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Additive Effect of Some Medicinal Plants Extracts Used for the Treatment of Malaria in Sokoto Metropolis, Nigeria

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Abstract

Malaria remains a threat and exert economic and social burdens in most communities in Africa. Medicinal plants have played an important role in the treatment of malaria worldwide. Several studies have shown that, the efficacies of most antimalarial agents are compromised by the emergence of drug-resistant *Plasmodium* species. This research was aimed at evaluating the synergistic effect of some medicinal plants used for the treatment of malaria in Sokoto metropolis, Nigeria. Three (3) plants samples were collected and used for the study. They include *Azadirachta indica*, *Carica papaya* and *Psidium guajava* leaves. They were dried and extracted using 50% methanol. Qualitative phytochemical analysis of three (3) plant extracts was carried out using standard methods. The individual extracts and their combinations were subjected to *in vivo* antiplasmodial activity. Synergistic effect was determined by checkboard method. Phytochemical screening of *Azadirachta indica*, *Carica papaya* and *Psidium guajava* leaves methanol extracts all revealed the presence of phenols, tannins and glycosides in all the extracts. Extract combination of *Carica papaya* and *Psidium guajava* demostrated 100% reduction of parasitemia at both 100 and 200 mg/kg b. wt respectively after 72-hour treatment. Determination of synergistic activity showed that Fractional Activity Index (FAI) of *Carica papaya* and *Psidium guajava* is greater than 1.0, hence the effect was considered to be additive. The findings of this study had provided scientific bases supporting the traditional use of the plants for the treatment of malaria.

Keywords: Malaria; Medicinal plants; *Plasmodium falciparum; Azadirachta indica; Carica papaya; Psidium guajava;* Sokoto metropolis.

1. Introduction

Malaria is a mosquito-borne infectious disease of humans and other animals caused by parasitic protozoans of the genus *Plasmodium*. The five identified Plasmodium species responsible for inflicting malaria in humans are *Plasmodium vivax, Plasmodium ovale, Plasmodium malariae, Plasmodium knowlesi* and *Plasmodium falciparum. Plasmodium falciparum* is the most virulent and prevalent *Plasmodium* species [1]. The disease is widespread in tropical and subtropical regions around the equator, including much of Sub-Saharan Africa, Asia, and the America. In fact, Nigeria tops the list of malaria burdened countries [2].

Medicinal plants have played an important role in malaria treatment with the discovery of two major drugs, quinine and artemisinin that are used worldwide. Artemisinin, isolated from *Artemisia annua L. (Asteraceae)* has served as a skeleton for the synthesis of artemether, artesunate, dihydroartemisinin, etc used for the current treatment of chloroquine-resistant *Plasmodium falciparum* malaria [3]. Quinine is derived from the bark of the cinchona tree and was used for treating fevers as early as the 17th century [4]. It is appraised that 66-85% of the world's population relies on folkloric herbs and the search for drugs derived from plants has been increasing in the last two decades [5]. African medicinal plants are known to contain a large variety of bioactive compounds and extracts of these plants have been screened for various biological activities, in the pursuit for prospective novel therapeutic drugs against the different human and animal illnesses [6]. Scientists and traditional healers now agree that plants are sources of new drugs, and various concoction of plants and crude extracts are effectively used for the prevention and the treatment of malaria and other ailment in several parts of the world [7].

Several plant bioactive compounds such as tannins, flavonoids, glycosides, alkaloids, anthraquinones and alkaloids have been labeled with antiplasmodial and antitrypanosomal activities [8]. The structural diversity of natural products and their ability to interact with therapeutic targets justify their use in the search for new drugs. More than 40% of the authorized drugs on the market are of natural or semisynthetic origin [9]. A majority of traditional medicinal plants are thought to be safe based on the ethnomedical knowledge available. Drugs have been generated from plants because of their availability, efficacy, and their mode of action [10].

2. Materials and Methods

2.1. Malaria Parasites

Plasmodium falciparum parasites were obtained from the blood of symptomatic malaria children (0-5 years) at Specialist Hospital, Sokoto, Nigeria.

2.2. Experimental Animals

Albino rats aged 6-8 weeks and weighing 120-260g were purchased and kept for (2) two weeks to acclimatize to laboratory conditions, and had constant access to feed on a standard rodents diet and allowed water access. They were then placed in plastic cages with metal covers for free passage of air, and at room temperature [11].

2.3. Standard Drug

A drug with a combination of Artemether (20 mg) and Lumefantrine (120 mg) was used as a standard in this study.

2.4. Plant Samples

Fresh leaves of *Azadirachta indica*, *Carica papaya* and *Psidium guajava* were collected within Sokoto metropolis, Sokoto State, Nigeria. The samples were authenticated at the herbarium of the Department of Plant Science and Biotechnology, Faculty of Life Sciences, Kebbi State University of Science and Technology, Aliero (KSUSTA), Nigeria.

2.5. Plant Samples Preparation and Extraction

The collected leaves were cleaned with water and air-dried under shade, pulverized using pestle and mortar. Three hundred grams (300g) of each sample was measured and soaked in 1L of 50% methanol. The mixture was then kept at room temperature for 48h and filtered twice; initially with a muslin cloth and later with a Whatman filter paper. The extract was stored in screw capped vials in refrigerator and later used for the study.

2.6. Malarial Parasite Inoculation

Blood from previously infected mice was collected and diluted with 0.9% saline in EDTA container. Then 0.2ml was injected intraperitoneal into each mouse for four days [12].

2.7. Preliminary Qualitative Phytochemical Screening

Qualitative phytochemical screening was carried out using standard procedures to identify the constituents as described by Sofowara [13], Trease and Evans [14] and Harborne [15].

Test for Tannins: A 0.5g of the dried powdered samples was boiled in 20ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride was added and observed for browrish green or a blue-black colouration.

Test for Saponins: A 2.0g of the powdered sample was boiled in 20ml of distilled water in a water bath and filtered. A 10ml of the filtrate was mixed with 5ml of distilled water and shaken vigorously for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously, formation of emulsion was observed which indicates a positive result.

Test for Flavonoids: A 5ml of dilute ammonia solution were added to a portion of the filtrate of each plant extract followed by addition of concentrated H_2SO_4. A yellow colouration was observed which indicates the presence of flavonoids. The yellow colouration disappeared on standing.

Test for Terpenoids (Salkowski test): A 5ml of the extract was mixed in 2ml of chloroform, then 3ml concentrated H_2SO_4 was carefully added to form a layer. A reddish brown colouration of the interface is formed to shows positive results for the presence of terpenoids.

Test for Alkaloids: A 1ml of the extract was dissolved in distilled water and 2ml of Wagner's reagent (Iodo-potassium iodide) was added. Formation of a reddish-brown precipitate indicates the presence of alkaloids.

Test for Phenol: Lead acetate test: A 3ml of 10% lead acetate solution was added to 5ml of extract and mixed gently. The production of bulky white precipitate indicates positive for phenols.

Test for Phlobatannins: An aqueous extract of each plant sample was boiled with 1% aqueous hydrochloric acid. Deposition of a red precipitate indicates the presence of phlobatinins.

Test for Anthraquinones: A 5ml of the extract solution was hydrolysed with diluted Conc. H_2SO_4 extracted with benzene. 1ml of dilute ammonia was added to it. Formation of rose ink coloration indicated the presence of anthraquinones.

Test for Glycosides: A 25ml of dilute sulphuric acid was added to 5ml extract in a test tube and boiled for 15 minutes, the solution was cooled and neutralized with 10% NaOH, then 5ml of Fehling solution was added. Formation of brick red precipitate indicates the presence of glycosides.

Test for Steroids (Liebermann-Burchard's test): A 0.5g of the extract was dissolved in 10ml anhydrous chloroform and filtered. The solution was divided into two equal portions. The first portion of the solution was mixed with 1ml of acetic anhydride followed by the addition of 1ml of concentrated sulphuric acid down the side of the test tube to form a layer underneath. Formation of green colouration indicates the presence of steroids.

2.8. *In vivo* Synergistic Antiplasmodial Activity of the Extracts Combinations

Group 1: (Normal control) administered with normal saline, 5ml/kg.

Group 2: (Negative control) administered with *P. falciparum* infected RBCs only.

Group 3: (Positive control) administered with *P. falciparum* infected RBCs and then treated with Amatem (Artemether 20 mg & Lumefantrine 120 mg).

Group 4: Administered with *P. falciparum* infected RBCs and treated with *Azadirachta indica* and *Carica papaya* extracts at 100 mg kg^{-1} b.wt.

Group 5: Administered *P. falciparum* infected RBCs and treated with *Azadirachta indica* and *Carica papaya* extracts at 200 mg kg^{-1} b.wt.

Group 6: Administered with *P. falciparum* infected RBCs and treated with *Azadirachta indica* and *Psidium guajava* extracts at 100 mg kg^{-1} b.wt.

Group 7: Administered with *P. falciparum* infected RBCs and treated with *Azadirachta indica* and *Psidium guajava* extracts at 200 mg kg^{-1} b.wt.

Group 8: Administered with *P. falciparum* infected RBCs and treated with *Carica papaya* and *Psidium guajava* extracts at 100 mg kg^{-1} b.wt.

Group 9: Administered with *P. falciparum* infected RBCs and treated with *Carica papaya* and *Psidium guajava* extracts at 200 mg kg^{-1} b.wt.

Group 10: Administered with *P. falciparum* infected RBCs and treated with *Azadirachta indica*, *Carica papaya* and *Psidium guajava* extracts at 100 mg kg^{-1} b.wt.

Group 11: Administered with *P. falciparum* infected RBCs and treated with *Azadirachta indica*, *Carica papaya* and *Psidium guajava* extracts at 200 mg kg^{-1} b.wt.

At 0, 24, 36, 48, 60, and 72 hour, blood sample was collected from the tail of each mouse and smeared onto a microscope using clean, non-greasy slides and thin films were made slide to make a thin film. The blood films were fixed with methanol, stained with 10% Giemsa, and examined microscopically using X100 objective. The percentage parasitemia and percentage reduction were analysed by counting the number of parasitized RBCs in three random microscopic fields. The percentage parasitemia and percentage reduction of parasitemia were calculated using equation 1 and 2.

$$\% \text{ Parasitemia} = \frac{\text{Number of infected RBCs}}{\text{Total number of RBCs}} \times 100 \quad \text{Eq. 1}$$

$$\% \text{ Reduction} = \frac{\text{Parasitemia of negative control} - \% \text{ Parasitemia of treated group}}{\% \text{ Parasitemia of negative control}} \times 100 \quad \text{Eq. 2}$$

2.9. Determination of Synergistic Effect of the Extracts

The checkerboard method was used to determine the synergistic effect of the combined extract activity as described by Odds [16]. The data obtained from the *in vivo* antiplasmodial activity assays were analysed in terms of the fractional activity (FA) and fractional activity index (FAI). The fractional activity of a plant extract is equal to the activity of that extract in combination with another extract divided by the activity of the extract acting alone as presented in equation 3 & equation 4. The fractional activity index is equal to the cumulative number of fractional activities as presented in equation 5.

$$FA_A = \frac{\text{Activity of extract A in combination with extract B}}{\text{Activity of extract A acting alone}} \quad \text{Eq. 3}$$

$$FA_B = \frac{\text{Activity of extract B in combination with extract A}}{\text{Activity of extract B acting alone}} \quad \text{Eq. 4}$$

$$FAI_{AB} = FA_A + FA_B \quad \text{Eq. 5}$$

If FAI is ≤ 0.5, the effect is synergistic.

If FAI > 0.5 to 4.0, the effect is additive.

If FAI > 4.0, the effect is antagonistic.

2.10. Statistical Analysis

The data obtained from the study were analyzed using one-way ANOVA followed by Duncan's multiple comparison test with the aid of a Statistical Package (SPSS version 20).

3. Results

The phytochemical composition of the plants samples is presented in Table 1. Phenols, tannins and gltycosides were detected to be present in all the leave extracts. While only phlobatannins were found to be absent in all the extracts.

Table-1. Qualitative Phytochemical Composition of the Leaves Extracts

Phytochemicals	*Azadirachta indica*	*Carica papaya*	*Psidium guajava*
Flavonoids	+	-	+
Phenols	+	+	+
Tannins	+	+	+
Saponins	+	-	+
Phlobatannins	-	-	-
Alkaloids	+	-	-
Terpenoids	+	+	-
Steroids	+	-	+
Anthraquinones	+	-	-
Glycosides	+	+	+

(+) means detected, (-) means not detected

Table-2. Effects of the Leaves Extracts on Percentage Parasitemia in *Plasmodium* infected Albino Rats

Treatments	Percentage Parasitemia (%)						
	0HR	12HR	24HR	36HR	48HR	60HR	72HR
Normal control	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
Negative Control	95.83±0.78^{b}	100.00±0.00^{f}	100.00±0.00^{g}	100.00±0.00^{c}	100.00±0.00^{d}	100.00±0.00^{c}	100.00±0.00^{c}
Positive Control	97.03±1.70^{c}	85.62±2.54^{ef}	79.38±2.02^{f}	89.42±5.87^{b}	46.32±7.50^{c}	25.30±8.06^{b}	4.78±2.64^{b}
Plant AB (100mg/kg)	91.25±0.22^{b}	63.63±3.75^{cd}	41.49±1.44^{c}	26.86±2.71^{a}	14.01±2.96^{ab}	5.56±1.26^{a}	1.84±0.94^{ab}
Plant AB (200mg/kg)	92.81±2.04^{b}	37.13±2.33^{b}	10.37±2.70^{ab}	18.42±2.71^{a}	12.57±3.67^{ab}	2.78±1.65^{a}	3.00±1.62^{ab}
Plant AD (100mg/kg)	97.95±1.03^{c}	74.68±4.79^{de}	18.39±4.81^{b}	10.58±2.69^{a}	20.74±7.54^{b}	4.08±0.90^{a}	0.81±0.81^{ab}
Plant AD (200mg/kg)	95.89±2.06^{b}	49.16±11.16^{bc}	38.16±2.64^{c}	16.23±5.03^{a}	12.68±3.74^{ab}	3.13±3.13^{a}	2.02±1.01^{ab}
Plant BD (100mg/kg)	93.50±0.71^{b}	80.14±6.67^{de}	61.61±9.07^{de}	16.47±4.71^{a}	20.40±6.02^{b}	6.60±1.98^{a}	2.08±2.08^{ab}
Plant BD (200mg/kg)	94.82±2.70^{b}	72.72±4.36^{de}	44.18±4.49^{cd}	17.42±4.71^{a}	12.86±4.17d^{ab}	3.00±1.75^{a}	0.00±0.00^{a}
Plant ABD (100mg/kg)	95.83±2.09^{b}	86.80±4.88^{ef}	67.74±9.98^{ef}	21.80±6.06^{a}	15.63±4.60^{ab}	6.67±3.51^{a}	0.00±0.00^{a}
Plant ABD (200mg/kg)	94.82±0.98^{b}	76.90±5.61^{de}	50.74±10.68^{cde}	18.17±6.14^{a}	13.87±4.49^{ab}	9.73±2.98^{a}	0.00±0.00^{a}

A = *Azadirachta indica* Leaves Extract, B = Carica *papaya* Leaves Extract, C = *Psidium guajava* Leaves Extract.
Values are presented as mean ± SEM (n = 3). Value having same superscript in columns are not significantly different at (*P*>0.05) using One-Way ANOVA, followed by Duncan multiple comparison test with SPSS version 20.0.

The percentage parasitemia of infected control, positive control and all extract treated groups significantly (P<0.05) increases compared to normal control at zero hour (0hr). Similarly, at 12hr and 24hr a significant (P<0.05) increases in parasitemia of induced control, positive control and all extract treated groups compared to normal control. However, at 36, 48, 60 and 72hr, a non-significant difference (P>0.05) in percentage parasitemia was observed in all combined extract treated groups compared to normal. Although time dependent decrease was observed in drug control, however the reduction was significantly (P>0.05) different from normal control at all the time intervals.

Table-3. Effects of the Leaves Extracts on Percentage Reduction of Parasitemia in *Plasmodium* Infected Albino Rats

Treatments	Percentage Reduction of Parasitemia (%)						
	0HR	12HR	24HR	36HR	48HR	60HR	72HR
Normal control	100.00±0.00^{c}	100.00±0.00^{f}	100.00±0.00^{g}	100.00±0.00^{c}	100.00±0.00^{d}	100.00±0.00^{c}	100.00±0.00^{c}
Negative Control	0.00±0.00^{ab}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
Positive Control	0.00±0.00^{a}	10.67±2.66^{ab}	17.17±2.11^{b}	6.69±6.13^{b}	51.67±7.83^{b}	73.60±8.41^{b}	95.01±2.75^{b}
Plant AB (100mg/kg)	4.50±0.33^{b}	33.60±3.92^{cd}	56.71±1.48^{e}	71.97±2.5^{c}	85.38±3.09^{bc}	94.19±1.31^{c}	98.08±0.99^{bc}
Plant AB (200mg/kg)	3.14±2.13^{ab}	61.25±2.44^{e}	89.18±2.82^{fg}	80.79±2.83^{c}	86.88±3.98^{bc}	97.10±1.72^{c}	96.87±1.69^{bc}
Plant AD (100mg/kg)	0.00±0.00^{a}	22.08±5.00^{bc}	80.80±5.02^{f}	88.96±2.80^{c}	78.35±7.86^{c}	95.74±0.94^{c}	99.15±0.85^{bc}
Plant AD (200mg/kg)	0.00±0.00^{a}	48.70±11.65^{de}	60.18±2.75^{e}	83.06±5.25^{c}	86.77±3.90^{bc}	96.74±3.26^{c}	97.89±1.06^{bc}
Plant BD (100mg/kg)	2.43±0.74^{ab}	16.52±6.44^{bc}	35.71±9.47^{cd}	82.81±4.91^{c}	78.72±6.28^{b}	93.10±2.07^{c}	97.83±2.17^{bc}
Plant BD (200mg/kg)	1.05±2.82^{ab}	24.11±4.54^{bc}	53.89±4.68^{de}	81.81±4.91^{c}	86.58±7.54^{bc}	96.87±3.16^{c}	100.00±0.00^{c}
Plant ABD (100mg/kg)	0.00±2.18^{a}	9.42±5.09^{ab}	29.31±10.42^{bc}	77.25±6.32^{c}	85.15±9.13^{bc}	92.93±6.35^{c}	100.00±0.00^{c}
Plant ABD (200mg/kg)	1.05±1.02^{ab}	19.75±5.86^{bc}	47.05±11.14^{cde}	81.03±6.41^{c}	85.52±4.69^{bc}	89.85±3.11^{c}	100.00±0.00^{c}

A = *Azadirachta indica* Leaves Extract, B = Carica *papaya* Leaves Extract, C = *Psidium guajava* Leaves Extract.
Values are presented as mean ± SEM (n = 3). Value having same superscript in columns are not significantly different at (*P*>0.05) using One-Way ANOVA, followed by Duncan multiple comparison test with SPSS version 20.0.

The percentage reduction of parasitemia revealed significant (P<0.05) differences upon comparing infected control, positive control and all combined extract treated groups compared to normal control at 0 and 12hr. However, at 24hr only group treated with combined extract AB (200mg/kg b.wt) was not significant (P>0.05) different from normal control. While, at 36hrs the percentage decrease in parasitemia of groups treated with all combined extracts was not significant (P>0.05) different from normal control. At 48hr, none of the combined extract percentage reduction was comparable (P<0.05) to normal control. However, at 60 and 72hr, there was no significant (P>0.05)

differences in percentage parasitemia reduction of group treated all combined extract treated groups compared to normal control respectively.

Table-4. Fractional Activity Index of the Extracts Combinations

Indices	Value	Remark
$FAI_{AB(100)}$	1.96	Additive
$FAI_{AB(200)}$	1.94	Additive
$FAI_{AD(100)}$	2.03	Additive
$FAI_{AD(200)}$	1.96	Additive
$FAI_{BD(100)}$	1.99	Additive
$FAI_{BD(200)}$	2.00	Additive
$FAI_{ABD(100)}$	3.04	Additive
$FAI_{ABD(200)}$	3.00	Additive

FAI value ≤ 0.5 means the effect is synergistic, FAI value > 0.5 to 4.0 means the effect is additive, while FAI value > 4.0 means the effect is antagonistic.

4. Discussion

Methanol extracts have also been attributed to contain many of the bioactive compounds from plant materials which acts on the *plasmodia* parasites [17]. According to Mojab [18]: Parasitemia count is the major biomarker used in determining the degree of malarial infection. A mean parasitemia level ≤ 90% to that of mock-treated control animals usually indicates that the test compound is active in standard screening studies [19]. A study conducted on a 5-day curative test, the aqueous methanolic crude extract of *Psidium guajava* leaf demonstrated a significant (P<0.05) antiplasmodial activity in a dose-dependent pattern indicating that the plant has antimalarial activity [20]. Another study on the use of *Citrus limon* extract for treating mice malaria infections indicated increased red blood cell lyses. When the RBCs of another group of mice that were not infected with *P. berghei* but fed with *C.limon* extract were examined erythrocyte lysis was also observed, though with lesser intensity. This phenomenon of plant extract-induced RBCs lysis has been reported by Laser, *et al.* [21]. Lysing of RBCs could be attributed to the mechanism of action of the plant extracts, as has earlier been asserted by Laser, *et al.* [21]. Eluu, *et al.* [22] reported that antimalarial drugs target different stages of the *Plasmodium* life cycle and could express different stage-specific mechanisms of action during parasite clearance. The schizonticides have been reported to exert their chemotherapeutic effects by inducing premature lyses of parasitized red cells.

The use of polyherbal remedy is a commonly employed procedure by traditional healers in Africa. There is little or no scientific documentation of therapeutic benefits, safety information of such combination or its advantage over monotherapy of the individual plants [23]. The number of medicinal plants that constitutes a polyherbal mixture or combination could be dependent on patient presentation or traditional health giver discretion [24].

Combining two drugs that have different mechanisms of action is likely to reduce the emergence of drug resistant strains. Synergy may allow two drugs, both less than 50% efficacious, to be combined to achieve a very high efficacy [25].

Similar records of combined extracts giving good clearance and suppression have been reported by several authors which include Kingsley, *et al.* [26], Paula, *et al.* [27] and Osei-Djarbeng, *et al.* [24].

Multidrug strategy in therapeutic applications is expected to increase efficacy of two or more anti-infective agents [28], improve clinical cure, shorten the duration of therapy so as to minimize the risk of recrudescence, and provide a way in which resistance can be delayed [29]. Combination therapy, which has been a strategy approved for other multidrug resistance infections such as HIV and tuberculosis, is widely recommended for malaria treatment [29, 30]. Over the past decades combination therapy has gradually replaced single drug treatment due to the rapid spread of drug resistance by *Plasmodium* parasites globally [31].

5. Conclusion

The finding of this study has confirmed the traditional use of the plants for the treatment of malaria. The result can serve as a step towards the development of safe and effective herbal therapy against *Plasmodium* parasites.

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