

Phytochemical Analysis and In-vitro Anti Plasmodia Activity of *Chrozophora Senegalensis* Extracts on *Plasmodium falciparum*

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ABSTRACT

Chrozophora Senegalensis is traditionally employed in the treatment of malaria in Northern Nigeria. Its extracts were prepared by cold maceration with 4 solvents, n-hexane, ethylether, methanol, and aqueous. Phytochemical screening of the extracts showed the presence of tannins, alkaloids, saponins, flavonoids and phenolic in the methanol and aqueous extracts, while ethyl ether and n-hexane extracts contains terpenes, tannins and phenolics. Ethylether has flavonoids and n-hexane has traces of alkaloids. Quantitative phytochemical screening of the methanol extract indicated highest content of tannins with 3.12mg/100g, followed by alkaloids 3.10, flavonoids 2.51, phenolics 2.24, saponins 1.69 and then terpenes 1.61mg/100g. The clinical strain of *Plasmodium falciparum* were obtained and cultivated in-vitro for seven days, the parasite level increases consistently throughout the cultivation. The extracts were tested in vitro against cultured *Plasmodium falciparum*. The highest growth inhibition of the *P.falciparum* was demonstrated by the methanol extract with an IC_{50} of 2.37 μ g/ml followed by aqueous extract with IC_{50} of 13.36 μ g/ml, ethylether 32.47 μ g/ml and least by n hexane 37.68 μ g/ml. Methanol and aqueous extracts could therefore be very good potential antimalarial if further investigated.

Key words; parasitaemia, Chrozophora Senegalensis, in-vitro cultivation, plasmodium falciparum, inhibition concentration

INTRODUCTION

Malaria, a mosquito-borne disease with high global mortality threat, caused by a parasite in the blood called Plasmodia. Five species 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. The disease is mainly characterized by fever in uncomplicated cases but can develop into severe malaria within 24 hours after it first appears, if left untreated¹. Malaria is an Infectious disease that continues to be associated with considerable morbidity and mortality. It was estimated that there were

over 300 million cases of malaria every year in developing countries especially in Africa Sub-Sahara (90%) and other developing countries. Malaria kills over one million people a year-mainly children under five years and pregnant women². Malaria is a major health problem in Nigeria. It constitutes 30% of all attendance to health facilities. In all malaria endemic countries, plants are used in traditional medicine for treatment of the disease. The emergence and spread of plasmodium resistance to antimalarial drugs is a great challenge facing the global effort to control malaria³ Challenges such as the increased cost of

anti-malaria, resistance of the parasite to drugs and lack of an effective vaccine, exacerbated and frustrated the global efforts in the control of malaria³. Therefore this research is aimed at phytochemical analysis and invitro antiplasmodia activity of the *Chrozophora senegalensis* plant extracts which is a medicinal plant that is used for the treatment of different kind of illnesses in most of the African countries but also yet to be fully exploited.

MATERIALS AND METHODS

Plant Sample Collection

The plant material was collected fresh from Hussainiyya area, Zaria Local Government, Kaduna State in the month of July 2014 and identified at the herbarium unit Biological science department Ahmadu Bello University Zaria where a voucher specimen with a voucher number 689 was deposited

Chemical/Reagents

N-benzoyl- DL-arginine b-naphthylamide hydrochloride (BANA) and RPMI 1640 were obtained from Sigma Aldrich.

Preparation of Extracts

The whole plant of *C. Senegalensis* was collected fresh shade dried at room temperature (25°C) for two weeks then grounded to powder. The extraction was performed by maceration process. This involved soaking 100g of the crude powder of the plant in each of 1000mls of aqueous, methanol, ethyl ether and n-hexane, covered with intermittent shaking for 72 hours. The resultant extract was filtered using cheese cloth and number one

whatman filter paper. The extract was concentrated using pressured controlled rotary evaporator at 40°C. 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.

Phytochemical Analysis

Phytochemical tests were carried out on the extracts using standard procedures to identify the constituents as described by Sofowora⁴ and Trease and Evans⁵.

Antimalarial Assay

Selection of Patients

The inclusion criteria for the patient selection according to WHO 2001⁶ were patients having mono infection with *P.falciparum*, parasitaemia density not less than 1000 and not more than 80 000 asexual parasites/1ml of blood following microscopic examination were adapted for the screening. Three fresh blood specimens were collected from patients suffering from fever and other malaria symptoms who were further confirmed infected by *P. falciparum* using the RDT method and microscopic method of confirmation of malaria using vacumtainer.

Preparation of Culture Medium for Cultivation of Plasmodium falciparum

The cultivation of the plasmodia parasite was carried out using the technic described by Trager & Jensen (1976)⁷ in which one packet of RPMI 1640 (containing 25 mM of HEPES buffer, glucose) dissolved in 960 ml of double distilled water. 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.

Preparation of Washing Medium (Incomplete medium)

Exactly 4.2 ml of 5 % sodium bicarbonate (5 gms 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.

Serum Preparation

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.

Complete Medium

Normal inactivated O+ human serum (10 ml) was added to 90 ml of incomplete media to make complete malaria media(CMM).

Preparation of Erythrocytes (RBCs) for Culture

O+ blood was collected in anticoagulant into centrifuge tubes and centrifuged at 1500 x g for 10 min at room temperature. Plasma and buffy coat were removed with sterile Pasteur pipette. Washing media was added for further washing, centrifuged at 1500 x g for 10 min and supernatant was removed. The process was repeated thrice with equal amount of CMM added to the pellet to make 50% hematocrit and stored at 4°C.

Invitro Cultivation of P.falciparum.

Suspension of (50 % hematocrit) of uninfected 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 to with 5% CO₂ 5% O₂ and 90% Nitrogen at 37°C for 30 24 hours.

Monitoring Culture Growth

After every 24 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. Two ml of Fresh complete media (with 10 % serum) was added, mixed properly subjected to the same gas mixture in the candle jar, and kept back in the incubator. The prepared thin film was stained and examined for parasitaemia.

Invitro Anti-plasmodia 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)^[6]. RPMI 1640 (Sigma Company, USA) was the culture medium used for cultivation of *P. falciparum* ^[8]. Dilution was prepared from the crude plant extract with concentrations 200, 100, 50, 25, 12.5, 6.25 $\mu\text{g/ml}$ with DMSO and use to pre-dose the micro-titer plate. . Negative controls treated with solvent and positive controls (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°C 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

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

Statistical Analysis

The results were analyzed using Prism 5 software for the analysis of the anti-plasmodia screening and calculation of IC₅₀. The data obtained were expressed as means and standard deviation of the mean (\pm SD).

RESULTS

The Percentage Yield of C.Senegalensis Extracts

The percentage yields of the *C. Senegalensis* extracts are shown in Table 1. the percentage yield (w/w) of the various extract have methanol fraction 29.95 \pm 0.60% as the highestyield, followed by aqueous fraction with 26.87 \pm 0.53%, ethylether fraction with 15.48 \pm 0.57% and n-hexane has the lowest with 9.93 \pm 0.29%.

Phytochemical Analysis of C. Senegalensis Extracts

The preliminary qualitative phytochemical screening of the aqueous, methanol, ethyl ether and n-hexane extracts of the whole plant *C. seregalensis* revealed the presence of tannins, alkaloid, saponins, flavonoid and phenolic in aqueous and methanol extract while terpenes are absent in both the extracts

as shown in table 2. In the ethylether and n-hexane extracts the preliminary qualitative phytochemical screening shows the presence of the tannins, terpenes, and phenolics. Alkaloids are only present in ethylether extract and flavonoids only in n-hexane but saponins are absent in both the extracts.

In the quantitative phytochemicals of the extracts Table 3, the aqueous extracts of *C. senegalensis*, phenolics content was high with 3.24mg/100g and least was Tannins 0.21mg/100g. In the methanol extract, tannins content is highest with 3.12mg/100g and least was terpenoids 1.61mg/100g. Ethyl ether extract has higher concentration of terpenoids 6.21mg/100g and lower concentration of tannins 4.09mg/100g. The n-hexane extract that contain only 4 phytochemicals has highest concentration of terpenes 4.25mg/100g and lowest concentration of phenolics with 0.98mg/100g.

Percentage Parasitaemia in the Culture for Seven Days during Cultivation

The three slides (Plate I-III) have shown the microscopic visualization of the parasite for 3 days in the cause of cultivation. The 1st day, the culture was initiated with 0.5 %

parasitaemia (plate I). On the 3rd day (plate II) there was significant increase in the level of parasitaemia with 0.61% and it raised up to 1.87% parasitaemia on the 7th day (plate III). Figure 1 shows the increase in the parasites level for 7 days. The result shows constant increase in the level of parasitaemia throughout the cultivation which was even more prominent at day 4 to day 7. The Percentage parasitaemia of *plasmodium falciparum* in the culture for 7 days was represented graphically from the first day of cultivation with 0.5% parasitaemia up to the last day that was day seven (1.87 %).

Anti-Plasmodial Activity of Aqueous, Methanol, Ethylether and N-Hexane Extracts of C.Senegalensis.

The result for the anti-plasmodial activity of the extracts at different concentrations of the

plants was represented in Figure 2 which shows higher activity in methanol extract at all the 4 different concentrations. Ethyl ether and n-hexane extracts showed no significant difference at (P<0.05) in the anti-plasmodial activity when compared to negative control. Methanol extract showed large significant difference in the anti-plasmodial activity when also compared to negative control, but they all have significant difference with the positive control.

In Figure 3, 1C₅₀ value which is the concentration require to inhibit 50% of the parasite show that the methanol extract has the lowest 1C₅₀ of 2.37 µg/ml which made it the most potent among the 4 extracts and n hexane that has the highest 1C₅₀ of 32.47 µg/ml is the least potent

Table I: Percentage yield of *C.Senegalensis* Extracts

Fractions	Percentage yield (%) of <i>C. Senegalensis</i>
Aqueous	26.87 ± 0.60 ^a
Methanol	29.95 ± 0.53 ^a
Ethyl ether	15.48 ± 0.57 ^b
N-hexane	9.93 ± 0.29 ^c

Values are Mean ± SD for triplicate determinations. Values with different superscripts down the column are significantly different P (<0.05)

Table II: Qualitative Phytochemical Composition of Aqueous, Methanol, Ethylether and N-hexane Extracts of *C.Senegalensis*.

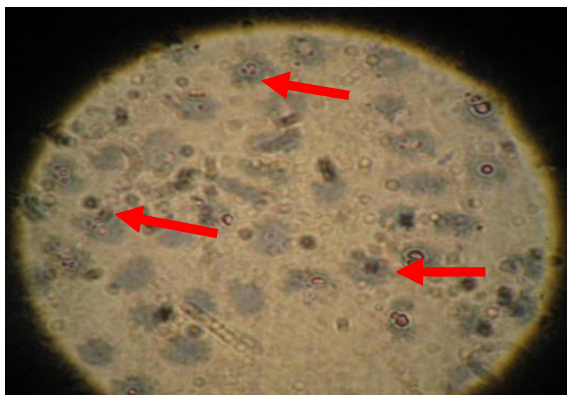
Solvents	Phytochemicals					
	Tannins	Alkaloids	Saponins	Terpenes	Flavonoid	Phenols
Aqueous	+	+	+	+	+	+
Methanol	+	+	+	+	+	+
Ethyl-ether	+	+	-	+	-	+
N-hexane	+	-	-	+	+	+

(+ve)=Present: (-ve) =absent

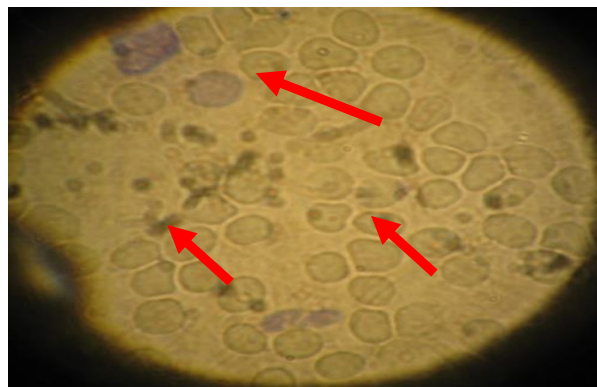
Table III: Quantitative Phytochemical Composition of Aqueous, Methanol, Ethylether and N-hexane Extracts of *C.Senegalensis*.

Phytochemical	Concentration (mg/100g)			
	Aqueous	Methanol	Ethylether	N-hexane
Alkaloids	2.88 ± 1.24 ^a	3.10 ± 0.01 ^a	0.11 ± 0.15 ^b	ND
Flavonoids	2.24 ± 0.09 ^a	2.51 ± 0.03 ^a	ND	1.53 ± 0.02 ^b
Saponins	2.69 ± 0.02 ^a	1.69 ± 0.02 ^b	ND	ND
Phenols	3.24 ± 0.02 ^a	2.24 ± 0.02 ^b	2.84 ± 0.01 ^b	0.98 ± 0.05 ^c
Terpenes	1.09 ± 0.06 ^a	1.61 ± 0.03 ^a	6.21 ± 0.17 ^b	4.25 ± 0.03 ^c
Tannins	0.21 ± 1.09 ^a	3.12 ± 0.01 ^b	4.09 ± 0.65 ^c	2.68 ± 0.03 ^d

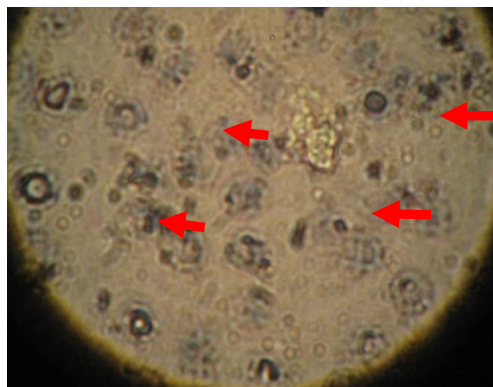
Value are mean ± standard deviation n=3 values with different superscripts in the row differ significantly (P<0.05)



A



B



C

PLATES A-C: Microscopic Visualization of *P. falciparum* on the 1st, 2nd and 3rd Days of Cultivation Respectively (x100 magnification)

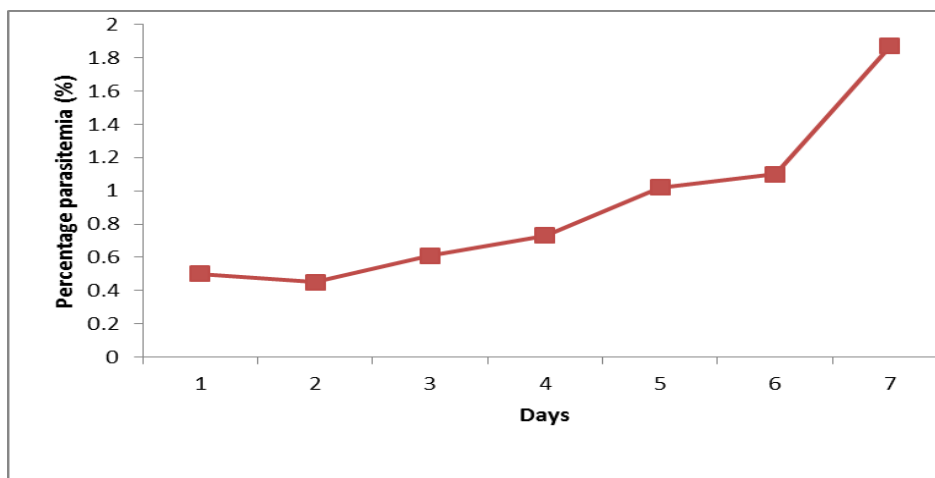


Figure I: Percentage Parasitaemia in the Culture for Seven Days during

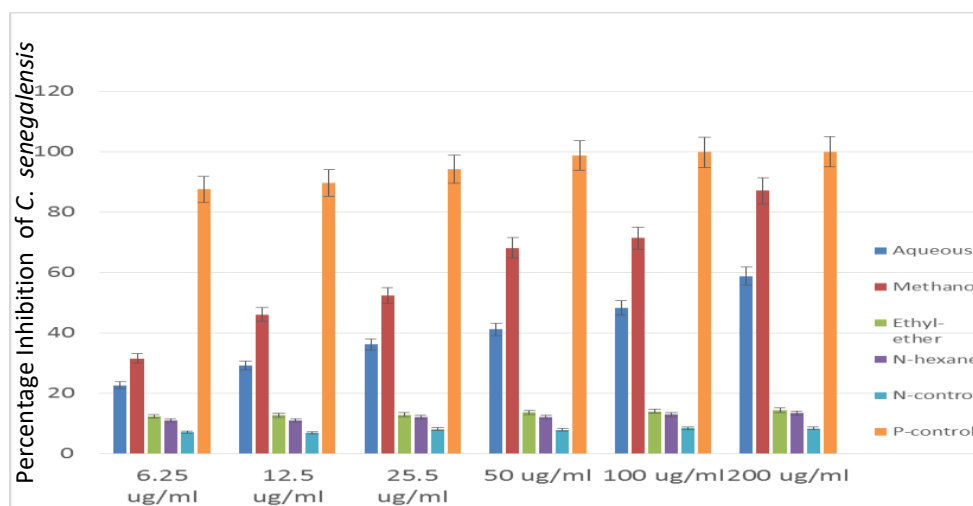


Figure II: Anti-Plasmodial Activity of Aqueous, Methanol, Ethylether and N-hexane Extracts of *C. senegalensis*.

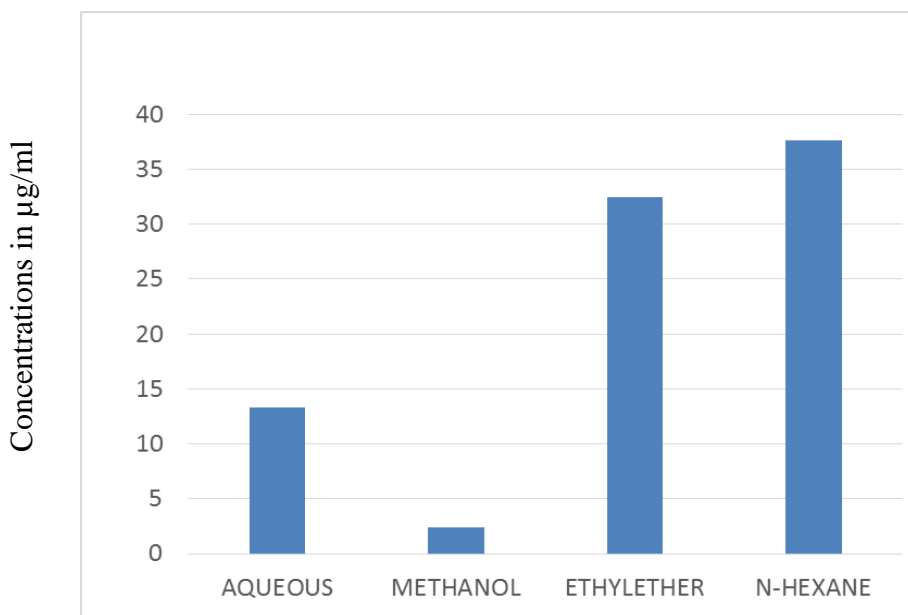


Figure III: Median Inhibitory Concentration (IC₅₀) of Aqueous, Methanol, Ethylether and N-hexane Extract of *C. Senegalensis*

DISCUSSIONS

The *Chrozophora Senegalensis* plant material used for this research was dried, ground and stored at low temperatures. This was done to preserve the presence and the quality of the compounds found within the plant. According to Makkar⁹ the moisture content of fresh plant material changes the chemical composition and properties of the plant over time. Grinding of the plant material into fine particles was to create a larger surface area to allow higher extraction of bioactive compounds.

The dried plant material was extracted using water and some organic solvents (methanol, ethyl-ether and n-hexane) to obtain the bioactive compounds present in the plant under pharmacological investigation.

The percentage yield (w/w) of the various extracts of the plant has methanol fraction $29.95 \pm 0.60\%$ as the highest yield, followed by aqueous fraction with $26.87 \pm 0.53\%$, ethylether fraction with $15.48 \pm 0.57\%$ and n-hexane has the lowest with $9.93 \pm 0.29\%$. The extract yield of the plants was higher in the aqueous and methanol extracts than for the ethylether and the n-hexane organic extracts. Parekh et al.,¹⁰ maintains that these observations can be explained due to the polarity of the compounds extracted. The high polarity of water and methanol probably accounted for the high yield in the dry extracts because both polar and some less polar compounds were extracted. Hence, the extract yield increased when solvents of polarity were used. The preliminary qualitative phytochemical screening of the aqueous, methanol, ethyl ether and n-hexane extracts of the whole plant *C. senegalensis* revealed

the presence of tannins, alkaloid, saponins, flavonoid and phenolic in aqueous and methanol extract while terpenes are absent in both extracts. In the ethylether and n-hexane extracts the preliminary qualitative phytochemical screening shows the presence of the tannins, terpenes, and phenolics. Alkaloids are only present in ethylether extract and flavonoids only in n-hexane, but both the extracts have absence of saponins.

In the quantitative phytochemicals of the extracts, in the aqueous extracts of *C. Senegalensis* phenolics content is high with 3.24mg/100g followed by saponins 2.69mg/100g, alkaloids 2.88mg/100g, flavonoids 2.21mg/100g then the least is terpenoids with 1.09mg/100g then the least is Tannins 0.12mg/100g then. In the methanol extract, Tannins content is highest with 3.10mg/100g followed by Alkaloids with 3.12mg/100g flavonoids 2.51mg/100g, phenolics, 2.24mg/100g, saponins 1.69mg/100g and then least is terpenoids 1.61mg/100g. Ethyl ether extract has higher concentration of terpenoids 6.21mg/100g and lower concentration of tannins 4.09mg/100g and the alkaloid content also is not as high as it is in both the aqueous and methanol extract only 0.11mg/100g was estimated. Lastly, the n-hexane extract has contain only 3 phytochemical has highest concentration of terpenes 4.25mg/100g followed by tannins phenolics 2.68mg/100g, flavonoid 1.53mg/100g and then phenolics with 0.98mg/100g. Generally majority of the secondary metabolites are higher in the aqueous and methanol extracts than in the ethyl ether and n-hexane extracts.

The results for cultivation of *plasmodium falciparum* parasite revealed that at the initial stage of cultivation, percentage parasitaemia was 0.5%, the second day it decreases a little bit to 0.45% which may be due to instability of the parasite, the 3rd day it increases to 0.61, to 0.73% on the 4th day from 0.73% it raises of to 1.02 the 5th day and this might be due to then acclimatization of the environment by the parasite. The 6th day it increases to 1.10% and then to 1.87% on the 7th day. The image in plate I-III shows the microscopic visualization of the parasite with their percentage parasitaemia on each day. The plant extracts were screened for anti-plasmodial activity when the parasitaemia level was at its peak using the *in vitro* model at different concentrations, it was found that in all the different extracts, the least activities is at concentration of 6.25 µg/mL as represented in Figure 1. The activities of the extract are concentration dependent since all the extracts demonstrated highest activity at higher. Past studies have revealed that compounds with antimalarial activity are commonly polar¹¹. In a research conducted by Hamilton (2004)^[12], terpenoids have an important role in producing antimalarial activity, proved by the high inhibition percentage of *Diaspyros Jarica* stem bark extract which only contained terpenoids however, result in inhibition percentage greater than 50%. Terpenoids as an antimalarial acts by inhibiting the growth phase of the plasmodium parasite from ring form to trophozoites and inhibited nutrient intake by the parasites by inhibiting the permeation pathway. Flavonoids were thought playing an important role in generating antimalarial

activity. The proof was the inhibition percentage of extracts containing high flavonoids in *Alectryon Senratus* leaves extract. The plant contains flavonoids and has inhibition percentage of 81.1%. Flavonoids work by inhibiting the influx of L-glutamine and myoinosol into the infected red blood cell¹². Anthraquinone could kill the parasites through various mechanisms, resulting from aldehyde at C-2. Alkaloids are one of the major classes of natural products that exhibit antimalarial activity, indeed, quinines; the first antimalarial activity drug belongs to the class

Among the 4 extract methanol has highest activity in all concentration probably due to the higher concentrations of all the phytochemical (alkaloids, terpenoids, flavonoids) that have the inhibition capacity of the malarial parasite activity in different ways. The activity is followed by aqueous extracts, ethyl ether and n-hexane has the least. This explains way the IC₅₀ value of methanol extracts is lower than that of all the 4 extract. The IC₅₀ value is the concentration require to inhibit 50% of the parasite and that as the concentration increases the activity also increases. Since methanol extract can have up to 87.10% inhibition at concentration of 200mg/mL, it means a little quantity is needed for its IC₅₀. N-hexane that has the lowest percentage inhibition capacity among the 4 extracts therefore has the highest IC₅₀ value.

CONCLUSIONS

C. senegalensis revealed the presence of tannins, alkaloid, saponins, flavonoid and phenolic phytochemicals in aqueous and methanol extract. These phytochemicals

were found to be implicated in the anti-malarial activity of the plants and could be what account to such effects in *C. senegalensis*. Methanol and aqueous extracts could therefore be very good potential antimalarial if further investigated

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