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Comparative *in vivo* antiplasmodial effects of cold methanol and hot aqueous infusion extracts of different combinations of *Parquetina nigrescens* and *Spathodea campanulata* leaves in mice

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Abstract

Solvents and dosage forms can influence therapeutic efficacy of herbal remedies. The antiplasmodial activities of cold methanol (ME) and hot aqueous infusion extracts (HI) from similar ratio combinations of Spathodea campanulata (S) and Parquetina nigrescens (P) were compared in mice. Powdered leaves of S and P were separately macerated in methanol. Also, same powdered leaves were combined in the ratios, S: P (0:1, 1:0, 1:1, 2:1, 1:2, 1:3 and 3:1, infused in boiled water. ME, combined in similar selected ratios above and HI were subjected to a four-day chemosuppressive antiplasmodial test against chloroquine-sensitive Plasmodium berghei berghei in mice. The S and P of both ME and HI were also combined with chloroquine and similarly tested. Normal saline and chloroquine (10 mg/kg) were negative and positive controls, respectively. Values of percentage parasitemia, percentage chemosuppression, survival times, and mice survivors' percentages obtained were statistically analysed. All extracts of HI gave higher survival times, while all extracts combined with CQ produced better survivor percentages. The S: P (3:1) of ME and S: P (2:1) of HI elicited the highest parasite chemosuppression. Individuals of all extracts, combined with CQ except S of ME gave lower chemosuppression than CQ. Upon GC-MS analysis, most active S: P (3:1) of ME contains Benzoic acid methyl ester, while P and S: P (2:1) of HI had Hexanoic acid methyl ester and methyl stearate, as the most prominent antimalarial components. Scientific evidence should guide the selection of plant combinations, solvent, and extraction methods for antimalarial recipes in ethnomedicine.

Keywords: *Cold methanol, hot aqueous infusion, Parquetina nigrescens, Spathodea campanulata, Antiplasmodial.*

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1. Introduction

The combination of medicinal plants, solvent extraction methods, and dosage forms used in ethnomedicine is a topic of significant interest [1,2]. Solvent selection dictates the extent of extraction, while dosage form influences the bioavailability of active ingredients [3]. The impact of dosage form and solvent on therapeutic efficacy is crucial in both orthodox and traditional medicine [3]. For medicinal plant preparations, oral dosage forms include decoctions, infusions, macerations (tisanes), tinctures, and powders, with solvents like water, alcohol, and edible oils playing a vital role [1]. Infusions are suitable for soft plant materials like leaves and flowers, extracting essential oils, vitamins, and enzymes, whereas decoctions, which involve boiling, are more effective for harder plant parts like roots and seeds [4]. Decoctions tend to be preferred when biological activity is prioritized over flavor or aroma [4]. Water is the most commonly used solvent in ethnomedicine, but alcohols and edible oils can also be beneficial in some cases [5].

The necessity of combining orthodox drugs in malaria treatment is well established, as is the combination of medicinal plants in traditional medicine (TM). Artemisinin combination therapy (ACT) was introduced to combat parasite resistance in orthodox medicine [6], while plant combinations have been utilized in traditional medicine for centuries. Several studies have confirmed the synergistic effects of plant combinations in malaria treatment [7,8]. In Kenya, [9,10] demonstrated synergy between multiple plants in both *in vivo* and *in vitro* studies. Artemisinin from *Artemisia annua* prevents malaria recurrence and prolongs survival in mice [11]. Similarly, curcumin combined with leaf extracts of *Andrographis paniculata* and *Hedyotis corymbosa* exhibited significant synergistic effects in both *in vitro* and *in vivo* studies [12].

In Mali, *Nauclea latifolia*, *Guiera senegalensis*, *Mitragyna inermis*, and *Feretia apodanthera* combinations have been reported for malaria treatment [13]. In Nigeria, multiple plant combinations are used against malaria, including *Chrysophyllum albidum* (leaf and bark) and *Citrus aurantium* (leaf and fruit) [14]. These combinations have demonstrated significantly higher curative, prophylactic, and chemosuppressive antimalarial activities compared to single extracts [15]. Other reported combinations include *Alstonia boonei* bark with *Capsicum fructescens* fruit, and *Gossypium barbadense* leaf with *C. aurantium* fruit [14]. A critical question remains: which dosage form whether decoction, infusion, or maceration would be appropriate for preparing ethnomedicinal antimalarial remedies with optimal activity. Also, important is, what factors and which solvents would determine the choice of suitable dosage forms in Traditional medicine. This will usually that would depend on scientific evaluations that can compare medicines prepared using different dosage forms and various solvents. *Spathodea campanulata* (leaf and stem bark) has been used in Ghana for malaria treatment [16], while *Parquetina nigrescens* (aqueous leaf extract) and *Spathodea campanulata* leaf have shown antimalarial activity [16-20]. The antiplasmodial activities of the individual leaf extracts of *S. campanulata* and *P. nigrescens* have been compared in a separate study [21]. In this study, however, their different ratio combinations prepared as hot aqueous infusions and maceration in methanol are being compared in order to obtain insights into their relative therapeutic potential, as antimalarial agents.

2. Methods and Preparation

2.1 Plant collection and authentication

The leaves of *Spathodea campanulata* and *Parquetina nigrescence* were collected from the premises of Oduduwa Hall, near Hezekiah Oluwasanmi Library and Faculty of Health Sciences, Obafemi Awolowo University, Ile-Ife respectively. The leaves of both plants were identified and authenticated at the Faculty of Pharmacy Herbarium, by Mr. I. I. Ogunlowo of the Pharmacognosy Department, Obafemi Awolowo University, Ile-Ife. Voucher specimens were deposited at the herbarium with the numbers FPI2318 and FPI2317 allocated for *Spathodea campanulata* and *Parquetina nigrescence* respectively.

2.2 Plant material and preparation of plant extracts

2.2.1Maceration (ME)

The leaves were separately air dried inside the screen house of the Faculty of Pharmacy, Obafemi Awolowo University, Ile-Ife and powdered. Powdered leaves of *Spathodea campanulata* and *Parquetina nigrescence*, (600 g each) were macerated separately in 2 L methanol for 72 hours inside a macerating tank with intermittent shaking. The resultant extracts were filtered and concentrated *in vacuo* using the rotary evaporator. The percentage yields were then calculated using the formula: $(100\times W_1)/W_2$; where W_1 is the weight of dried extract; W_2 is the weight of plant material. The dried extracts were kept inside a desiccator.

2.2.2Hot Infusion (HI)

The mixtures of the powdered leaves of *Parquetina nigrescens* and *Spathodea campanulata* for the different selected ratios of the infusion were constituted as follows: (1:1): 20.0 g of P + 20.0 g of S; (1:2): 13.3 g of P + 26.7 g of S; (2:1): 26.7 g of P + 13.3 g of S; (3:1): 30.0 g of P + 10.0 g of S; (1:3): 10.0 g of P + 30.0 g of S respectively. Also, (P) 40.0 g of P, (S) 40.0g of S. Boiling water (500ml) was added to each of the separately constituted mixtures, stirred and covered and allowed to stand for 30mins after which the resultant extracts were filtered, concentrated and freeze dried. The percentage yields were as follows: 1:1 (13.0), 1:2 (18.0), 2:1 (19.6), 3:1 (14.5), 1:3 (19.1), P (25.0), S (23.0).

2.2.3Preparation of test extracts (ME) and standard drugs

The methanol extracts of each plant were combined in different ratios according to their median effective doses [21], and prepared as follows: (1:1) 125.47mg of S +122.28mg of P; (1:2), 125.47mg of S +244. 57mg of P; (2:1), 250. 54mg of S +122.28mg of P; (3:1) 376.01mg of S +122.28mg of P; (1:3) 125.47mg of S +376.01mg of PN in 5.0 ml of normal saline respectively. Also, (P), 122.28mg of P, (S), 125.47mg of SC; (P +CQ) 122.28mg of P +10.0 mg/kg of CQ; (S +CQ) 125.47 mg of S +10.0 mg/kg of CQ. Normal saline was administered to the negative control group while chloroquine (10.0 mg/kg) was administered to the positive control. These stock combinations were administered to each group of five (5) mice according to their body weights.

2.2.4Preparation of the infusion extracts (HI) and the standard drugs

For each of the ratios 1:1, 1:2, 2:1, 3:1, 1:3, of the infusion extract, a 400 mg/kg of each combination was prepared and administered to each experimental mouse during the experiment. Each of the individual infusion extracts was also combined with 10 mg/kg of chloroquine, the positive control drug. Normal saline and

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chloroquine (10 mg/kg) were administered to the negative and positive control groups respectively.

2.3 Animals

A total of one hundred and ten (110), healthy, seven weeks old Swiss mice of either sex with weight ranging between 18 and 22 g were obtained from the Animal House of the Department of Pharmacology, Faculty of Pharmacy, Obafemi Awolowo University, Ile-Ife. They were randomly divided into groups of five animals each, then housed in aluminum cages with wood shavings as beddings and allowed free access to food and water. They were allowed to acclimatize for a period of five before the experiment commenced.

2.4 Rodent parasite

Chloroquine sensitive *Plasmodium berghei berghei* NK65 infected mouse were ~~was~~ obtained from the Institute of Advanced Medical Research and Training (IMRAT), University College Hospital, Ibadan. They were maintained by serial passaging in mice with close monitoring of the parasitaemia level. The parasitized animal (30% parasitaemia) was anaesthetized with dichloromethane. Blood was withdrawn by cardiac puncture into heparinized bottle and diluted with normal saline to contain 1×10^7 parasitized red blood cells in 0.2 mL of inoculum.

2.5 Preparation of blood smears

The tip of the tail of each mouse was gently excised to get drops of blood. The blood was collected on the surface of a clean glass slide which had been carefully labeled on the other side with a paper tape. The blood was carefully spread across the slide using another slide placed on the spot at an angle of $45^0 45^0$. The blood was allowed to dry and fixed with a few drops of methanol and air-dried.

2.5.1Staining of blood smear

The air-dried blood films were stained using a 4% Giemsa stain diluted with water at a ratio 1:9. The slides were allowed to stand for about 30 minutes, rinsed with water and allowed to dry.

2.6 Assessment of parasitaemia level and percentage chemosuppression

Each of the glass slides containing the stained blood film was mounted on the microscope and examined using immersion oil (×100) objective to observe the distribution of red blood cells. Ten fields of view with uniform distribution of red blood cells were counted for each slide. Each view was observed for the number of parasitized red blood cells (PRBC) as well as the unparasitized red blood cells (UPRBC). The percentage parasitaemia for each field of view was calculated using the formula: 100 (PRBC)/ (PRBC+UPRBC) where, PRBC=Total number of parasitized red blood cells, UPRBC=Total number of unparasitized red blood cells.

The percentage chemosuppression was also calculated using the formula:

100(PNC – PTD)/PNC where PNC= Average percentage Parasitaemia in the negative control; PTD= average percentage parasitaemia in the test dose. The values were recorded as % parasitaemia ± SEM and % chemo suppression ±SEM [22].

2.7 Determination of survival time and percentage survivor

The mice in each of the above experiments were observed for 28 days post drug administration to determine their survival time and percentage survivor. The survival times of each of the groups were determined and recorded in days ±SEM while percentage survivor was also recorded [23].

2.8 Chemosuppressive antimalarial test model for the methanol extract (ME) combinations

The chemosuppressive antiplasmodial test was carried out on the methanol extract (ME) at a dose of 400mg/kg as follows: The extract (as combined for each of the combinations: S:P 1:1, 2:1, 1:2, 1:3, 3:1, the individual drugs (S and P), P+CQ, S+CQ, CQ obtained above were each administered to a group of five acclimatized mice each on the first day (Day 0) of the experiment, two hours after the inoculation with parasite and subsequently daily for three days (days 1-3), the temperature being taken daily before the administration of the extract. The parasitaemia was assessed on the fifth day (day 4) after a blood smear has been made from the tail vein blood of each of the mice as in section 2.6 above and viewed under the microscope thereafter. Percentage chemosuppression was assessed from the percentage parasitaemia in order to determine the activities of each combination.

2.9 Chemosuppressive antimalarial test model for the infusion extract (HI) combination

The chemosuppressive antiplasmodial test was carried out on the infusion extract as in section 2.9 above. The extract (400 mg/kg) of each combination, 1:1, 2:1, 1:2, 1:3, 3:1, the individual drugs (SC and PN), P+CQ, S+CQ, CQ obtained above was administered to a group of five acclimatized mice on the first day (Day 0) of the experiment each, two hours after the inoculation with parasite and subsequently daily for three days (days 1-3), the temperature being taken daily before the administration of the extract. The parasitaemia was assessed on the fifth day (day 4) after a blood smear has been made from the tail vein blood of each of the mice under test as in section 2.6 above and viewed under the microscope thereafter. Percentage chemosuppression was assessed from the percentage parasitaemia in order to determine the activities of each combination.

2.10 Statistical analysis

Values were expressed as mean ±SEM and analyzed statistically using One-way Analysis of Variance (ANOVA) followed by Student Newman Keul's *post-hoc* test for comparisons to determine the source of significant difference for all values. Values of $p < 0.05$ were considered to be of statistical significance.

2.11 Gas-Liquid Chromatographic analysis of the individual extracts and the most active combinations

The 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. The GC-MS operating by MSD in Scan mode, to ensure all levels of detection of the target constituents, was used to determine the levels of phytochemicals in the sample. The already established criteria were used to check the abundance of m/z 69, 219, 502 and other instrument's optimal & sensitivity conditions before analysis after the MS has been auto-tuned to perfluorotributylamine (PFTBA). 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%

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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), interface temperature 300°C, initial nominal pressure 1.4902 psi, out time 1.8 mins; 1µL of the samples were injected in spitless 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 2.0 s/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 compounds 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.

3. Results

3.1 Combination Experiment

3.1.1 Percentage parasitaemia and percentage chemosuppression elicited by the extracts of *S. campanulata* and *P. nigrescens*, in combinations ratios and with chloroquine

Chloroquine elicited the highest percentage chemosuppression of 74%. Hence it is the most active of all the test agents in this work. Also, neither of the combinations nor the single extracts were as active as chloroquine. All the ratios of combination tested were comparable in activity to the individual extracts and were significantly different ($p < 0.05$) in activity from the negative control. Also, both individual extracts inhibited the activity of chloroquine.

Table 1. Percentage parasitaemia and percentage chemosuppression elicited by the methanol and infusion extracts of *S. campanulata* and *P. nigrescens* and their combinations

Ratios	Methanol extract		Infusion extract	
	% Parasitaemia	% Chemosuppression	% Parasitaemia	% Chemosuppression
NC	7.34±0.21^{c}	0.00±00^{a}	5.29±0.35^{c}	0.00 ± 0.00^{a}
SP 1:1	2.07±0.08^{a,b}	71.80±1.88^{b,c}	3.08±0.1^{b}	39. 37±6.2^{b}
SP 1:2	2.58±0.12^{b}	64.85±1.33^{b}	3.52±0.16^{b}	31.61±5.95^{b}
SP 2:1	2.44±0.19^{b}	66.75±1.93^{b}	2.66±0.27^{b}	47.87±5.4^{b}
SP 1:3	2.48±0.25^{b}	66.21±4.40^{b}	3.34±0.11^{b}	34.83±5.66^{b}
SP 3:1	1.99±0.06$^{a, b}$	72.89±1.36$^{b, c}$	3.25±0.16^{b}	35.77±7.76^{b}
S + CQ	1.40±0.01^{a}	80.92±0.48^{d}	3.62±0.14^{b}	28.52±7.81^{b}
P + CQ	2.30±0.08^{b}	68.66±1.66^{b}	3.30±0.05^{b}	35.29±5.75^{b}
S	1.98±0.10$^{a, b}$	73.02±0.23^{c}	2.82±0.23^{b}	46.02±3.77^{b}
P	1.95±0.11$^{a, b}$	73.43±0.21^{c}	2.96±0.21^{b}	42.89±4.51^{b}
CQ	1.89±0.04$^{a, b}$	74.25±1.18^{c}	1. 34±0.1^{a}	73.22±4.01^{c}

Table Abbreviations: NC; Negative control, CQ; Chloroquine (10 mg/kg), S; *Spathodea campanulata*, P; *Parquetina nigrescens*, Combination ratio in the order of S and P (S: P). Superscripts a, b or c are statistical notations to depict significantly different activities in the values).

3.1.2 Table 2. Average Survival time and Percentage survivor elicited by the methanol and infusion extracts of *S. campanulata* and *P. nigrescens* in combinations studies

Ratios	Methanol extract		Infusion extract	
	Average Survival time(days)	Percentage survivor (%)	Average Survival time(days)	Percentage survivor (%)
NC	12.0±0.00^{a}	40	9.4±1.57^{a}	40
SP 1:1	10.3±1.9^{a}	60	10.2±2.4^{a}	40
SP 1:2	11.0±1.0^{a}	80	9.2±2.18^{a}	60
SP 2:1	11.0±0.0^{a}	75	9.8±2.33^{a}	40
SP 1:3	8.0±3.0^{a}	40	15.2±3.57^{a}	60
SP 3:1	10.3±0.8^{a}	60	14.2±2.4^{a}	80

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S + CQ	19.2±4.6 [b]	80	19.2±4.6 [b]	60
P+ CQ	28.0±0.0 [b]	100	21.0±4.41 [b]	60
S	17.6±5.05 [a]	40	16.4±3.72 [b]	60
P	17.4±1.50 [a, b]	60	23.0±2.49 [b]	80
CQ	28.0±0.00 [b]	100	28.0±0.00 [b]	100

Table Abbreviations: NC; Negative control, CQ; Chloroquine (10 mg/kg), S; *Spathodea campanulata,* P; *Parquetina nigrescens,* Combination ratio in the order of S and P (S: P). Superscripts a, b or c are statistical notations to depict significantly different activities in the values).

3.2 Table 3. Some compounds identified in the methanol (ME) and hot infusion extracts (HI) of *S. campanulata* and *P. nigrescens* and their combinations using GC-MS.

S/N	Compounds Identified/ Mol Formula and Weight	EXTRACTS				
		SME	**PME**	**SHI**	**PHI**	**SPI**
1	Undecane $CH_3(CH_2)_9CH_3$, 156.31	Rt: 4.701 PA: 5.09. QL: 90	Rt: 4.701 PA:7.17 QL: 74	Rt: 4.701 PA:8.79 QL: 83	Rt: 4.706 PA:2.04 QL: 87	-
2	Benzoic acid, methyl ester, $C_6H_5COOCH_3$, 136.15	Rt: 4.804 PA: 49.78 QL: 94	Rt: 4.810 PA:57.67 QL: 81	Rt: 4.810 PA:71.92 QL: 76	Rt: 4.804 PA:15.46 QL: 76	-
3	Pentadecanoic acid, 14-methyl $C_{17}H_{34}O_2$, 270.45	Rt: 13.576 PA: 13.61 QL: 96	-	-	-	-
4.	Methyl stearate $C_{19}H_{38}O_2$, 298.5	-	-	-	Rt: 15.149 PA:23.16 QL: 97	Rt: 15.126 PA:9.0 QL: 99
5.	1,3-Thiazolidin-2-one C_3H_5NOS, 103.14	Rt: 3.825 PA: 2.77 QL: 42	-	-	-	-
6	Phenol, 2,5-bis (1,1-dimethylethy $C_{14}H_{22}O$, 206.32	Rt: 9.69 PA: 1.16 QL: 83	-	-	-	-
7	Hexadecanoic acid, methyl ester $C_{17}H_{34}O$, 270.4507	-	-	-	Rt: 13.513 PA: 52.37 QL: 98	Rt: 13.53 PA: 24.03 QL: 97
8	Naphthalene1, 4-dihydro-1-oxo-2,4- $C_{10}H_8O_2$; 160.1693	-	Rt: 6.154 PA: 1.17 QL: 31	-	-	-
9.	3-cyclohexane-1-nitrile-6-methyl $C_8H_{11}N$, 121.18	-	Rt: 3.831 PA: 2.18 QL: 49	-	-	-
10.	1,3, pentadiene, (E)— C_5H_8; 68.1170	-	Rt: 3.986 PA: 4.25 QL: 27	-	-	-
11	Anthranilic acid, N-methyl-, butyl	-			-	-

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	$C_{12}H_{17}NO_2$; *207.2689*			Rt: 6.177 PA: 1.39 QL: 35		
12.	[1,3] Dioxolo[4,5-g] quinolin-6(5H) -one, 7,8-dihydro $C_{10}H_9NO_3$; 191.182	-		Rt: 9.696 PA: 1.19 QL: 53	-	-
13	1H-isoindol-1- One,2,3- dihydro-3- hydroxy-2-methyl one, $C_9H_9NO_2$; 163.176	-	Rt:8.123 PA: 1.25 QL: 38		-	-
14	4- hydroxy-2-methylbenzoic acid, $C_8H_8O_3$; 152.15	-	Rt: 9.834 PA: 1.10 QL: 16	-	-	-
15	4- fluoro-1methy-1H-indazol-3-ylamine $C_8H_8FN_3$; *165.17*	-	Rt: 11.361 PA: 1.14 QL: 38	-	-	-
16	3-methylene-7,11-dimethyl-1-dodecene $C_{15}H_{28}$; *206.37*	-	Rt: 12.752 PA: 4.83 QL: 50	-	-	-
17	Undecanal,2-methyl- $C_{12}H_{24}O$; 184.32	-	Rt: 12.85 PA: 4.13 QL: 38	-	-	-
18	Undec-10-ynoic acid, tetradecyl ester $C_{25}H_{46}O$; *378.6315*	-	Rt: 12.963 PA: 1.05 QL: 20	-	-	-
19	1H-exadecyne $C_{16}H_{30}$; 222.41	-	Rt: 12.986 PA: 1.04 QL: 27	-	-	-
20	2,5,5,6,8a-Pentamethyl- trans4a,5,6,7,8,8a-hexahydro-gamma-chromene $C_{14}H_{26}O$; 210.348	-	Rt: 15.344 PA: 1.72 QL: 35	-	-	-
21	2-(n-Propyl) oxybenzylidene acetophenone $C_{20}H_{22}O_2$; *294.4*	-	-	Rt: 15.355 PA: 1.45 QL: 47	-	-
22	1-[alpha-(1-Adamantyl) benzylidene] Thiosemicarbazide $C_{18}H_{23}N_3S$; 313.5	-	-	Rt: 3.837 PA: 2.03 QL: 43	-	-
23	Cyclohexane-1,3-dione, 2-allylaminomethylene-5,5-dimethyl	-	-		-	-

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	$C_{12}H_{17}NO_2$; *207.273*			Rt: 3.974 PA: 1.87 QL: 46		
24	2-Methyl-4-ethoxycarbonyl-3H-imidazo[1,5-b] pyridazine-5,7-(6H)-dion $C_{13}H_{16}N_4O_4$; 292.298	-	-	Rt: 9.834 PA: 1.34 QL: 38	-	-
25	Heptadecanoic acid, 16-methyl-ester $C_{19}H_{38}O_2$; 298.5038	-	-	-	Rt: 15.327 PA: 2.11 QL: 70	
26	9,12-Octadecadienoic acid (Z, Z)-, methyl ester $C_{19}H_{34}O_2$; 294.4721	-	-	-	-	Rt: 15.258 PA: 1.18 QL: 98
27	6-Octadecenoic acid, methyl ester, $C_{19}H_{36}O$; 296.4879	-	-	-	-	Rt: 14.966 PA: 19.21 QL: 99
28	9,12-Octadecadienoic acid methyl ester $C_{19}H_{34}O_2$, 294.4721	-	-	-	-	Rt: 13.53 PA: 35.57 QL: 99
29.	Limonene $C_{10}H_{16}$, 136.23	-	Rt: 3.957 PA: 3.24 QL: 46	-	-	-
		70	88	89	94	88

Table Abbreviations: PME: Methanol extract of *Parquetina nigrescens;* SME: Methanol extract of *Spathodea campanulata*, SHI: Hot Infusion extract of *Spathodea campanulata*, PHI: Hot Infusion extract of *Parquetina nigrescens* SPI: most active infusion extract of combination of *Spathodea campanulata and Parquetina nigrescens*, Rt: Retention time; PA: Peak Area; QL: Quality.

4. Discussion

Neither the combination of extracts nor the combination of medicinal plants to treat ailments especially malaria is new to science and medicine. In ethnomedicine, plants are often combined to produce medicines, and most plant preparations consist of various medicinal plants, either of the same or different morphological parts [14]. Such mixtures or combinations are often obtained randomly or by intuition. Additionally, drugs derived from medicinal plant extracts, either directly or indirectly, are often combined with others to create medicines used in disease management [24]. The principle is to obtain a mix of compounds that effectively treat or alleviate sickness or ailments [25]. In both orthodox and herbal treatments of malaria, combination therapy is widely practiced. Many plants have been evaluated, in combination, in the search for improved antimalarial activity. For example, [14] reported several plant combinations in the Oke-Igbo community, believed to be effective antimalarial remedies. There are also reports of certain plants being used in malaria management when combined [15,26,27]. Furthermore, plants have been evaluated in combination with standard drugs [28] to observe the effects of combining plant extracts with standard drugs, as patients may do so either deliberately or inadvertently.

The leaf and stem bark of *S. campanulata* have been used in the treatment of malaria in ethnomedicine [16] while that of *Parquetina nigrescens* have been used in the treatment of parasitic infections, headache and pains [29,30]. These ethnomedicinal uses suggest that a combination of the leaf of the two plants can be used in the management of the plasmodial infections and the attendant headache and pains [14,31] and so the combination may yield an antimalarial herbal remedy. More so, the adoption of artemisinin combination therapy (ACT) by WHO in combating resistant malaria is a motivation for scientist to investigate combinations of drugs including those of ethnomedicinal plants. The empirical investigations on medicinal plants and their combination will not only validate ethnomedicinal uses, it will also prepare the ground for standardized medicinal preparation for the treatment of such diseases and others. As at today, many plants have been investigated for possible synergistic, complimentary or antagonistic (working 'hand in hand' with each other or against each other) actions individually and in combination [12,13,15]. This study evaluated and compared the *in vivo* antiplasmodial

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potential of the cold methanol (ME) and hot infusion (HI) extracts of the leaves of *S. campanulata* and *P. nigrescens* individually and in combination in mice. This will serve to validate or invalidate the ethnomedicinal uses and identify which of the methods of extraction is better in preparing a proposed herbal remedy and also give an empirical data for the best ratio combination possible.

Other parts of *S. campanulata* and *P. nigrescens* had been singly investigated on many activities based on ethnomedicinal uses. For example, the stem bark of *S. campanulata* is a known antimalarial remedy validated by experiment [18], while the leaf of *P. nigrescens* had also been tested for antimalarial activities and both have afforded many chemical constituents[20,32-34]. However, neither of them has been combined together in an antimalarial test neither in an antimalarial remedy. However, it is necessary to investigate these various mixtures of combinations as to validate their usage as this will serve as leverage for such mixtures in scientific combinations [35].

The median lethal doses obtained for both plants indicate their safety levels and was a guide for the doses to be used in the preliminary and subsequent experiments [21,23]. It is on this premise that the extracts of these two plants were combined together in five (5) ratios as follows: *S. campanulata* (S): *P. nigrescence* (P); 1:1, 1:2, 2:1, 1:3, 3:1, and with chloroquine, S+CQ, P+CQ in addition to those of single plants (S, P), standard drug (CQ) and negative control. The infusions of the above combinations in water and maceration in methanol were subjected to chemosuppressive antimalarial test, each at 400mg/kg, being the optimal dose obtained in a previous chemosuppressive test of the composite plants [21] and evaluated using the various parameters of percentage parasitaemia, percentage chemosuppression, survival times and percentage survivor.

4.1 Percentage Parasitaemia and Percentage chemosuppression:

For ME and HI, the percentage parasitaemia obtained for all the combinations and the single drugs were significantly ($p<0.05$) lower than that elicited by the negative control (Table 1), an indication that both extracts and the combinations reduced the parasitaemia in mice. For the combinations, For ME, it appears that tripling the proportion of S over P was more effective than doubling P over S when compared with the other tested combinations of S and P and the individual extracts (Table 1). The combinations tested definitely did not give significantly higher activities than the individual drugs which were comparable in activities to each other indicating that individual plants are better used as antimalarials remedies than combinations of the two plants at least in the ratios tested. Those other combinations, S: P 1:2, 2:1, and 1:3 elicited values that were comparable to each other showing that maximally increasing the proportion of P over S may not be able to increase activity beyond the maximum obtained vice versa for S over P. It also confirms that combinations are better obtained scientifically in reasonable proportions rather than arbitrarily. Also, from the results, it could also be deduced that PN reduced the activity of CQ while S enhanced it. It was thereby demonstrated that the combination of either of these extracts affects the activities of the standard drug. This may be hypothetically extrapolated to other orthodox drugs, an obvious caution on administering herbs with orthodox prescribed drugs. As a result, extra care must be taken in administering orthodox drugs with herbal recipes, as this may result in antagonistic or synergistic effect. In similar study of [28], the antagonistic effect in combining *Morinda* species with Artesunate was demonstrated, where the activity of the positive control drug was reduced drastically. Also, *Nauclea latifolia* root extract inhibited the effect of CQ when combined together in a similar chemosuppressive experiment [36]. The studies using the combined aqueous extract of powdered leaf of *Azadirachta indica* which reduced and maintained the level of parasitaemia significantly compared to the effects of individual crude active constituents in the curative tests validates the practice of combination of plants for therapeutic action in traditional medicine [37]. Though, the combination of the extracts of the ferns did not affect the activities of chloroquine in a similar experiment where extracts of *Nephrolepis undulata*, *N. biserrata Platycerium stemaria* and *P. angolense* were combined [38], the effect was different when *Chrysophyllum albidum* stem bark, leaf and *Citrus aurantifolia* leaf and fruit extracts were combined similarly. The four extracts inhibited the activities of chloroquine significantly [15]. Also, some degrees of antagonism or inhibition were exhibited when *Chromolaena odorata* with *Tithonia diversifolia* and *Lawsonia inermis* were combined in an *in vivo* 4-day suppressive antimalarial test [39]. The combination of *P. nigrescence* with CQ (P+CQ), gave comparable values to that of the other tested combinations except 1:1 and 3:1 (Table 1). Similar ratio combinations of *Eugenia uniflora* and *Artocarpus altilis* (1:1 and 3:1) with (1:2, 1:3, 2:1) gave very low and comparable percentage chemosuppression [40]. For ME, combination of P with CQ (P+CQ), couldn't have given any effect better than that of S in the different ratios tested. In effect, the order of parasitaemia elicited is 1:2=1:3=2:1= P+CQ<1:1=3:1< S=P =CQ < SC+CQ.

For HI, all the parallel combinations tested as done for ME gave higher percentage parasitaemia and consequently lower percentage chemosuppression values than those of ME (Table 1). Also, all the combinations were comparable ($p>0.05$) in activities to each other including the individual extracts and the combinations with Chloroquine. Doubling the proportion of S relative to P, in HI (2:1) gave better relative activities than vice versa (1:2). Higher relative chemosuppression for ME, may likely indicate that infusions cannot be exhaustive in attaining full extraction like maceration. Similar combinations of *Gongronema latifolium* and *Artocarpus altilis* prepared as infusions in the ratios 1:1, 1:2, 2:1, 3:1 gave higher activities than the individual infusion extracts, while 1:3 gave 42 % chemosuppression in a similar experiment and comparable % chemosuppression to single drugs [40].

4.2 Survival times

The combination ratios of the methanol extract, ME gave average survival times that were comparable to that of the negative control and significantly ($p < 0.05$) lower than that of chloroquine. This implies that the combinations were not able to increase survival times beyond that given by the negative control. Samples S+CQ and P+CQ gave average survival times that were significantly ($p < 0.05$) higher than that of the negative control but were comparable to that of chloroquine, the positive control. However, S and P like the combinations, gave an average survival time that were also comparable ($p >0.05$) to that of the negative control. The combination ratios of the infusion extract, HI gave average survival times that were comparable to that of the negative control and significantly ($p < 0.05$) lower than that of chloroquine. Samples S+CQ, P+CQ, S and P gave average survival times that were significantly ($p < 0.05$) higher than that of the negative control but were comparable to that of chloroquine, the positive control. The infusion extract, HI of P definitely possess better survival time

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profiles than that of ME; this may be due to some water-soluble compounds extracted with the hot infusion that were not found or extracted in cold methanol. Methyl stearate came with higher peak area in the hot infusion extract of *P. nigrescence* while the cold methanol extract of *P. nigrescence* did not present the same compound in the GS MS analysis. Identification of these compounds may give a sordid clue to the relatively better survival time profile of individual extract of HI relative to that of ME and probably, the higher percentage chemosuppression values obtained for ME relative to HI.

4.3 Percentage survivor

The percentage survivor as a parameter of activity showed that S gave 40 and P 60% while combinations S:P, at ratios 1:3, 2:1 and 1:2 of ME gave a % survivor of 40, 75 and 80 respectively while 1:1 and 3:1 gave values of 60 each (Table 2). *S. campanulata* was reported to possess immune boosting or radical scavenging / antioxidant activity of 1.32 mmolTE/g [41]. This did not reflect in the observed percentage survivor elicited. However, doubling the proportion of either of the individual plants (2:1 and 1:2) increased percentage survivor by similar amount (Table 2). In the infusion extract, S gave 60% and P 80 and tripling the proportion of *P. nigrescens* over *S. campanulata* (S: P, 1:3) maximized the percentage survivors like in *S. campanulata* (Table 2). Also, tripling the proportion of *S. campanulata* over *P. nigrescens* (S:P 3:1) gave similar percentage survivors like in *S. campanulata* (Table 2). Some combinations (2:1 and 1:2) gave enhanced percentage survivor for ME while only S: P, 1:3 elicited a percentage survivor of 80 for HI.

In summary, the leaves of *P. nigrescens* and *S. campanulata* in tested combinations, did not significantly enhance the antiplasmodial activities of each other in both extractions. This cautions against arbitrarily combining of ethnomedicinal antimalarials in ethno medicine for improved activities without being evaluated in an antiplasmodial and other relevant tests. Combinations or preparation methods that are chosen arbitrarily without evaluation may not be effective after all. Also, administration of ethnomedicinal antimalarials with standard drugs should be approached with utmost caution. However, the infusions seem to present a better survival time profile than the methanol extract which may be due to a preferential extraction of antioxidant compounds of any or both plants thus boosting immunity in mice.

4.4 GC MS analysis of ME, HI and most active combination ratios

The five different extracts: Individual S and P of HI and ME, including the most active infusion extract S: P 2:1 of HI (SPI), were further subjected to GCMS analysis in order to identify and appropriate the chemical constituents that may be responsible for the above observations. Individual S and P of ME showed a total of 30 and 21 compounds respectively while individual S and P of HI gave 18 compounds each. The ME must have extracted more compounds than HI. This could have accounted for higher percentage chemosuppression values observed in ME than HI extracts (Table 1). The combination infusion extract, S P 2:1 of HI gave a total of 40 compounds while the totality for S and P in combination for ME is 51 compounds. The number of compounds might have been responsible for the level of percentage chemosuppression as well. The number of compounds is understandable, being a combination of two plants. In both cases even in combination, infusion extracted lower number of compounds than ME accounting for the relatively lower percentage chemosuppression observed (Table 1). Of the compounds identified, both undecane and benzoic acid methyl ester are common to both individual extracts of ME and HI but were absent in the combination (Table 3). Methyl stearate and Hexadecanoic acid methyl ester were found in the combination, SPI (2:1 infusion ratio combination of S and P) and also in PHI, the infusion extract of *P. nigrescens* but with higher peak area in PHI. From the peak area, PHI must have contributed the methyl stearate (23.16) and hexadecanoic acid methyl ester (52.37) found in the most active combination of the infusion. Both PHI and SHI were comparable in activities. Also, PHI possess heptadecanoic acid, 16-methyl-ester (2.1) exclusively but also some significant proportion of benzoic acid, methyl ester (15.46). PHI elicited the highest survival times (Table 2). These constituents might have been partially or solely responsible for these observations. Also, benzoic acid methyl ester which was found to be common to individual SME and PME and SHI may be responsible for the high antiplasmodial activities observed. Both hexadecanoic acid, methyl ester and 9,12-octadecadienoic acid methyl ester have been observed in the antiplasmodial active fractions of some medicinal plants like the methanol extracts of *D. arborescens* root and are likely to be responsible for its antiplasmodial activities [21,42]. Also, GCMS of selected antimalarial agents, column chromatographic sub fractions of *Glyphaea brevis* leaf extract identified methyl stearate; hexadecanoic acid, methyl ester (also identified in HI), heptadecanoic acid, 16-methyl-, methyl ester (also identified in the most active combination ratio S: P 2:1) alongside dodecanoic 9-octadecenoic acid (Z)-, methyl ester (oleic acid), carotol all with their antimalarial reported activities [43]. There is growing evidence that fatty acids have antiplasmodial properties. This is attributed to the ability of these class of chemical constituents to inhibit the fatty acid biosynthetic machinery of *Plasmodium falciparum* parasite [44]. Polyunsaturated fatty acids especially the essential fatty acids have medicinal properties. Hexadecanoic acid methyl ester has been credited with anti-inflammatory properties [](Swiatek *et al.*, 2022), 9, 12-Octadecanoic (linoleic) acid methyl ester with anti-plasmodial and anti-inflammatory [45] and methyl stearate with anti-inflammatory properties [46]. Essential fatty acids (EFA) are a group of unsaturated fatty acids which are not produced in humans, but can be obtained in diet and are necessary for the proper functioning of the human body [47,48]. The antimalarial properties of n-3 and n-6 polyunsaturated fatty acids have been earlier reported [49]. The two main families of EFAs are the ω-6 and the ω-3 groups. Linolenic and linoleic acids are essential fatty acids which belong to the omega 3 and omega 6 fatty acids respectively. Essential fatty acids (EFA) were reported to be of benefit in the prevention and management of coronary heart disease, stroke, diabetes mellitus, hypertension, cancer, depression schizophrenia, Alzheimer's disease, and collagen vascular disease [50]. The likely effects of fatty acids in this work may validate such claims for malaria.

Conclusion

The study concluded that, methanol maceration of the plant extracted more constituents than the infusion and this might have been responsible for their relative activities. The enhanced the antiplasmodial activities of *Spathodea campanula* stem bark's methanol extract by chloroquine, and the fact that the tested combinations of PN and SC did not significantly enhance each other's activities in parasite suppression or percentage survivor

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enhancement in both solvents and methods employed suggest that caution must be exercised to avoid arbitrarily combining antimalarials in ethnomedicine without prior evaluation in an antiplasmodial test. Also, the observed role of fatty acids esters may suggest their effects on the malaria parasites, an assertion that can be validated in future work.

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