Bio-Guided Assay Fractionation of Methanol Extract on Cysteine Protease of *Plasmodium Falciparum*

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ABSTRACT

Many plants are employed traditionally in the treatment of malaria in Nigeria but very few are explored to the fullest. This research work is designed to study the antimalarial activity of Chrozophora senegalensis via cysteine protease inhibitory effect. The extracts of the plant were prepared by cold maceration with 4 different solvents, n-hexane, ethyl ether, methanol, and aqueous. Investigation against malarial cysteine protease with the four extracts shows highest inhibitory activity of the enzyme in the methanol extract with percentage inhibition of 80% and 76%, 29%, and 15% for aqueous, n-hexane and ethyl ether respectively. Fractionation of the most active extract (methanol extract) gave rise to 50 fractions which were pooled to ten different fractions according to their similarities in R_f values. The ten fractions were further tested against the enzyme malarial cysteine protease. Fraction 3 showed highest inhibitory activities which on gas chromatography/mass spectroscopy characterization which revealed the presence of nitro-benzoic acid and ellagic acid alone side other fatty-acids and their derivatives. The best inhibitory activity through the anti- plasmodia effect of the methanol extract of the plant could be due to its cysteine protease inhibitory activity. Further work on fraction 3 will be required to characterized and isolate the active lead compound.

Key words: *chrozophora Senegalensis*, percentage inhibition, anti-plasmodia effect chromatography/mass spectroscopy, inhibitory concentration

INTRODUCTION

Malaria is caused by a parasite in the blood called Plasmodia and a notorious global threat. Five species of Plasmodia genus are implicated in human malaria, of which the most deadly is *Plasmodium falciparum*. They are usually transmitted via the bite of infected female Anopheles mosquitoes in the cause of her blood meal. The disease is principally characterized by fever in uncomplicated cases but become severe within 24 hours if left untreated[1]. Malaria is an Infectious disease that continues to be associated with considerable morbidity and mortality. It was estimated that over 300 million cases of malaria annually in developing countries especially in Africa Sub-Sahara up to 90% case recorded. Malaria kills over one million people yearly mainly children under five years and pregnant women [2]. It is a major health problem in Nigeria and constituted 30% of all out patient attending to health facilities in Northern Nigeria [2]. In most malaria endemic countries, plants are employed in traditional medicine for treatment of the disease. The emergence and spread of plasmodium resistance to antimalarial drugs also possess greater challenge in the global effort to control malaria [3]. Other challenges such as the increased cost of anti-malaria, parasite resistance to most potent orthodox drugs and lack of an effective vaccine, exacerbated and frustrated the global efforts in the control of malaria[3] Therefore this research is designed to study the to study the

antimalarial activity of *Chrozophora senegalensis* via cysteine protease inhibitory effect.

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^{o}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^{O}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.

In-vitro Cultivation of P. falciparum

In-vitro cultivation of P. falciparum is required for adequate parasite for the isolation of the cysteine protease enzyme for the enzyme activity assay and inhibition screening of the plant extracts. The process involved preparation of Culture Medium for Cultivation;

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)[4] 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.

Cultivation, Synchronization and Monitoring of Culture's Growth of P. falciparum

Suspension of (50 % hematocrit) of un-infected cells with CMM (with 15 % serum) was prepared. Appropriate number 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% CO_2 5% O_2 and 90% Nitrogen at 37°C for 24 hours.

After 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 at different stages of development (early and late rings (trophozoites), and early and late schizonts). Synchronization is performed as described by Lambros and Vanderberg[5], by initial Pre-warm of an aliquot of 5% D-sorbitol with complete medium at 37˚C. The culture was centrifuged at 1800 rpm for 5 minutes and the supernatant discarded. The pellet is further re-suspended in 5 volumes of pellet of pre-warmed sorbitol solution, Homogenized and incubated for 5 minutes at 37˚C and further Centrifuged for 5 minutes at 1800 rpm at room temperature. The supernatant Removed, and the residual pellet was washed twice with complete medium. Finally, the supernatant is removed and the pellet is adjusted to a hematocrit of 50%. The resultant mixture was inoculated for further cultivation in a culture flask, adding fresh RBCs to maintain hematocrit at 5% for the synchronous cultures.

Isolation of Cysteine Protease from the Parasites

Triton X-100 temperature-induced phase separation procedures were used in the extraction of plasmodium parasite using Wannapa et al;[6] protocol with modifications as described briefly. The parasite obtained from saponin washed whole blood 5% in-vitro cultivation of clinical isolate of *P. falciparum* was mixed with 0.5 % Triton X-100 in Tris-buffered saline and incubated at 4^{o}C for 90 min. The supernatant obtained on centrifugation at 10,000 x g for 30 min at 4^{O}C was layered on 6% sucrose containing 0.06 % Triton X-100 followed by incubation at 37°C for 5 min. At the end of the initial centrifugation at 900xg for 5 min at 37^{o}C the cytosolic phase was collected and precipitated with cold acetone. Pellets of each preparation were suspended in 6 ml of 50 mM phosphate buffered saline at pH 7.2[7].

Cysteine Protease Activity Assay

The detergent phase of the malaria parasite was used for the enzymatic activity assays as described by[7,8] 50 µl of the enzyme lysate with 500 µl of 100 mM sodium acetate buffer, pH 4.5, and 100 µl of 1% (w/v) N-benzoyl- DL-arginine b-naphthylamide hydrochloride (BANA), The reaction volume was adjusted to 1 ml using distilled water and the Assays was carried out at 37 ºC for an hour. The assay activity was stopped by the adding 200 µl of 20 % (v/v) trichloroacetic acid. Centrifugation process was carried (10,000 x g for 5 min at room temperature) to remove the protein precipitate and absorbance of the supernatant was read at 405 nm.

Cysteine Protease Inhibitor Assay:

The extract inhibitory activity against cysteine protease was assay using N-benzoyl- DL-arginine b-naphthylamide hydrochloride (BANA), as substrate. Aliquots of 500µl from fractions was pre-incubated for 10 min at 37^{0}**C** with 500 µl crude enzyme lysate. The assays was initiated by the addition of 1ml substrate solution (1% (w/v) BaNA), incubated for 20 min at 37^{0}**C**, and 2 ml of trichloroacetic acid (TCA, 20% w/v) was added to terminate the reaction. After 20 min at room temperature, the mixture was centrifuged at 10,000g for 10 min and the absorbance of the supernatant was measured at 405nm.

Thin Layer Chromatography (TLC)

A strip of the pre-coated silica gel was cut out and a spot of the sample was applied on the plate with a dropper about 1.0 cm from the edge. It was then dried using hot air dryer. The strip was lowered into a small chromatographic jar containing the solvent system. The jar was covered with a glass lid and the solvent was allowed to ascend until the solvent front was about ¾ of the length of the strip. The strip was removed and dried by a hot air dryer and viewed under UV light to identify the fluorescing spot. The fluorescent spot was marked and then sprayed with spray reagent: vanillin in concentrated tetraoxosulphate (IV) acid. The strip was placed in hot oven at 110^{0}**C** for 5 seconds for visibility of fluorescent bands. The dark blue colour reaction was recorded and the relative Retention factor (R_f) value was calculated based on the formula described by Patra et al;[11].

$$R_f = \frac{\textit{Distance traveled by the streak from the starting point}}{\textit{distance traveled by the solvent from the starting point to the solvent front}}$$

Column Chromatography

Silica gel was used as the stationary phase while varying solvent combinations of increasing polarity was used as the mobile phase [8, 9]. The slurry was prepared by Mixing 150 g of silica gel and 350 ml of hexane was poured down carefully into the column. The tap of the glass column was left open to allow free flow of solvent into a conical flask below. At the end of the packing process, the column was allowed

24 h to stabilize. The sample was prepared by adsorbing 8.0 g of the extract to 20 g of silica gel 60G in methanol and dried on a hot plate. Sample was continuously stirred to dryness by using Spatula, while guarding against getting it burnt. Dry powder was allowed to cool and gently layered on top of the column. The column tap was opened to allow the eluent to flow at the rate of 40 drops per minute. Elution of the extract was done with solvent systems of 0.2:9.8 methanol to chloroform; the eluted fractions were then collected in aliquots of 1 ml in test tubes. . Similar fractions were pooled together according to the TLC profile, which were then used for further in-vitro anti-plasmodia activity. The lead fraction obtained was characterized using GC/MS methods.

RESULTS

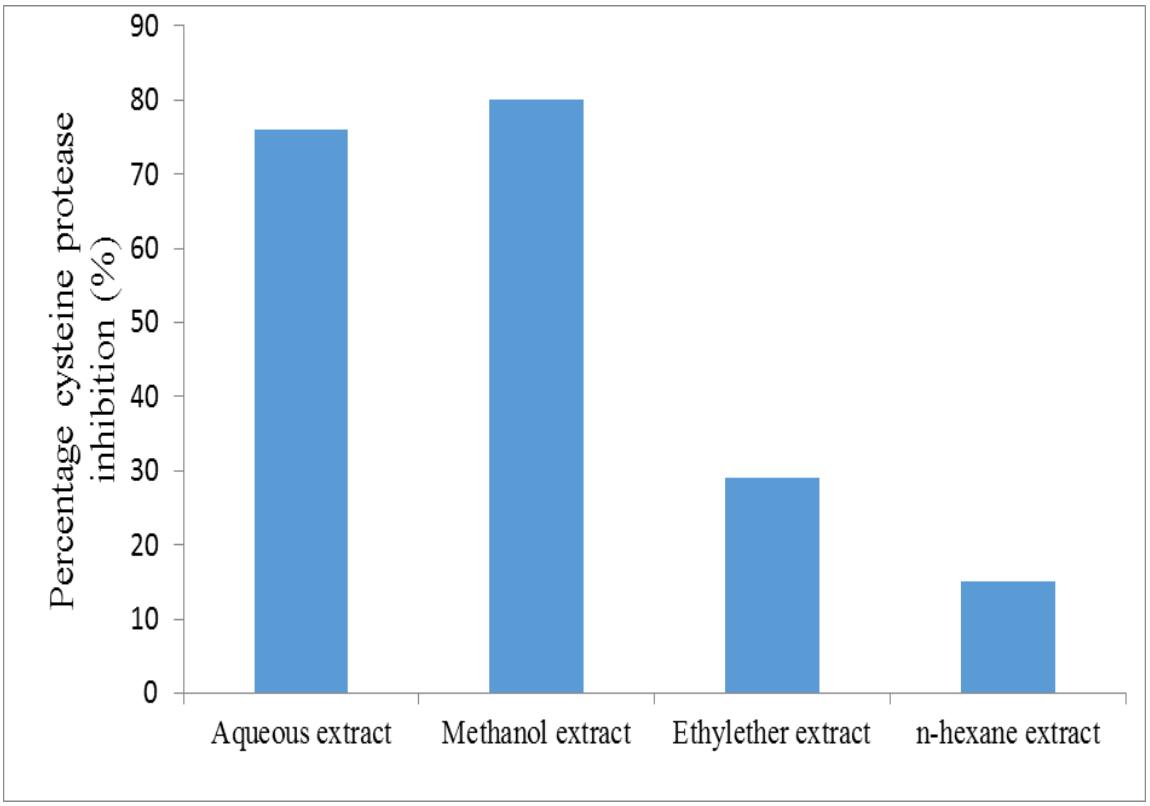

Figure I: Percentage Cysteine Protease Inhibition of Aqueous, Methanol, Ethyl ether and N-hexane Extracts of *C. Senegalensis*

Table I: R_f Values for the 50 Fractions of the *C. Senegalensis* Methanol Extracts and the Pooling to 10 Sub-fraction.

	Initial fraction	R_f 1	R_f 2	R_f 3	R_f 4
Fraction 1	1	0.78	0.75	0.64	0.6
	2	0.79	0.72	0.67	0.58
	3	0.81	0.71	0.6	0.56
	4	0.86	0.8	0.59	0.54
	5	0.81	0.76	0.59	0.55
	6	0.84	0.73	0.64	0.58
Fraction 2	7	0.71	0.68	0.59	0.44
	8	0.68	0.55	0.49	
	9	0.72	0.67	0.61	0.48
	10	0.72	0.61	0.57	0.46
Fraction 3	11	0.58	0.45	0.33	0.29
	12	0.58	0.42	0.35	0.3
	13	0.6	0.47	0.29	0.27
	14	0.56	0.49	0.32	
	15	0.55	0.51	0.36	
Fraction 4	16	0.57	0.29		
	17	0.61	0.32	0.26	
	18	0.57	0.27		
Fraction 5	19	0.36	0.31	0.19	
	20	0.36	0.32	0.21	
	21	0.41	0.35	0.26	
	22	0.44	0.33	0.18	
	23	0.39	0.29		
	24	0.38	0.31		
Fraction 6	25	0.22	0.17		
	26	0.27	0.19		
	27	0.3	0.21	0.19	
Fraction 7	29	0.19	0.15	0.12	
	30	0.17	0.15	0.11	
	31	0.19	0.17		
	32	0.18	0.14		
	33	0.2	0.16	0.12	
Fraction 8	34	0.13	0.09	0.05	
	35	0.14	0.08		
	36	0.14	0.08	0.05	
	37	0.12	0.06		
Fraction 9	38	0.07	0.05		
	39	0.06	0.04		
	40	0.06	0.03		
	41	0.06	0.04		
	42	0.08	0.05		
	43	0.09	0.05		
Fraction 10	44	0.04			
	45	0.03			
	46	0.04			
	47	0.05			
	48	0.03			
	49	0.05			
	50	0.04			

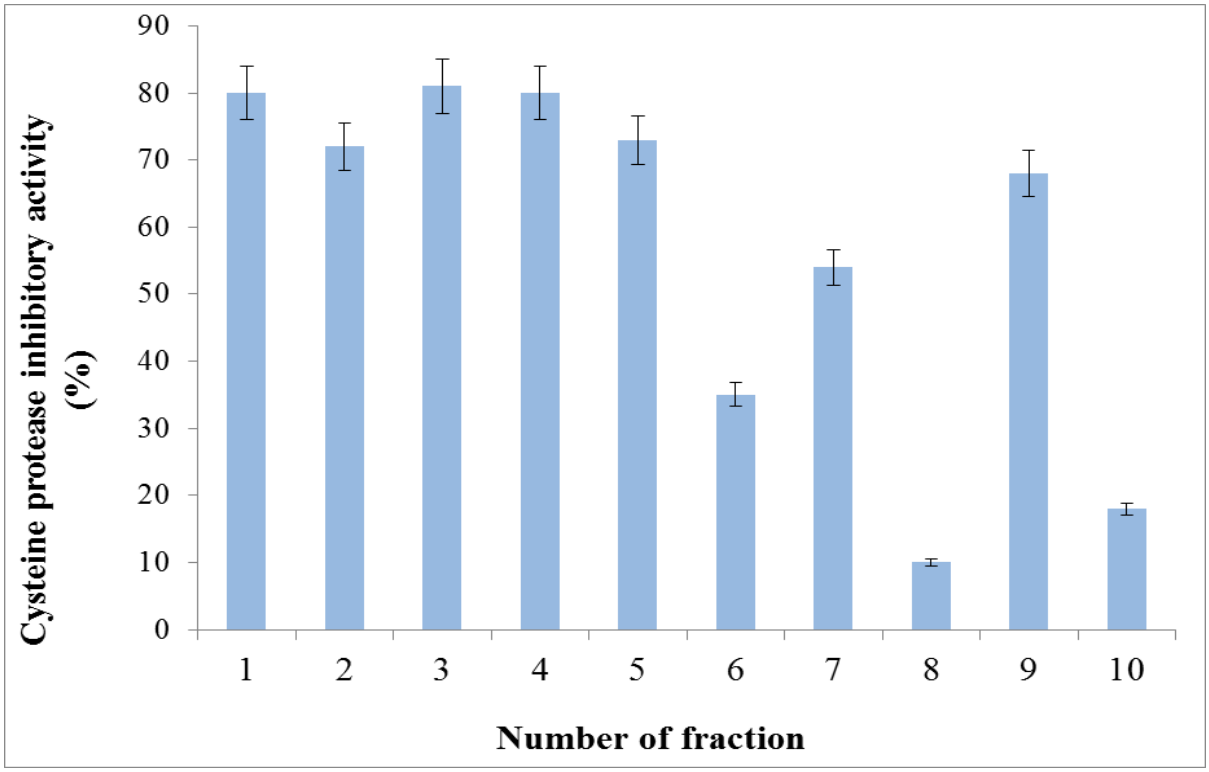

Figure II: Percentage Cysteine Protease Inhibitory Activity of the 10 Fractions of Methanol Extracts of *C. Senegalensis.*

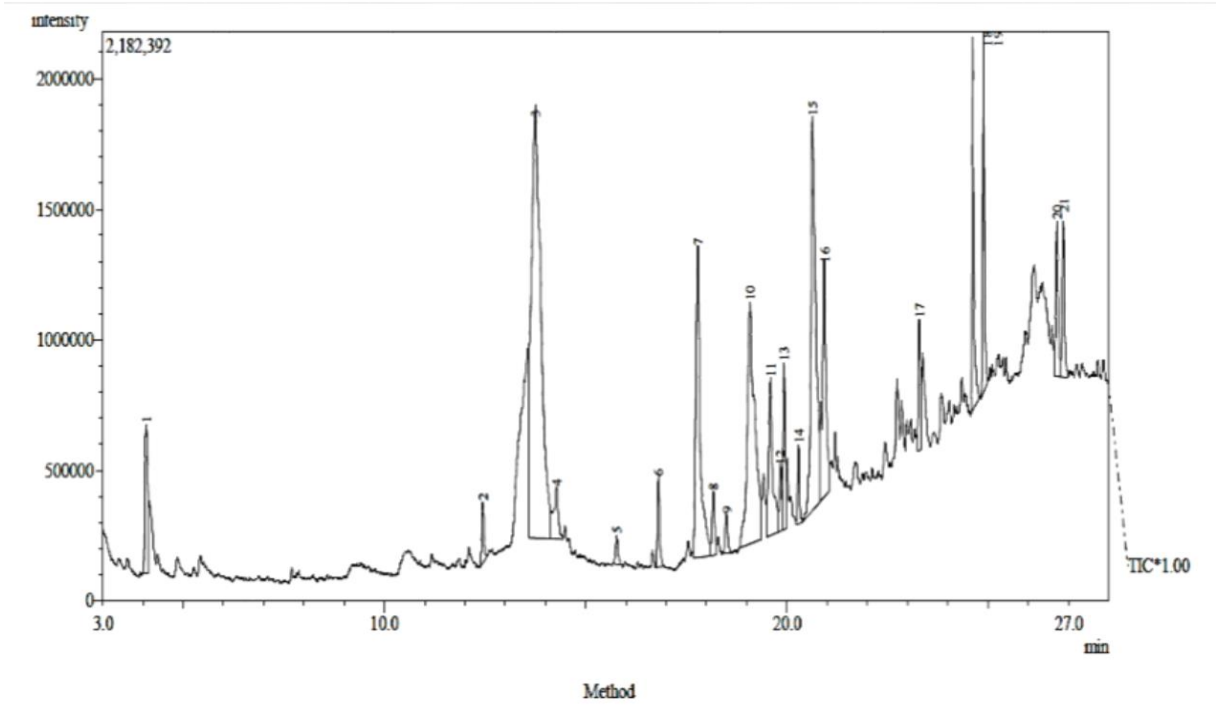

Figure III: the GC/MS Spectra of the Most Potent Fraction of Methanol Extract of *C. senegalensis.*

Table II: The Acids in the GC/MS result of the Methanol Most Active Extract

S/No	Retention time	Common name	Similarity Index
1	12.45	Palmitic acid	89
2	14.26	Petroselinic acid	80
3	16.81	Isopalmitic acid	92
4	18.51	Stearic acid	89
5	19.08	Oleic acid	94
7	19.83	Linoleic acid	88
9	20.29	Hexyamine	80
10	23.29	Phthalic acid	83
11	24.88	Nitrobenzoic acid	84
12	26.88	Ellagic acid	97

Cysteine Protease Inhibition of the Aqueous, Methanol, Ethyl Ether and N–Hexane Extracts of C. Senegalensis

The result of the inhibition of the enzyme cysteine protease by the crude extracts of the 4 solvents, aqueous, methanol ethyl ether and n–hexane of *C. Senegalensis* is represented in Figure 1. It shows that methanol extract has the highest enzyme inhibition activity, while that of n-hexane was the least. However, mean values when compared statistically there was no significant difference (P>0.005) between the aqueous and methanol extracts, but significant difference exist between the mean values of ethyl ether and the n-hexane extracts (P<0.005)

R_f Values for the 50 Fractions of the C. Senegalensis Methanol Extracts Pooled to10 Fractions.

The result of the thin layer form the column chromatography fractions are represented in Table 1: the result shows the R_f values of the 50 fractions of the most active methanol extract of C. Senegalensis. The fractions

were pooled based on their similarities in R_f values. Fractions 1-6 have 4 different R_f values ranging between 0.71-0.86, R_F 2 from 0.68-0.8, Rf3 from 0.59-0.67 and Rf 4, 0.44 - 0.68. While, fractions 7-10 were pooled as a single fraction 2, with different R_f values ranging between 0.46 - 0.74, fraction 11-15 on the other hand have values between 0.27 - 0.58 which were all pooled together as fraction 3 (Table 1).

Similarly, Fractions 16-18 were pooled as fraction 4 with R_f values of 0.26-0.61, fractions 19-24 have R_f values of 0.18-0.44 and are pooled as fraction 5, fraction 25-28 were pooled as fraction 6 and the R_f values are 0.19-0.27, while fraction 29-33 gives fraction 7 with R_f values of 0.11-0.19. Fraction 8 was pooled from fractions 34-37 with R_f values of 0.05-0.14, while fractions 38-43 and 44-50 were pooled as fraction 9 and 10 with R_f values 0.03-0.09 and 0.03-0.05 respectively (Table 1).

Cysteine Protease Inhibition of the Methanol Fractions

The result of cysteine protease inhibition tested for fractions collected and pooled after column chromatography of the methanol extract the most active shown in figure 2. The result indicated Fraction 3 to have the 81% inhibition the highest inhibition value. However, no significant difference ($p<0.05$) observed statistically between the mean percentage values of Fractions 1,2,3,4 and 5 in their anti- cysteine protease activity however, their values differ significantly with the rest of the fractions.

DISCUSSIONS

Effective extraction from the dried plant material was achieved using different solvent. The dried plant material was extracted using water and some organic solvents system (methanol, ethylether and n-hexane) to obtain the bioactive compounds present in the plant under pharmalogical investigation.

Extensive evidence suggests that the degradation of hemoglobin is necessary for the growth of erythrocytic malaria parasites, apparently to provide free amino acids for parasite protein synthesis [9, 10]. In *P. falciparum*, hemoglobin degradation occurs predominantly in trophozoites and early schizonts, the stages at which the parasites are most metabolically active. Incubation of cultured *P. falciparum* parasites with the protease inhibitor leupeptin caused trophozoite food vacuoles to fill with apparently un-degraded erythrocyte cytoplasm [11][12]. Analysis of the leupeptin-treated parasites showed that they contained large quantities of un-degraded globin, while minimal globin was detectable in control parasites[13]. Leupeptin inhibits both cysteine and some serine proteases, but the highly specific cysteine protease inhibitor E-64 also caused un-degraded globin to accumulate. After parasites were incubated with inhibitors of other classes of proteases including the aspartic protease inhibitor pepstatin[14], globin did not accumulate. More recent studies indicated that used non-denaturing electrophoretic methods demonstrated that cysteine protease inhibitors not only blocked malarial globin hydrolysis, but also inhibited earlier steps in hemoglobin degradation, including denaturation of the hemoglobin tetramer and the release of heme from globin[15]. Another study showed that E-64, but not pepstatin, inhibited the production of hemozoin (the malarial product of heme) by cultured parasites [16]. These results suggest that a cysteine protease is required for initial steps in hemoglobin degradation by *P.*

falciparum. In this research results, even though the exact mechanism of action of inhibition is not known, the methanol extract had the highest activity in cysteine-protease inhibition test. This shows the tendency that the plant extract anti-plasmodia activity in a research conducted by Isyaku et al;[17] is due to cysteine protease enzyme inhibition so also the level of activity is in conformity as observed by Isyaku et al;[17] that of anti-plasmodial activity in their work that is from methanol to aqueous, ethylether and then n-hexane had the least activity.

Bio guided assay and sequential fractionation of the methanol extract increases the specific activity of the enzyme which could be attributed to the removal of interacting protein components attached to the enzyme. Such observed inhibition implies that the group of compounds in the extract through individual or synergistic action inhibited the enzyme activity. The fractions of the most active fraction of the methanol extract on column chromatography collected and pooled after has exhibited 81% inhibition, which give the highest cysteine protease inhibition but exhibited no significant difference at ($p<0.05$) with the average percentage anti-cysteine protease activity of other fraction. The non-significant difference between the fractions and the most active fraction signal possible anti- cysteine protease activity potential of other fractions and the possible composition of similar lead compounds in the fractions. The inhibition activities of cysteine protease demonstrated dose dependent activity which implies that the higher the concentration of the compounds in the fractions the higher the inhibition of the enzyme activities and the lower the virulence posed by the parasite. Characterization of fraction three that shows the best inhibitory activity through gas chromatography/mass spectroscopy revealed the presence of nitro-benzoic acid and ellagic acid alone side other fatty-acids and their derivatives which shows a high binding strength towards the enzyme cysteine protease. Ellagic acid was tested for antiprotozoal activity against *Entamoeba histolytica* and *Giardia lambia* and was found to be very effective[18]. These compounds could serve as a good source of antidote for malaria and could as well guide the design of a novel drug of malaria.

CONCLUSIONS

The result shows that methanol extract fractions were found to have some level of inhibition in the activity of the cysteine protease enzyme with 80.1% inhibition capacity, this study has scientifically validated probable principle and site of action in the use of *Chrozophora senegalensis* in the treatment of malaria and having explored the antimalarial properties in the plant extracts with the active compounds identified which may be effective therapeutic agents for the treatment and management of malaria.

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