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In vitro antiplasmodial activity of aqueous leaves extract of *Azadirachta indica* (Juss), *Senna occidentalis* (Linn) against combination therapy of both plants extract on the multiplication of *Plasmodium falciparum* (Laveran)

Alkali K[1], Abdullahi K[2], Mshelia HE[3], Yahaya MA[4], Abubakar N[5], Tarbutu AG[6]
[1-6] Mohammed Goni College of Legal and Islamic Studies, Usmanu Danfidiyo University, Sokoto, Nigeria

Abstract
This study investigated the antiplasmodial effects of leaves extract of *Azadirachta indica*, *Senna occidentalis* and Combination therapy of both plans extracts using RPMI 1640 culture media (*In vitro*). Parasite density was determined by counting the number of *Plasmodium falciparum* infected erythrocyte in 5,000 erythrocytes of the culture, thin blood smear were prepared and stained with Giemsa stain. Varying concentrations of the extracts such as 10, 20, to 100 mg/mL were prepared, the effect of the leaves extracts against the growth of *schizonts* were dose dependant. *A.indica* showed highest growth inhibition (96.92%) at 70m g/mL. However, the *schizonts* were found to be inhibited by the leaves extract of *S. occidentalis* at the highest concentration (100 mg/mL) with growth inhibition of 98.46%, there was no significant difference in the anti-malaria efficacy among the leave extracts and the Combination therapy ($p<0.05$) at 100 mg/mL. The results of phytochemical screening indicated *A.indica* and *S.occidentalis* contain Alkaloids, Flavonoids, Saponins, Saponins glycosides, Steroids and Terpenoids. The result of the study showed that *Azadirachta indica* and *Senna occidentalis* contain pharmacologically active compounds, hence they are potential antimalaria.

Keywords: Antiplasmodial Activity, *Azadirachta indica*, *Senna occidentalis*, combined therapy, *Plasmodium falciparum*

Introduction
Malaria remains an important public health concern in countries where transmission occurs regularly, as well as in areas where transmission has been largely controlled or eliminated (WHO 2001) [27]. The classic symptom of malaria is fever with spikes on alternating days, headaches, malaise, fatigue, nausea, and anemia are also common. Severe forms of the disease can result in organ failure, delirium, impaired consciousness, and generalized convulsions, followed by persistent coma and death (Bleakley, 2007) [2]. the World Health Organization (WHO) estimated that 216 million cases of malaria in 91 countries in 2016, more than the 211 million cases reported in 2015, Malaria continues to claim a significant number of lives: in 2016,445 000 people died from malaria globally, compared to 446 000estimated deaths in 2015, Children under five are particularly susceptible to malaria. The disease claims the life of a child every two minutes (WHO 2018) [29]. The African Region continues to bear 90% of malaria cases and 91% of malaria deaths worldwide and Nigeria, the continent's most populous country, accounted for 27% of malaria cases and 24% of malaria deaths globally in 2016 (WHO 2018) [29]. *Plasmodium falciparum* the most widespread etiological agent for human malaria has become increasingly resistant to standard antimalarials (*e.g.* chloroquine and antifolates), Artemisinin combination therapies (ACTs) are the recommended treatment for uncomplicated malaria. However, their uptake remains relatively low – in part due to availability issues, but also due to the high cost of ACTs in relation to cheaper, less effective alternatives. This is of great concern to all parties with an interest in access to medicines and the control of malaria, WHO (2010) [28]. Medicinal plants have been the focus for the search of new antimalaria drugs in various parts of the world (Schuster, 2001) [20] and the present global situation indicates a recent resurgence in the severity of malaria, due to the resistance of malaria parasites to antimalaria drugs (Peter, 1988) [16]. Hence, there is a need to intensify research in the development of new, cheap and effective antimalaria drugs from medicinal plants.

Plants are important source of drugs; especially in traditional medicine (Bako *et al.*, 2005) [1]. It is a common practice in Nigeria and other parts of the world to use plant in the form of crude extracts, decoction, infusion or tincture to treat common infection and chronic conditions. According to WHO, over 70% of the world populations rely on medicinal plants for primary health care2 and there are reports from various researchers on natural substances of plant origin which are biologically active, with desirable antimicrobial and antioxidant properties (Hamid *et al*., 2010) [10].

Materials and Method
Collection and Identification of Samples
Fresh and mature leaves of *Azadirachta indica* were obtain behind new postgraduate hostel Usmanu Danfodiyo University Sokoto (permanent site). While *Senna occidentalis* fresh mature leaves were obtained behind First Bank plc, Usman Danfodio University Sokoto, (permanent site). The samples were collected separately in a clean sterile polythene bag and brought to the herbarium of the Department of Biological Science, Usman Danfodio University Sokoto, for

identification and authentication. Voucher specimen UDUS/ANS/0115 and UDUS/ANS/0168) were prepared and deposited in the same herbarium.

Fifty grams (50 g) from each sample of *Azadirachta indica* was extracted with 1500mL of distilled water in 2000mL beaker. The soaked sample was stirred and covered with aluminum foil and keep for twenty four hours. The resultant extract was filtered using muslin cloth and each filtered was evaporated separately to dry using hot plate set at 40̊ C to obtain crude extract. The extract was weighted and stored in the refrigerator until use. Artemether and Lumefantrine reference standard were purchased from Sigma-Aldrich, USA.

Qualitative photochemical screening of plant extract

The leaves extract of the plant were screened for metabolites such as alkaloids, tannins, flavonoids, saponins, balsams, anthraquinones, cardiac glycosides, glycosides, and steroids, using Dragendorff's Test for alkaloid, Ferric Chloride Test for tannins, H_2SO_4 Test for flavonoid, Frothing Test for saponins, Liebermann Burchard's Test for terpenoid Steroids, Borntrager's Test for Anthraquinone, Fehling's Test for Glycosides, Keller-Kiliani's Test for Cardiac Glycosides, Fehling's Test for Saponins Glycosides and Ferric Chloride Test for Balsams (El-Olemy *et al.*, 1994) [7] and (Evans, 1999) [8].

Media Preparation

P. falciparum originally obtained from a positive patient from specialist hospital Sokoto, was continuously cultured based on a modified method previously described by Trager (1976) [23]. The parasites were maintained in continuous culture on human erythrocytes (blood group O^+ obtained from the Hematology Department, Sokoto specialist hospital, in RPMI 1640 medium supplemented with 10% human AB+ serum, 25 Mm N-2-hydroxyethylpiperazine- N-2-ethanesulfonic acids (HEPES), 2g $NaHCO_3$ and 60 mg/ml gentamicin sulfates, at pH 7.2. The assay was performed in a culture flaks, the cultures were incubated at 37° C in an atmosphere of CO_2 in a candle jar for 24 hours. Parasite cultures were synchronized to the ring stage by treatment with 5% Sorbitol.

Inoculation procedure for efficacy test

plant extracts 30µL each were dropped in to different wells, each containing different concentration of 10 mg/mL, 20 mg/mL, 30 mg/mL, 40 mg/mL, 50 mg/mL, 60 mg/mL, and 70 mg/mL, of *A.indica* and standard antimalaria were also screened in 96 well microtiter plates. Culture media (100µL) was parasitized with erythrocyte parasitemia at 0.5% and then inoculated in to the wells. The control well contains no treatment. The cultures were incubated at 37°C in an atmosphere of CO_2 in a candle jar for 24 hours (Rieckmann, 1978) [18]. Schizont growth inhibitions per 200 asexual parasites were counted in 25 microscopic fields. The control parasite culture was considered as 100 % growth. The percentage inhibition per concentration was calculated using the formula:

$$\% \text{ inhibitions per concentration} = \frac{\% \text{ parasitemia in control} - \% \text{ parasitemia in test well}}{(\% \text{ parasitemia in control})} \times 100$$

X 100 (Ngemenya *et al*., 2006) [12].

Statistical analysis

Data obtained from the study were subjected to statistical analysis using statistical package for social science (SPSS) version 20.0. Analysis of variance (two ways ANOVA) was carried on the data, at 95% level of significant and mean generated from this study were separated using New Duncan Multiple Range Test (DMRT).

Results

The phytochemical screening of plants material showed the presence of saponins, tannins, flavonoids, terpenoids, cardiac glycosides, alkaloids, steroids and Saponin glycosides

Table 1: Qualitative Phytochemical screening of *A. indica* and *S. occidentalis* leaves extracts

Constituent	*A.indica*	*S.occidentalis*
Alkaloids	+	++
Saponin glycosides	+	+
Terpenoid steroids	+	+
Tannins	+	-
Saponins	+++	+++
glycosides	-	+
Flavonoids	+	+
balsams	+	-
Cardiac glycosides	+	-
volatile oils	-	+
anthraquinones	-	-

Key; - Not ditected, + Identified in a trace amount, ++ Identified in moderate amount, +++ Identified in high amount.

The results of the antimalaria activity of aqueous extract of *A.indica* on the *schizonts* growth of *P.falciparum* are presented in Table 4.3. The result show complete *schizont* growth inhibition when *P. falciparum* is treated with 70 mg/mL of *A.indica* with the mean growth inhibition of 96.92% (0.67) and the lowest inhibition was recorded at 10 mg/mL, with the mean growth inhibition of 22.72% (17.0). Similarly, complete *schizont* growth inhibition of 98.46% (0.33) was recorded when *P. falciparum* is treated with 100 mg/mL of *S.occidentalis* and the lowest *schizont* growth inhibition of 19.68% (17.67) at 10 mg/mL. Standard antimalaria inhibited *schzont* growth 100% at all concentration.

Table 2: Antimalaria activity of aqueous leaves Extract of *Azadirachta indica*, *Senna occidentalis* and Combination therapy on trophozoit growth of *Plasmodium falciparum*

Concentrations (mg/ml) of plant extract	*Azadirachta indica*		*Senna occidentalis*		Combination therapy	
	Mean growth ±SE	Inhibition (%)	Mean growth ±SE	Inhibition (%)	Mean growth ±SE	Inhibition (%)
control	22.00	0	22.00	0	22.00	0
10	17.0^{c}±0.57	22.72	17.67^{c}± 1.45	19.68	10.67^{b} ± 1.20	51.1
20	15.33^{c}±1.53	30.31	16.00^{c}± 0.58	27.27	07.33^{b}± 1.20	66.68
30	08.67^{b}±0.88	60.59	15.00^{c}± 0.57	31.81	04.33^{b}± 0.88	80.31
40	03.67^{b}±0.67	83.31	14.67^{c}± 1.20	33.31	02.67^{b}± 0.67	87.86
50	02.67^{b}±0.88	87.85	14.00^{c}± 0.58	36.36	01.00^{a}± 0.57	95.45
60	01.33^{a}±0.88	93.95	12.33^{b}±0.33	45.45	00.00^{a}± 0.00	100
70	00.67^{a}±0.67	96.95	06.33^{b}± 0.88	71.22	00.00^{a}± 0.00	100
80	00.00^{a}±0.00	100	03.33^{b}± 0.58	84.86	00.00^{a}± 0.00	100
90	00.00^{a}±0.00	100	02.00^{b}± 0.58	90.90	00.00^{a}± 0.00	100
100	00.00^{a}±0.00	100	00.33^{a}±0.33	98.45	00.00^{a}± 0.00	100

Values are mean ± standard error 3 replication with different superscripts are significantly different (P<0.05)

Discussion

The phytochemical study of *A.indica* and *S.occidentalis* revealed the presence of tannins, saponins alkaloids, glycosides, flavonoids, steroids, balsams, volatile oil, anthraquinones, saponin glycosides and cardiac glycosides. Qualitative phytochemical analysis of *Azadirachta indica* and *Senna occidentalis* indicated that the plants are rich sources of bioactive compounds similar bioactive compounds were also earlier observed on whole plant of *S. occidentalis* Egharevba *et al.* (2013) [5]. Reported the presence of carbohydrates, saponins, sterols, flavonoids, resins, alkaloids, terpenes, anthraquinones, glycoside and balsam in *S. occidentalis.* And seed back and leaves of *Azadirachta indica* (Niyi, 2011) [13]. The presence of bioactive compound in *Azadirachta indica* and *Senna occidentalis* is an indication that they have medicinal potentials due to the fact that each of the bioactive compounds identified has one or more uses therapeutically (Nonita *et al.,* 2010) [14] and (Garba *et al.,* 2012) [9]. Other study include (Odeja *et al.,* 2014) [15], (Ronan *et al*., 2009) [19] (Chukwujekwu *et al*., 2006) [4] and (Sheeba *et al*., 2009) [21].

The antimalarial study revealed the activity of *A.indica and S.occidentalis* leaves *extracts.* The study revealed that *A.indica* is the most effective against *schizonts* growth of *P.falciparum* followed by *S.occidentalis*. *A.indica* leaves extract significantly inhibited the growth by 22'72% of at 10mg/ml, this differs significantly compare to the Combination therapy which inhibited the growth by 51.1%. At 70 mg/mL *P.falciparum* growth was inhibited significantly by 96.92%, which shows no significant difference compare to the Combination therapy with 100% inhibition. This findings conforms to the report of in which similar constituents was found to exhibits antiprotozoal and antibacterial activities (Anyanwu and Dawet, 2012) and Usha *et al.* (2001) [26], who tested aqueous extracts of *Azadirachta indica* (bark), *In vivo* against *P. berghei* following Peter‟s 4-day test and recorded about 70% parasitemia inhibition. Extracts from Nigerian neem leaves (*Azadirachta indica*) have been earlier reported to have anti-malarial activities (Ekanem, 1971) [6], (Jadhav *et al.* 2014) [11]. But Udeinya *et al*. (2004) [25] demonstrated that acetone/water mixture is a more efficient solvent than water alone for the extraction of anti-malarial activity from Nigerian neem leaves. Its anti-malarial activity has been reported to be superior to chloroquine (Puri, 1999) [17], gametocytocidal (Udeinya *et al*., 2004, 2006) [24, 25] and schizonticidal (Puri, 1999) [17], against *falciparum* malaria parasite. The effectiveness of *A.indica* is not has been surprising as the plant shows to posses antimalarial activity (Ekanem, 1971) [6]. This also explains the rampart use of *A.indica* by the people. *S.occidentalis* leaves extract significantly inhibited the growth by 19.68% at 10 mg/mL, this indicate there is significant difference compare to the Combination therapy. At 100 mg/mL *P.falciparum* growth was inhibited significantly by 98.46%, this indicates there is no significant difference compare to the Combination therapy. These findings are in conformity with that of Tona *et al.* (2001) [22], who worked on *Cassia occidentalis* against rat model *Plasmodium* and reported 60% inhibition. Choudhary and Nagori, (2013) [3] reported 63% inhibition of *Cassia occidentalis leaves extracts In vitro* antimalarial activity. Although much data was not found about antimalarial activity of *S.occidentalis*. The study revealed that there is no significant difference between *A.indica and S.occidentalis* leaves *extracts* at 10 mg/mL, but they differ significantly at 70 mg/mL. There was significant difference across concentration of all plants extracts, indicating that *A.indica and S.occidentalis* leaves possess antimalaria potential against *P.falciparum.*

Conclusion

Based on the present study, it can be concluded that the extracts of *Azadirachta indica* and *Senna occidentalis* possess antiplasmodial activity. The phytochemical screening revealed the presence of bioactive constituents that could be the reason for pharmacological activity. Even though they are not as effective as the Combination therapy at low concentration, both plants showed promising activity against schizons growth. Both *Azadirachta indica* and *Senna occidentalis* antimalarial activities were found to be dose dependant. Therefore, the observed antiplasmodial activity of both plant extract can be a positive attributes in the malaria control.

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