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In Vitro Antimalarial Activity of Extracts of Some Indigenous Plant Species in Kebbi State

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Abstract

The search for antimalarial compounds has been necessitated by the resistance of *Plasmodium falciparum* to almost all antimalarial drugs. The aim of this research was to determine in-vitro antimalarial activity of extracts of some indigenous plants species in Kebbi State. Plant extraction was carried-out by maceration using ethanol and water as solvent. The antiplasmodial activity of the extracts was evaluated against fresh clinical isolates of *P. falciparum* using WHO method of in-vitro micro test. Phytochemical screening was also carried out on the extract to deduce the active chemicals present in the plant extract. All plant extracts demonstrate dose dependent antimicrobial activities with IC50 Less than 50%. However highest growth inhibition of the *P. falciparum* was demonstrated by aqueous and ethanol extract of *A. indica* with IC50 7.4µg/ml and 8.6µg/ml respectively followed by ethanol and aqueous extract of *C. occidentalis* with IC50 15.3µg/ml and 18.0µg/ml respectively. Least antimalarial activity was demonstrated by aqueous extract of *M. oleifera* with IC50 33.5µg/ml while ethanolic extract of *M. oleifera* demonstrated IC50 of 20.50µg/ml. *M. indica* ethanolic and aqueous extract also demonstrated moderate antimalarial activity with IC50 18.8µg/ml and 24.5µg/ml. The phytochemical screening of medicinal plants showed the presence of tannins, saponins, alkaloids, flavonoid, phenol and cardiac glycosides in the extracts, which may be responsible for the antiplasmodial activity. This result justifies the traditional use of the plant in malaria treatment and further research is suggested to identify and characterize the active principles from the plants.

***Keywords: Antimalaria**, Invitro, Medicinal Plants, Malaria, Kebbi*

INTRODUCTION

Malaria is a disease is of global importance that results in 300-600 million cases annually and an estimated 2.2 billion people are at risk of infection according to Singh *et al* (2011). Despite more than a century after identification of the causative parasites and more than half a century of finding effective drugs and insecticides, the disease is still as deadly as ever (Mehta and Desai, 2013).

Malaria is caused by a parasite in the blood called Plasmodia. Five species (*Plasmodium falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi)* of this genus are implicated in human malaria, with the most deadly to be *Plasmodium falciparum*. They are usually transmitted via the bite of infected female Anopheles mosquitoes (Umar *et al.*, 2017). Numerically the most important of the life threatening protozoan disease is malaria, which is responsible for at least 750,000 death a year, mostly in young children in Africa (Greenwoodv *et al.*, 2012; WHO, 2015). Clinical symptoms of malaria ranges from acute febrile illness with fever associated with chills, headache, and vomiting to deadly complications like severe anaemia, respiratory distress in relation to metabolic acidosis, or cerebral malaria which can eventually lead to death (Singh, 2011).

Transmission of the disease occur all year round in the Southern part of the country while in the Northern part, the disease is more seasonal, killing children every 30 seconds, hence, pregnant women and their unborn children

become vulnerable to malaria which serves as the major cause of maternal anemia, low birth weight and increase in child mortality (Liadi *et al.,* 2016). Over half of the world's population is at risk from catching malaria. Malaria is currently endemic in 109 countries in four continents and of the 500 million cases of malaria estimated to occur annually, approximately one million result in death. Most of the fatalities are in children under the age of five years old and pregnant women(Lamb, 2012).Africa accounts for 91% of all malaria deaths and, with an estimated 57.5 million cases and 225,000 deaths per year. Nigeria accounts for 27% of the total African malaria burden (WHO, 2017). There are estimated 100 million cases of malaria with over 300,000 deaths and 50% of the population has at least one episode of malaria every year (FMOH, 2015). Within Nigeria, malaria is a major cause of illness, death, and poverty, and a significant drain on the economy and wellbeing of the nation. It is estimated that 50% of Nigeria's adult population will have at least one episode of malaria each year and children under five will have 2–4 attacks annually (Oladepo *et al.*, 2019).The nation loses over 132 billion naira annually in form of treatment costs, prevention, loss of man-hours etc. (FMOH, 2015; Uzochukwu and Emma-ukaegbu, 2010). Children under five and pregnant women are most at risk for malaria-related morbidity and mortality, with 11% of maternal and 20% of under-five deaths attributed to malaria (NMEP, 2016). Poorer and more rural populations are also at greater risk, with malaria prevalence higher among the lowest wealth quintile and among rural populations (Oladepo *et al.,* 2019).

MATERIALS AND METHODS

Ethical Consideration

The study was approved by Kebbi State Ministry of Health with ethical letter reference MOH/KSREC/VOL.I/56.

Plant identification

The plant sample was collected fresh from various communities in the study area with the help of informants who participated in the survey carried out and authenticated by Plant Taxonomist at Herbarium of Kebbi State University of Science and Technology, Aleiro where voucher numbers were obtained.

Plant Sample Collection

Most frequently mentioned plants from survey result were selected to determine the antimalarial potentials of the plant extract. Selected plants are: *Cassia occedentalis, Azadirachta indica, Moringa oleifera and Magnifera indica*. The plant sample already collected and identified in Herbarium Unit was used.

Extraction

Fresh leafs of the selected plant was obtained. The leaves were washed and air dried at room temperature. The extraction was performed by maceration process. This involved soaking 100g of the crude powder of the plant in each of 1000mls of aqueous and ethanol, covered with intermittent shaking for 72 hours. The resultant extract was filtered using muslin cloth and number one Whatman filter paper. The extract was concentrated using pressured controlled rotary evaporator at 40^0C. The percentage yield of all the crude extracts were determined as percentage of weight (g) of the extract to the original weight (g) of the dried sample used(Umar *et al.,* 2017).

Phytochemical Test

Phytochemical screening was carried out on the extract to detect the presence of tannins, saponins, alkaloids, flavonoid, phenol and cardiac glycosides in the plant extract. These tests were carried out using standard procedures to as described by Sofowora(1993) and Trease and Evans (1989).

Tests for Tannins

0.5ml of the extract was put in a test tube and a few drops of 0.1% ferric chloride were added and the mixture was observed for brownish green or blue black colouration.

Test for Alkaloids

0.5ml of the extract was stirred in 5ml of 1% HCl on a steam bath for 5 minutes. The mixture was filtered using Whatman's filter. To the filtrate, 2-4 drops of Dragendoff's reagent was added to 1ml of the filtrate. An orange colouration indicated the presence of alkaloids.

Test for Flavonoids

1ml of the extract was heated with 10ml of ethyl acetate over a steam bath for 3 minutes. The mixture was filtered and 4ml of the filtrate was shaken with 1ml of the dilute ammonia solution. A yellow colouration indicated the presence of flavonoids.

Test for Cardiac Glycosides (Keller-Killani test).

0.5ml of the extract was treated with 2ml of glacial acetic acid containing one drop of ferric chloride solution. It was under laid with 1ml of concentrated H_2SO_4. A brown ring at the interface indicated deoxysugar characteristics of cardenolides. A violet ring may appear below the brown ring, while in the acetate acid layer, a

greenish ring may form just gradually throughout

Test for Saponin

10ml of distilled water was added to 0.5ml of each extract in a test tube. It was shaken vigorously for 2 minutes. The presence of fronting or bubbling indicated the presence of saponin.

Test for Phenol

A small amount of the extract was taken with 1 mL of water in a test tube and 1 to 2 drops of Iron III chloride ($FeCl_3$) was added. A blue, green, red or purple color is a positive test

Antimalarial Assay

Preparation of Culture Medium for Cultivation of *Plasmodium falciparum*

The cultivation of the plasmodium parasite was carried out using the technic described by Trager and Jensen (1976) in which one packet of Rosewell Memorial Park Institute (RPMI) 1640 medium (containing 25 mM of HEPES buffer, glucose) was dissolved in 960 ml of double distilled water into which 40 µg/ml of gentamycin sulfate (1.2 ml of Gentamycin/L) was added. This solution was passed through a Millipore filter of 0.22 µm porosity and store at 4°C as 96 ml aliquots in glass media bottle. Exactly 4.2 ml of 5 % sodium bicarbonate (5gms of sodium bicarbonate dissolved in 100 ml double distilled water and filtered through a Millipore filter of 0.22 µm porosity and store at 4°C) was added to 96 ml of stock RPMI 1640 media (Incomplete media).

O+ blood was collected in centrifuge tube without anticoagulant and kept at 4°C. It was centrifuged at 10000 x g for 20 min at 4°C next day. Serum collected was separated aseptically and kept in aliquots. The serum was inactivated by keeping using water bath at 56°C for half an hour. Normal inactivated O+ human serum (10 ml) was added to 90 ml of incomplete media to make complete malaria media(CMM).

In-vitro Cultivation of *P.falciparum*

Suspension of (50 % hematocrit) un-infected cells with CMM (with 15 % serum) was prepared. Appropriate amount of uninfected cells was added to an initial 0.75 % of parasitaemia and diluted with CMM to get 0.5 % cell suspension (5 % hematocrit). The culture was kept in a candle jar in an atmosphere of CO_2 at 37°C for 48 hours. After every 48 hours the media was removed using a sterile Pasteur pipette without disturbing the cells that settled down. Then the cells mixed without frothing and a drop of blood was placed on the slide to make a thin film. The prepared thin film was stained and examined for parasitaemia.

thin layer.

In-vitro Anti-plasmodial Activity of the Plant Extracts

The assay was performed in triplicate in a 96-well microtiter plate, according to WHO method [in vitro micro test (Mark III)] by assessing the inhibition of schizont maturation (WHO, 2001). RPMI 1640 (Sigma Company, USA) was incorporated. Dilution was prepared from the crude plant extract at concentrations 100, 50, 25, 12.5, 6.25, 3.13µg/ml with DMSO and use to pre-dose the micro-titer plate. Negative control treated with solvent and positive control (Chloroquine phosphate) was added to each set of experiments. Two hundred microliters from blood mixture with media was added to each well in plate and incubated in CO2 condition at 37^0C for 30 –40 hours. The contents of the wells were harvested smear on slides and stained for 30 min in a 2% Giemsa solution at pH 7.2, after which the developed schizonts with three or more nuclei per 200 asexual parasites were counted in triplicate. The control and the test wells were compared for the determination of the percentage inhibition. The inhibitions of parasite growth in the drug treated groups were calculated as follows:

Percentage inhibition =

$$100 - \frac{\text{Number of schizonts in test well}}{\text{Number of schizonts in control wells}} \times 100$$

Statistical analysis

The anti-plasmodial activities of the extracts were expressed by the concentrations of the drug that inhibited 50% parasites (IC50) relative to the negative control. These IC50 values were calculated by plotting the inhibition (%) against the logarithm to the base 10 of the concentrations of the extracts (log10 concentration) using regression equation ($y = aLn(x) - c$) where y= percentage inhibition, Lnx= anti-log of concentration of extract, a= slope, c= constant. ANOVA test was used to compare means of plant extracts with p-value ≤0.05 considered significant.

RESULTS

Antimalarial Activity

The results of the antimalarial activity of *Azadirachta indica, Cassia occidentalis, Moringa Oleifera* and *Mangifera indica* on the schizonts growth of *P. falciparum* are presented in Table 1. All plant extracts demonstrated varying antimalarial activity with different parasite count and percentage inhibition as compared with the positive and negative controls.

The highest level of parasite inhibition was observed with *Azadirachta indica and Cassia occidentalis*. *Azadirachta indica* inhibited 100% both aqueous and ethanolic extract with 100µg/ml. *Cassia occidentalis* inhibited 99% ethanolic extract and 98% aqueous extracts with 100µg/ml. *Mangifera indica* Ethanol and Aqueous extract inhibited the growth of *P. falciparum* by 89.86% and 81.30 respectively when treated with 100µg/ml. *M. indica* and *M. oleifera* show lowest antimicrobial activity among all the plant extract with percentage inhibition of 2.7% and 6.6% respectively at the lowest concentration of 3.13µg/ml..

The result of the anti-plasmodial activity of each plant extracts at different concentrations is shown in Figure 1 to 4 which shows higher activity in ethanol extract at all the 6 different concentrations except for *Azadirachta indica* in which the aqueous extract shows more inhibition than ethanolic extract. *Azadirachta indica* aqueous, ethanol extract and *Cassia occidentalis ethanolic* extract showed no significant difference at ($P<0.05$) in the anti- plasmodial activity when compared to positive control. Other plant extract showed slight to large significant difference in the anti- plasmodial activity compared to positive control.

In Figure 5, 1C50 value which is the concentration require to inhibit 50% of the parasite show that the aqueous extract of *Azadirachta indica* has the lowest 1C50 of 7.39µg/ml which made it the most potent among the 8 extracts and *Moringa oleifera* aqueous extract with the highest 1C50 of 33.54µg/ml is the least potent.

Phytochemical Screening

Qualitative phytochemical investigation of aqueous and ethanolic leaf extracts of *Azadirachta indica, Cassia occidentalis, Moringa Oleifera* and *Mangifera indica* are presented in Table 5. The result revealed the presence of alkaloids, cardiac glucosides, flavonoids, saponins and tannins in both aqueous and ethanolic leaf extracts.

Table 1: Antimalarial activity of plant extracts

Extract	Concentration (mean ± SD)											
	3.13 (µg/ml)		6.25 (µg/ml)		12.5 (µg/ml)		25.0 (µg/ml)		50.0 (µg/ml)		100.0 (µg/ml)	
	Parasite count	% Inhibition	Parasite count	%Inhibition	Parasite count	%Inhibition	Parasite count	%Inhibition	Parasite count	% Inhibition	Parasite count	% Inhibition
N Control	230.3±13.4	0.0±5.8	230.3±13.4	0.0±5.8	230.3±13.4	0.0±5.8	230.3±13.4	0.0±5.8	230.3±13.4	0.0±5.8	230.3±13.4	0.0±5.8
AZA_ETH	176.±3.0	23.6±1.3	146.7±4.2	36.3±1.8	83.0±1.7	64.0±0.8	36.0±2.0	84.4±0.9	14.0±1.0	93.9±0.4	0.0±0.0*	100.0±0.0
AZA_AQ	164 ±3.6	28.8±1.6	142.3±2.1	38.2±0.9	71.0±1.7	69.2±0.8	32.0±1.0	86.1±0.4	11.3±1.5	95.1±0.7	0.0±0.0 *	100.0±0.0
SEN_ETH	197.0±2.0	14.5±0.9	157.7±1.5	31.5±0.7	133.0±2.0	42.2±0.9	91.0±1.0	60.5±0.4	63.7±1.5	72.4±0.7	2.0±2.0 *	99.1±0.9
SEN_AQ	206.0±2.0	10.6±0.9	168.0±1.0	27.1±0.4	143.0±2.0	37.9±0.9	102.0±4.4	55.7±2.0	73.3±1.5	68.2±0.7	4.0±2.0	98.3±0.9
MOR_ETH	215.0±3.0	6.6±1.3	178.0±2.0	22.7±0.9	154.0±1.7	33.1±0.8	97.0±1.0	57.9±0.4	55.7±1.5	75.8±0.7	39.0±1.0	83.1±0.4
MOR_AQ	224.0±1.0	2.7±0.4	186.3±2.1	19.1±0.9	168.0±4.0	27.1±1.7	122.7±2.5	46.7±1.1	109.0±3.0	52.7±1.3	57.0±0.0	75.2±0.0
MAG_ETH	208.0±1.0	9.7±0.4	172.0±1.0	25.3±0.4	156.7±2.1	32.0±0.9	92.0±2.0	60.1±0.9	59.0±1.0	74.4±0.4	23.3±2.1	89.9±0.9
MAG_AQ	213.0±2.0	7.5±0.9	179.0±3.0	22.3±1.3	162.0±2.0	29.7±0.9	114.7±2.5	50.2±1.1	76.0±4.0	67.0±1.7	43.0±2.0	81.3±0.9
CQ C	20.3±1.5C	91.2±0.7	16.7±1.5 C	92.8±0.7	9.0±2.0 C	96.1±0.9	2.7±1.2 C	98.8±0.5	0.7±1.2 C	99.7±0.5	0.0±0.0 C	100.0±0.0

SD = standard deviation; % Inh. = percentage parasite inhibition; *= statistically the same antimalarial activity with control (CQ) using one-way ANOVA post hoc (donnet) at $p < 0.05$, others had statistically significant lower antimalarial activity with the control (CQ); C = control;
(CQ) C = control, N control= Negative control, AZA_ETH=*Azadirachta indica* ethanol, AZA_ETH=*Azadirachta indica* Aqueous, SEN_ETH=*Cassia occidentalis* Ethanol, SEN_AQ=*Cassia occidentalis* Aqueous, MOR AQ= *Moringa oleifera* Aqueous, MO_ETH= *Moringa oleifera* Ethanol, MAG_AQ=*Mangifera indica* Aqueous, MAG_ETH=*Mangifera indica* Ethanol

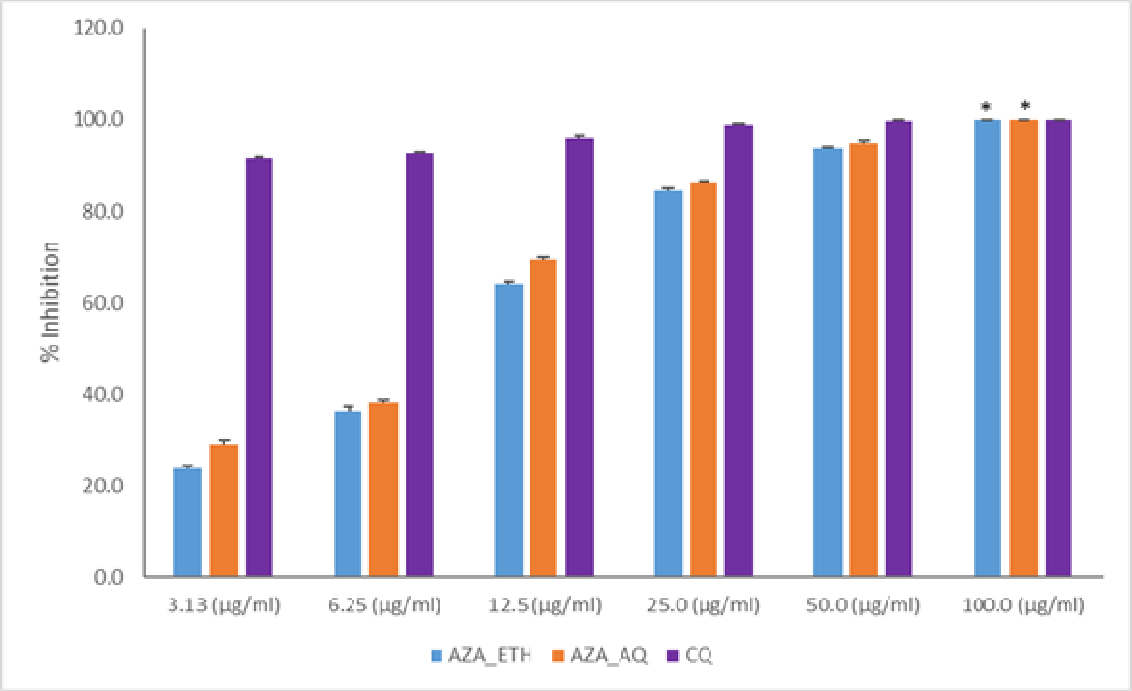

Figure1: Antimalarial activity of Aqueous and Ethanol extracts of *Azadirachta indica*

* = Statistically the same antimalarial activity with chloroquine
AZA_ETH=*Azadirachta indica* ethanol
AZA_ETH=*Azadirachta indica* Aqueous
CQ= Control (Chloroquine)

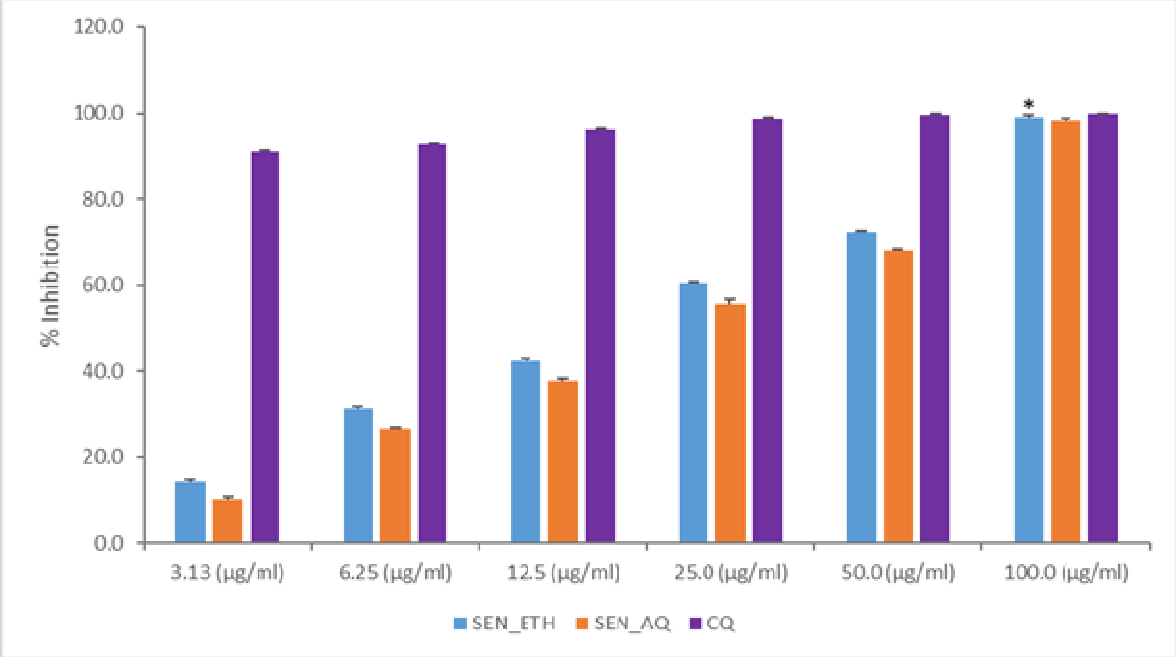

Figure2: Antimalarial activity of Aqueous and Ethanol extracts of *Cassia occidentalis*
* = Statistically the same antimalarial activity with chloroquine, SEN_ETH=*Cassia occidentalis* Ethanol, SEN_AQ=*Cassia occidentalis* Aqueous, CQ= Control (Chloroquine)

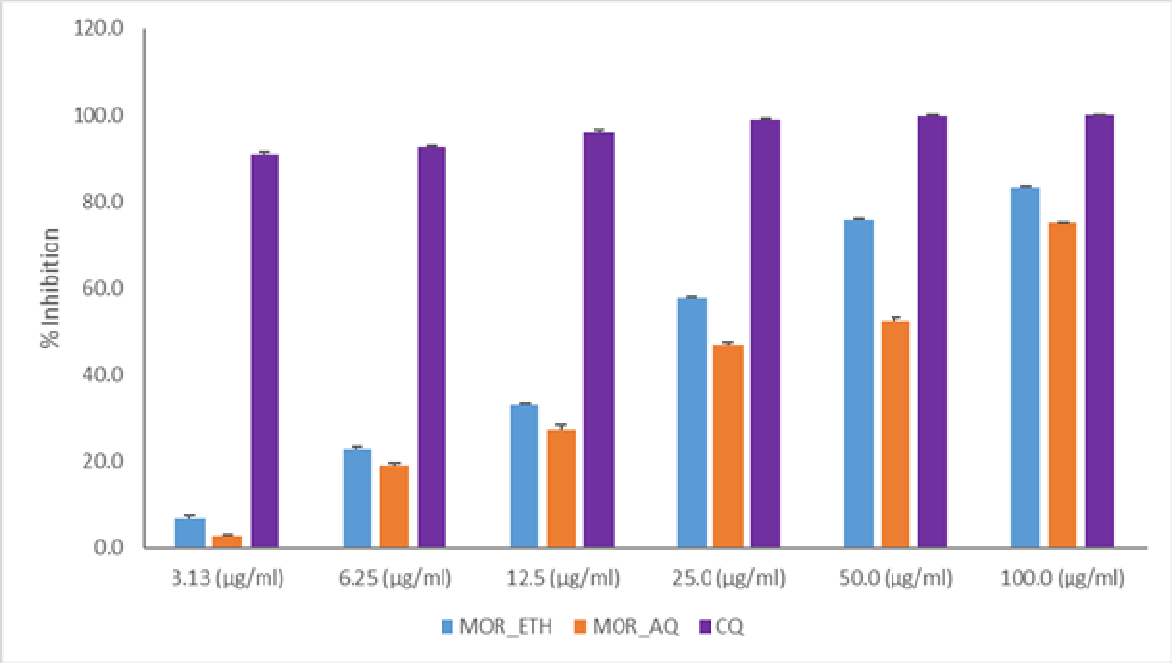

Figure 3: Antimalarial activity of Aqueous and Ethanol extracts of *Moringa oleifera*
MOR AQ= *Moringa oleifera* Aqueous, MO_ETH= *Moringa oleifera* Ethanol, CQ= Control (Chloroquine)

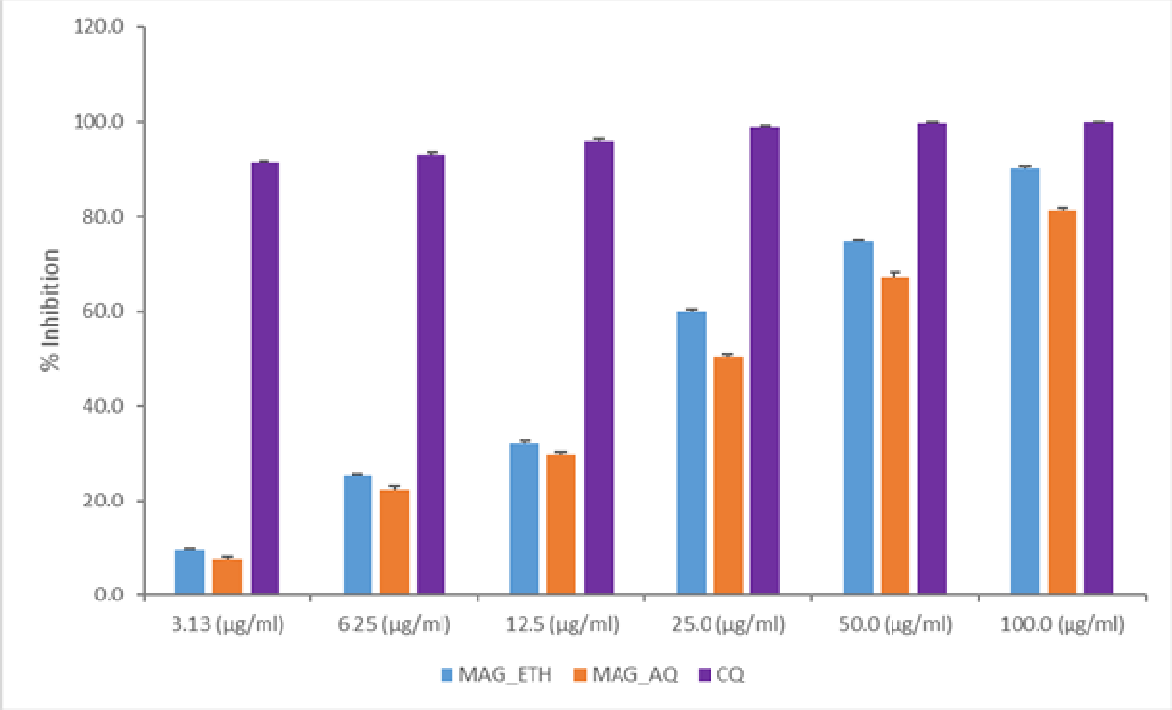

Figure 4: Antimalarial activity of Aqueous and Ethanol extracts of *Mangifera indica*
MAG_AQ=*Mangifera indica* Aqueous, MAG_ETH=*Mangifera indica* Ethanol, CQ= Control (Chloroquine)

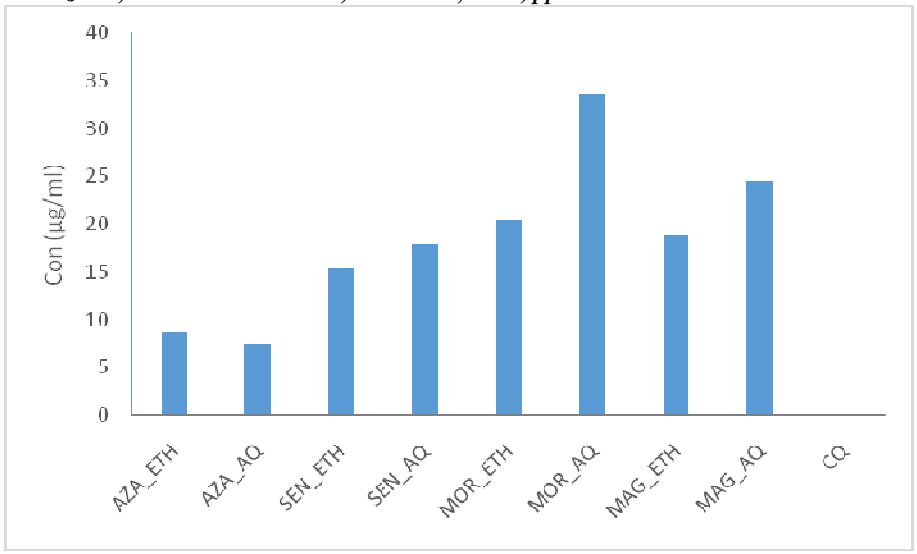

Figure 5: Median inhibitory Concentration of plant Extracts

AZA_ETH=*Azadirachta indica* ethanol, AZA_ETH=*Azadirachta indica* Aqueous, SEN_ETH=*Cassia occidentalis* Ethanol, SEN_AQ=*Cassia occidentalis* Aqueous, MOR AQ= *Moringa oleifera* Aqueous, MO_ETH= *Moringa oleifera* Ethanol, MAG_AQ=*Mangifera indica* Aqueous, MAG_ETH=*Mangifera indica* Ethanol, CQ= Control (Chloroquine)

Table 2: Phytochemicals in Plant extract

Plant Name	Solvent	Phytochemicals					
		Alkaloid	**Tanin**	**Saponin**	**Flavonoid**	**Cardiac Glycosides**	**Phenol**
Azadirachta indica	Aqueous	+	+	+	+	+	+
	Ethanol	+	+	+	+	+	+
Cassia occidentalis	Aqueous	+	+	+	+	-	+
	Ethanol	+	+	+	+	-	+
Mangifera indica	Aqueous	+	+	+	+	-	+
	Ethanol	+	+	+	+	-	+
Moringa oleifera	Aqueous	-	+	+	+	-	-
	Ethanol	-	+	+	+	-	-

+: presence; −:absence

DISCUSSION

The result of antiplasmodial activity of plant extracts shows that Aqueous and ethanol extract of *A. indica* is the most effective against schizonts growth of *P. falciparum followed by S. occidentalis, M. indica* ethanol extract, *M. Oleifera* ethanol extract and then *M. indica* aqueous extract and *M. Oleifera* aqueous extract which demonstrated least antimalarial activity. *Azadirachta indica and Cassia occidentalis* both demonstrate complete schizont growth inhibition when *P. falciparum* is treated with 100 µg/ml concentration and there was no statistical significant difference in their result when compared with the positive control using one-way ANOVA post hoc (donnet) at significance level of $p<0.05$ while other plant extract gave a statistical significant difference when compare with the positive control (Chloroquine). Antimalarial activity can be classified into four broad categories according to previous studies which are; extract with IC50 less than 10 µg/ml is considered very good; 10 to 50 µg/ml considered moderate. over 50 µg/ml considered to have low activity and greater than 100µg/ml are considered inactive (Cudjoe, Donu, Okonu, Amponsah, and Amoah, 2020; Sha, 2011).Aqueous extract of *A. indica* leaves extract significantly inhibit the schizont growth with IC 50 = 7.39 µg/ml. The antimalarial activity of *A. indica* was previously reported by (Alshawsh *et al.*,2009). They found that the aqueous extract of *A. indica* leaves showed IC50 value 2.0µg/ml which is less than 10µg/ml which is in agreement with this study (IC50=7.39 µg/ml). Also, the ethanolic extract showed very good activity with IC50 =8.63µg/ml. The anti-plasmodial activity of the leaves of *Moringa oleifera* has been reported by Donkor *et al.,* 2015 against *P. falciparum* and their findings showed moderate antimalarial activity which is in agreement with this study.In this study, the result of antiplasmodial activity of *Moringa oleifera* gave an IC50 of 33.53µg/ml for the ethanolic extract and 20.50µg/ml for the aqueous extract. Tarkang *et al.,* 2014 reported the antiplasmodial activity of *Mangifera indica* with a different experimental method. The result of their ethanolic extract is in agreement with this study which gave 18.80 within the range of moderate antimalarial activity but the result of their aqueous extract is in contrast with this study. Their aqueous extract gave a low antiplasmodial activity while the result of this study demonstrate moderate antiplasmodial activity. Based on the classification of antiplasmodial activity reported in previous studies, result from this study of the water and ethanol extracts of *Azadirachta indica* with IC50 less than 10µg/ml have very good antimalarial activity while *Cassia occidentalis, Mangifera indica and Moringa oleifera* with IC50 range between 15.30µg/ml to 33.5µg/ml can be said to have moderate antimalarial activity which is in agreement with previous studies by Alkali *et al.,*(2018); Tarkang *et al.,*(2014).

The phytochemical screening of medicinal plants showed the presence of tannins, saponins, alkaloids, flavonoid, phenol and cardiac glycosides in the extracts, which may be responsible for the antiplasmodial activity. Alkali *et al.,*(2018) also reported the presence of saponins, flavonoids, alkaloids, terpenes, anthraquinones, glycoside and balsam in *S. occidentalis* and *A. indica*. Bioactive compound in these plants have reported to for various medicinal purpose and may also be responsible for the antiplasmodial activity of these plants. The presence of triterpenoids, limonoids, in *A. indica* may take part in the antimalarial activity of this traditional medicinal herb. It is known that the limonoid gedunin, isolated from *A. indica*, exerts antimalarial effect in vitro (Alshawsh *et al.,* 2009). Flavonoids and phenolic compounds in herbal plants have also been associated with antimalarial activity and difference in the antimalarial activity. Plants with higher level of these compounds may exhibit higher antimalarial activity than others.

CONCLUSION

All plant extracts showed dose dependent antimalarial activities and the study affirmed that selected plants (*Cassia occidentalis, Azadiracta indica, Moringa oleifera and Magnifera indica*)have great potentials to cure Malaria and justifies the reliance of indigenous rural communities on traditional health care system. The phytochemical screening revealed the presence of bioactive constituents that could be the reason for pharmacological activity. The selected plants for this study can be said to be future promising for the treatment of malaria. However, in vivo studies on these medicinal plants are necessary and should seek to determine toxicity of the active constituents, their side effects.

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