu FA, Nwafor PA, Nwoko NT and Umana US. *In vivo* Antiplasmodial Potentials of the Combinations of Four Nigerian Antimalarial Plants. *Molecules.* 2014; 13136 -13146.
7. Hafid AF, Septiani RP, Fabriana LH, Febrianty N, Ranggaditya D, Awaruyanti W. Antimalarial Activity of Crude Extracts of *Artocarpus heterophyllus, Artocarpus altilis*,

And *Artocarpus camansi Asian Journal of Pharmaceutical and Clinical Research* 2016; 9 (1) : 279-81

8. Al-Hindi B, Yusoff NA, Ahmad M, Atangwho IJ, Asmawi MZ, Al-Mansoub MA, Tabana- Bello I, Yam MF. Safety assessment of the ethanolic extract of *Gongronema latifolium* Benth. leaves: a 90-day oral toxicity study in Sprague Dawley rats. *BMC Complement Altern Med* 2019; 19:152.
9. Chintamunnee V, Mahomoodally MF. Herbal medicine commonly used against infectious diseases in the tropical island of Mauritius. *Journal of Herbal Medicine*, 2012; 2: 113–125
10. Odugbemi TO, Odunayo RA, Ibukun EA, Peter OF. Medicinal Plants Useful for Malaria Therapy in Okeigbo, Ondo State, Southwest Nigeria. *Africa Journal of Traditional, Complementary and Alternative Medicine*, 2007; 4(2): 191-198.
11. Oreagba IA, Oshikoya KA, Amachre, M. Herbal Medicine use among urban residents in Lagos, Nigeria. BMC Complementary and Alternative Medicine. 2011; 11: 117.
12. Djordjevic SM, From Medicinal plant raw materials to herbal remedies. Aromatic and medicinal plants : Back to nature Chapter 16 p 269-288 Doi: 10:5772
13. Rachuonyo HO, Ogola PE, Arika WM, Wambani JR, Gatheri GW, Nyamache AK. Combined Effect of Crude Leaf Extracts of Selected Medicinal Plants against Selected Enteric Bacterial Pathogens and Candida albicans. J Antimicro 2016; 2: 110. doi: 10.4172/2472-1212.1000110

14. Mustafa G, Arif R, Atta A, Sharif D, Jamil A. Bioactive Compounds from Medicinal Plants and Their Importance in Drug Discovery in Pakistan Matrix Science Pharma 2017; 1(1) : 17-26 DO - 10.26480/msp.01.2017.
15. Enemor VH, A, Nnaemeka JO, Okonkwo CJ. Minerals, vitamins and phytochemical profile of Gongronema latifolium: Indices for assessment of its free radical scavenging, nutritional and antinutritional qualities. Intern. Res. J. Biol. Sci., 2014; 3(1): 17-21.
16. Tarkang PA, Appiah-Opong R, Ofori MF, Ayong LS, Nyako AK. Application of multi-target phytotherapeutic concept in malaria drug discovery: a systems biology approach in biomarker identification. *Biomark Res* 2016; **4,** 25. https://doi.org/10.1186/s40364-016-0077-0.
17. Boadu AA, Asase A, Documentation of herbal medicines used for the treatment and management of human diseases in Southern Ghana Evidence-based Complementary and Alternative Medicine 2017; Article Volume 2017 ID 3043061 12 pages https://doi.org/10.1155/2017/3043061.
18. Mintah SO, Asafo-Agyei, MA, Junior PA, Boamah D, Kumadoh D, Appiah, A, Ocloo A, Boakye YD, Agyare, C. Medicinal Plants for the treatment of prevalent Diseases 2019; doi:10.5772/intecopen.82049.
19. Sardana S. Herbal drug development from natural sources J Adv Pharm Technol Res. 2012; 3(2); Apr-Jun 2012.
20. Saleh H, Azizollah J, Ahmadreza, H Raham, A, The application of medicinal plants in traditional and modern medicine: a review of *Thymus vulgaris. International Journal of Clinical Medicine.* 2015; 6: 635-642.

21. Autino B, Noris, A, Russo R, Castelli F. Epidemiology of Malaria in Endemic Areas. *Mediterranean Journal of Hematology and Infectious Diseases* 2012; 4(1): 6A 0

22. Samuelsson, G. (2004). Drugs of Natural Origin. Apotekarsocieteten. P 24

23. Karole S, Shirvastava S, Thomas S, Soni B, Khan S, Dubey J, Dubey SP, Khan N, Jain DK. Polyherbal Formulation Concept for synergistic action: a review 2019; 9:1-S.

24. Hill JA, Cowen LE. Using combination therapy to thwart drug resistance Future Microbiology. 2015; 10 (11).

25. Odediran SA, Awosode KE, Adegoke TA, Odebunmi KA, Oladunjoye BB, Obasanya AA. et al., Combinations of *Chrysophyllum albidum* and *Citrus aurantifolia* as Antimalarial Agents and their Effects on Orthodox Antimalarial Drugs in Mice. Annals of Complementary Alternative Medicine. 2020; 2(1): 1007.

26. Sofowora A. Medicinal Plants and Traditional Medicine in Africa, Spectrum book Limited, Ibadan. pp 8-9; 2012.

27. Ajayi CO, Elujoba, AA, Adepiti AO. Antiplasmodial Properties *of Alstonia bonnie* stem-bark and *Picralima nitida* seed in different combination. *Nigeria Journal of Natural Products and Medicine.* 2015; 19: 71-76.

28. Oloyede AM, Okpuzor J, Aina OO. Anti-pyretic and Antiplasmodial Activity of a Polyherbal Formula (Joloo). *Journal of Biological Sciences*2013;13: 717-721

29. Aliyu FA. *Murraya koenigii* and *Artocarpus altilis* in malaria and dengue fever control. M. Sc. Dissertation. OAU Ile Ife 2013; 185 pp.

30. Odediran SA, Elujoba AA, Adebajo CA. Influence of formulation ratio of the plant components on the antimalarial properties of MAMA decoction. Parasitology Research 2014; 113: 1977-1984.

31. Odediran SA, Optimisation and phytochemical Bioassay- monitored Investigation of MAMA Decoction PhD. Pharmacognosy Thesis, Obafemi Awolowo University, Ile- Ife. 210; 2016
32. Silva ACO, Santana EF, Saraiva AM, Coutinho FN, Castro RHA, Pisciottano MNC, Amorim ELC, Albuquerque UP. Which Approach is more effective in the Selection of plants with Antomicrobial Activity? Evidence-Based Complementary and Alternative Medicine, 2013; 2013, (Article ID 308980), 9 pages https://doi.org/10.1155/2013/308980.
33. Mukherjee PK, Quality control of herbal drugs: An Approach to Evaluation of Botanicals. First Edition, Elsevier. 784; 2019.
34. Adepiti AO, Iwalewa EO. Evaluation of the combination of *Uvaria chamae* (P. Beauv) and amodiaquine in murine malaria. Journal of Ethnopharmacology. 2016; 193:30 – 35.
35. Adesida AS, Odediran SA, Elujoba AA**.** Investigation on the Antimalarial Properties of *Plumeria Alba* Linn (Apocynaceae) Cultivated in Nigeria Nigerian Journal of Natural Products and Medicine. 2021; 25: 34-42.