International Journal of Biological and Pharmaceutical Sciences Archive

ISSN: 0799-6616 (Online)

Journal homepage: https://ijbpsa.com/

(Research Article)

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Phytochemical investigation, *in-vitro* antiplasmodial assessment and cytotoxic effect of ethanol extract of whole plant of *Crotalaria arenaria* (Benth)

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International Journal of Biological and Pharmaceutical Sciences Archive, 2024, 07(02), 013–023

Publication history: Received on 24 February 2024; revised on 05 April 2024; accepted on 08 April 2024

Article DOI: https://doi.org/10.53771/ijbpsa.2024.7.2.0033

Abstract

Crotataria arenaria (Benth) is a native plant from Northern part of Nigeria which is employed as antimalarial herb in traditional medicine in the communities where it grows. The phytochemical composition, *In-vitro* antiplasmodial activity and *in-vitro* cytotoxic effect of the extract and fractions of the whole plant of *Crotalaria arenaria* were investigated. The mature and fresh whole plant of C. arenaria were collected, air dried and pulverized. The pulverized form of the plant were soaked in ethanol for two weeks and later decanted, filtered and concentrated using rotatory evaporator at 40^{o}C and evaporated to dryness and labeled CRA-1. The dried ethanol solid extract (CRA-1) was patitioned/fractionated using water, chloroform, ethyl acetate, 18% aqueous methanol, n-hexane which afforded chloroform fraction (CRA-1-01), water fraction (CRA-1-02), ethyl acetate fraction (CRA-1-03) aqueous methanol fraction (CRA-1-04) and n-hexane (CRA - 1- 05) and they were dried and tested for their photochemicals and *In-vitro* antiplasmdial activity against Plasmodium falciparum strain (K1). The ethanol extract and its fractions were found to significantly inhibited/suppressed multi-drug resistant strain of P. falciparium (K1) in the range of 86.73% - 65.31% at varied concentrations of 5000 µg/ml, 2000 µg/ml, 1000 µg/ml and 500 µg/ml. The ethanol extract and its fractions showed the presence of tannins, saponins and alkaloids which could be responsible for the *In-vitro* antiplasmodial activity of the whole plant extract of C. *arenaria* and each of its fractions. Ethanol crude extracts of whole plant of *Crotalaria arenaria* and its fractions were investigated for cytotoxicity in Brine shrimp lethality Test (BSLT). Larvicidal activities were noticed with dosage concentration from 1000 µg/ml down to 10 µg/ml of the plant extract and all its fractions. The relative cytotoxic effects of extract and fractions obtained from whole plant of *Crotalaria arenaria* were found to be dose-dependent because higher dosage brought about an increase in mortality of *Artemia salina* Leach. Sea salty water of the crude ethanol extract and its fractions served as the negative control. The chloroform fraction was observed to be most cytotoxic killing 30% of brine shrimp larvae at lowest concentration of 10 µg/ml with average percentage lethality of 46.67%. Aqueous methanol and ethanol crude extract appears to show similar and closely related activities against brine shrimp larvae at highest concentration of 1000 µg/ml. The ethanol crude extract and aqueous methanol fractions are toxic at higher dosage as they were found to induce significant mortality effect (*In-vitro*) against *Artemia salina* Leach particularly at concentration of 1000 µg/ml and therefore not safe for oral consumption especially at higher uncontrolled doses but at relatively low/moderate doses. The findings in this present study demonstrated that the extract of *C. arenaria* (whole plant) and fractions have significant in vitro antiplasmodial activity and this has justified the traditional use of the whole plant extract of *Crotaria arenaria* in the folklore medicine to cure malaria related illness. The results obtained in this study may serve as key data for utilization of *Crotalaria arenaria* in the development of bioactive agents which could be used as antimalarial, anticancer, pesticidal, insecticidal and anti-proliferation agents. The study recommended in-vivo antiplasmodial assay of the extract of the whole plant of *Crotalaria arenaria* to further substantiate the findings of this present study.

Keywords: Phytochemical Investigation; *In-vitro* Cytotoxic Effect; *In-vitro* Antiplasmodial Assessment; Malaria; *Crotalaria arenaria* (Benth)

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1. Introduction

Malaria infects nearly one third of the population of the world in one hundred and four tropical countries and regions where it has been considered as endemic disease [1]. Malaria is caused by parasitic protozoans of the *Plasmodium* genus and is one of the frontline infectious diseases in multiple tropical regions which include Nigeria, where infection occurs all year round [2]. There are five known species in the *Plasmodium* genus responsible for malaria in human namely: *P. falciparum*, *P. ovale*, *P. ovivax*, *P. Knowles* and *P. malariae*. The precarious and commonest kind of malaria is caused by *Plasmodium falciparum,* and *Plasmodium vivax* do not cause malaria in Africa but are common causes of malaria in Oceania, Asia and Latin America. *P. malariae* and *P. ovale* malaria infections are rare [3]. *Plasmodium falciparum* has brought about the highest number of mortality from malaria [4] majorly due to two main complications: anaemia and cerebral malaria [5,4]. Malaria is of great public health importance being the most occurring infectious disease in the tropical and subtropical regions of the world [6]. The World Health Organization (WHO) reports that malaria is a deadly parasitic disease which causes almost 90% of mortalities in Africa [7]. In 2018, the WHO estimated that about 3.2 billion people across 91 nations are at risk of malaria infection [8,].

Treatment of malaria by the use of modern drugs such as chloroquine, primaquine amongst others have been reported to be ineffective as a result of the fact that they are being faced by resistance of the malaria parasite (*Plasmodium falciparum)*. Thus, new drugs with antimalarial activity exhibiting novel mode of actions are being investigated. Natural products have played a dominant role in the discovery of leads for the development of drugs to cure human diseases including malaria and this fact expects that new antimalarial drugs/leads may obviously spring forth from tropical plant sources with medicinal values/properties [10]. One of the plant sources with therapeutic properties is *Crotalaria arenaria* (Benth). Even though the use of plants with medicinal value to cure malaria disease and cancer is widely known throughout human history and despite the fact that their usage in Nigeria especially, northern part of the country has been reported [11,12,13]. Researches on plants for their biologically active secondary metabolites have become necessary as a result of the significant relationship between their applications in folklore medicine and the noticed bioeffects of their extracts [14]. This is due to the fact that the toxicity assessments of African plants with therapeutic properties have been carried out but not exhausted [15,16]. In our quest to identify natural products with bioactivities, the present study aimed at phytochemical investigation, *In-vitro* cytotoxic effect and antiplasmodial assessment of ethanol extract of whole plant of *Crotalaria arenaria* (Benth**)**. The special interest in this plant was due to its usage in Otukpo in Benue State, Kanuri and Hausa traditional medicine [17] for the treatment of malaria fever and coupled with the fact that few species of Crotalaria genus have been known for their high toxicity due to the presence of Pyrrolizidine alkaloids in the plant. Pyrrolizidine alkaloid, a poisonous/toxic compound has been found to be present in Crotalaria species and according to WHO report, many species of the genus have been reported to be toxic with epidemic outbreaks in some parts of the world [18,19]. It was reported that only few species of the genus Crotalaria have been studied and WHO have labelled the Pyrrolizidine alkaloid as a very important toxicant and have made a request to researchers to investigate all species of the plants that might contain the alkaloid [18]. Despite the medicinal importance of certain Crotalaria species, they might likely be poisonous due to the presence of Pyrrolizidine alkaloids as it has been acknowledged that Crotalaria poisoning is much under reported as a result of fewer information on the pharmacognostic parameters for identification of the plant. The toxicity of Crotalaria species appears to be known among veterinary and medical practitioners, however, patients have been advised to stop taking the herbs. Pyrrolizidine alkaloids (PAs) are considered to be important secondary metabolites largely on account of their biological activities, which include acute hepatotoxic [20] mutagenic [21], carcinogenic [22] teratogenic [23], anticancer [24] and neuroactive properties [25] amongst others. Brine shrine test (BST) which is employed in this study is well known to be utilized as a simple and rapid bioassay procedure to investigate extract from the plant and its entire fractions for their *In-vitro* cytotoxicity properties and potentials.

2. Materials and method

2.1. Chemical Reagents, Chemicals and Apparatuses/Equipment's

Analytically graded solvents, chemicals and chemical reagents used in this research work include; Ethanol (Aldrich), Petroleum ether (Aldrich), Chloroform (Aldrich), Ethyl acetate (Aldrich), Methanol (BDH), Potassium iodide, Silica gel powder, DMSO, Sodium hydroxide, Lead Ethanoate, Aqueous Ammonia , Ferric chloride, gentamycin injection, Gemsta stain, candle wax, Hydrochloric acid, Basic Bismuth Nitrate, Glacial acetic acid and Potassium hydroxide.

Apparatuses used include; Round bottom flasks, separation funnel, Conical flasks, Beakers, Test tubes. Measuring cylinder, Weighing balance, Chromatographic column, Spatula, media, glass slides, cover slides , anaerobic bell jar glass, microscope EDTA bottles, insulin syringes, Pasteur pipette, autoclave, centrifuging machine, and labeling sticker.

2.2. Plant materials

2.2.1. Collection, Authentication and Preparation of Plant Materials

Fresh and matured whole plant samples of *Crotalaria arenaria* (Benth) were collected on 10th of June, 2015 from Utukpo in Benue State. The plant was authenticated with voucher No. 068 at the herbarium of University of Agriculture, Makurdi, Benue state, Nigeria. The whole plant collected were thoroughly washed with clean water before being dried under shade at room temperature for 21 days until whole plant were completely crispy and dried. The whole plant sample was ground into powdered form and subjected to further analysis.

2.2.2. Preparation and Fractionation/Partitioning of Plant Extract

Two hundred and fifty grams (250 g) of fine powder/pulverized form of the whole plant leaves were separately weighted and soaked in 1.5 litres of 96% ethanol in a sample bottle with occasional shaking for 2 weeks. Ethanol liquid crude extract was then decanted and filtered using No. 1 Whatman filter paper of 110 mm diameter, and then concentrated with the aid of rotatory evaporator at 40 ^{0}C under reduced pressure in vacuum to give a residue (concentrate) which was allowed to dry properly. This was weighed and labeled as CRA-1. CRA-1 was partitioned between chloroform and distilled water (100ml, 1:1) in a separating funnel. After chloroform layer was removed, the aqueous layer was further washed with ethyl acetate (100 ml) and thereafter separated from ethyl acetate layer. The chloroform, water and ethyl acetate layer were labeled as CRA-1-01, CRA-1-02 and CRA-1-03 respectively. The ethyl acetate fraction CRA-1-

03 and chloroform fraction CRA-01 were evaporated to dryness at room temperature while water fraction (CRA-1-02) was freeze dried. Chloroform soluble fraction (CRA - l -01) was further partitioned between 18% methanol and n-hexane (100 ml, 1:1) in a separating funnel. The methanol and n-hexane layer in the separating funnel were separated by running methanol fraction layer first into a beaker followed by n- hexane layer into another separate beaker and were allowed to evaporate to dryness at room temperature and coded as CRA - l -04 and CRA-1-05 respectively. The extract and each of its dried fractions were weighed, stored in a refrigerator until screened for their phytoconstituents, *in vitro* antiplasmodial and cytotoxic activity.

2.3. Phytochemical Screening of Ethanol Extract and Fractions

2.3.1. Preparation of Test Solutions/Samples for Phytochemical Screening

Dried ethanol crude leaf extract and its fractions were separately dissolved in 25 ml of the organic solvent used for their extraction or partitioning/fractionation and the liquid solutions obtained constituted the test solutions. Thereafter, each of the test solutions/samples was subjected to phytochemical screening for the presence or absence of secondary metabolites using standard procedures [26,27,28] The secondary metabolites investigated were alkaloids, saponins, tannins, flavonoids and terpenoids.

2.4. Malaria Parasite Bioassay of the Extract and Fractions

2.4.1. Preparation of test solutions

A stock solution of 10,000 µg/ml was prepared by dissolving 20 mg of plant extract and fractions in 2 ml of Dimethyl sulphoxide (DMSO). Solution of 500 µg/ml, 1000 µg/ml, 2000 µg/ml, and 5000 µg/ml were prepared from the stock solution by serial dilution.

2.4.2. Source of Malaria Parasite for Assay

Infected human blood samples containing parasitaemia of *Plasmodium falciparum* were collected from Bayero Univesity Clinic, Murtala Mohammed Specialist Hospital, Kano and Aminu Kano Teaching Hospital, Kano in K3-EDTA coated disposable plastic sample bottles tightly fitted with plastic corks [8].

Venous blood samples from patients recommended for malaria parasites test (MP Test) using 5 cm^3 disposable plastic syringes and needles (BD and 20 SWG) was collected and the samples were immediately transferred into K3-EDTA disposable plastic sample bottles with tightly fitted plastic corks and mixed thoroughly and then transported to the Microbiology laboratory at Bayero University in a thermo flask containing water maintained at 40 ^{0}C as demonstrated by [29].

2.4.3. Determination of Plasmodium falciparum (Positive Blood Samples) Using Thin Smear Method)

The method followed was described by Lewis [29]. Using a clean capillary tube, a small drop of each blood sample was placed at the center of a clean glass slide at least 2 mm from one end. A cover slip was placed at angle 45 °C in front of each drop and drawn backwards to make contact with each drop. The drop was run along the full length of the edge of the cover slip. Smears were formed by moving the cover slip forward on glass slide. The thin smears were immersed in 30 ml of methanol contained in a Petri dish for 15 minutes. Geimsa stain was dropped on each smear and allowed to stay for about 10 minutes.

Excess stain was washed with clean tap water. The smears were air dried by hanging the glass slides inverted in a rack. Each dried smear was microscopically observed under a high power objective (x 100) using oil immersion after which an average parasitaemia was determined using the reading of 3 microscopic fields [30].

2.4.4. Separation of the Erythrocytes (5% Parasitaemia) from the Serum of the Blood Samples

50% Dextrose solution (0.5 ml) was added to each of 5ml defibrinated blood sample and then centrifuged at 2500 rpm for 15 minutes in a spectral merlin centrifugation machine. Supernatant layers were separated from the sediments. The later was diluted with 2-3 drops normal saline solution as demonstrated by Lewis [29] and further centrifuged at 2500 rpm for 10 minutes. The resulting supernatants were discarded. Samples with higher parasitaemia (above 5%) were diluted with fresh malaria parasite negative erythrocytes [30].

2.4.5. Preparation of Plasmodium falciparum Culture Medium

Venous blood (2 ml) from the main vein of white healthy rabbit's pinnae was withdrawn using a disposable 5ml syringe (BD 205 WG). This was defibrinated by allowing it to settle for at least one hour [28]. The defibrinated blood was centrifuged at 1500 rpm using spectre merlin centrifuge for 10 minutes and the supernatant layer was collected in a sterilized tube. The sediment was further centrifuged at 1500 rpm for five minutes, and the supernatant layer was added to the first test tube. The sediments were discarded and the serum collected was supplemented with the salt of RPMI 1600 medium (KCl 5.37mM, NaCl 10.27mM, $MgSO_4$ 0.4mM, $NaHPO_4$ 17.73mM, $Ca(NO_3)_2$ 0.42 mM, $NaHCO_3$2.5 mM, and glucose 11.0 mM. (BDH Ltd, UK) as demonstrated by Devo *et a*l[31]. The medium was sterilized by 40 µg/ml gentamicin sulphate [32].

2.4.6. In-vitro Bioassay of the Activity of Ethanol Extract and its Fractions on Plasmodium falciparum Culture

A 0.1 ml of test solution and 0.2 ml of the culture medium were added into a tube containing 0.1 ml of 5% parasitaemia erythrocytes and mixed thoroughly. The sensitivity of the parasites to the test fraction/samples was determined microscopically after incubation for 24, 48 and 72 hours at 32 ^{0}C. The incubation was undertaken in glass bell jar containing a lighted candle to ensure the supply of required quantity of Carbon (iv) oxide, CO_2 of about 5%, O_2 gas, 2% and nitrogen gas, 93% as demonstrated by Muktar *et al*[33].

2.5. Determination of the Activity

At the end of the incubation period of 72 hours (3 days), a drop of a thoroughly mixed aliquot of the culture medium was smeared on microscopic slides and stained using Giemsa staining techniques. The mean number of erythrocytes appearing as blue discoid cells containing life rings of the parasite (that appeared red pink) was estimated and the percentage elimination by the samples was determined. The activity of the tested samples was calculated as the percentage elimination of the parasites after incubation period of 72 hours, using the formula below:

$$\% = N/Nx \times 100$$

Where, % = Percentage Activity of the Extracts/Fractions
N = Total number of cleared RBC after 72hrs, Nx = Total number of parasitized RBC
Note: RBC= Red Blood Cells [33].

2.6. Brine Shrimp Lethality Test of Ethanol Crude Extract and its Fractions

Artermia salina brine shrimp lethality test was done to assess the cytotoxicity of the plant extract and each of its fractions. Brine shrine eggs were introduced to a hatching chamber which contains sea water (300 ml). The chamber contains the eggs which were positioned under artificial light emitted from an inflorescent bulb for twenty four hours, thereafter, which were expected to hatch into larvae. Twenty milligrams of each of the extract and its fractions (CRA-1,CRA-1-01, CRA-1-02, CRA-1-03, CRA-1-04, CRA-1-05) and positive control ($K_2Cr_2O_7$) was separately dissolved in methanol (2ml) and distilled water to form stock solutions of the crude extract, fractions and positive control

(Potassium heptaoxodichromate (VI)) respectively. A micropipette was used to measure and transfer 5 µl, 50 µl and 500 µl of each test samples of the extract, fractions and positive control into vials which are equivalent to 10 µg/ml, 100 µg/ml and 1000 µg/ml respectively and the solvent were allowed to evaporate to dryness completely. Each dose was assayed in triplicate in vials (9 per test solution/sample) containing each of the extract and its fractions. The negative control containing 5 µl, 150 µl and 500 µl of salty sea water as well as 5 µl, 50 µl, 500 µl of positive control containing $K_2Cr_20_7$ were all allowed to evaporate to dryness for forty eight hours at room temperature. Sea water (about 4.5 ml) was introduced to each test vial, followed by 1-2 drops of DMSO (Dimethyl suphoxide) to facilitate the solubility of the test extract and fractions. Ten (10) *Artemia salina* Leach larvae (taken 24-48hrs after the commencement of hatching of the eggs) were transferred using a Pasteur pipette to each of the vials containing test sample including the positive and negative control. The volume of solution in each of the test vials was finally topped up to 5 ml with salty sea water following the introduction of the larvae of the shrimps. The larvae of the shrimps were counted against lighted background. Counting of deaths of larvae began immediately 24 hours after commencement of bioassay/test. Naupli (Larvae) were regarded to be dead if they were not moving at the base of the vials. The total number and average percentage of mortality/death at each dosage of test sample (solution), positive and negative control were determined [34]. For each of the test samples/solutions, positive control and negative control, their percentage values for elimination of the *artemia salina* Leach larvae by test samples under study as well as positive and negative control were taken and recorded.

3. Results

The pulverized whole plant samples (250 g) yielded 6.5 g crude extract with percentage yield of 3.7%. From physical/colour point of view, it was observed that the ethanol crude extract, chloroform, methanol and n- hexane fractions were dark green with exception of water and ethyl acetate soluble fractions which has a brownish and greenish colour respectively. In terms of texture, the crude extract and fractions were sticky except ethyl acetate which was crystalline solid (Table 1).

Table 1 Weight, Appearance and Colour of Ethanol Extract and its Fractions

Extract & Fractions	Code	Weight	Nature of appearance	Colour
Ethanol Extract	CRA-1	17.5g	Sticky	Dark-green
Chloroform fraction	CRA-1-01	3.0g	Sticky	Dark-green
Water fraction	CRA-1-02	2.6g	Sticky	Brownish
Ethyl acetate fraction	CRA-1-03	2.4g	Crystalline solid	Greenish
Aqueous Methanol fraction	CRA-1-03	2.8g	Sticky	Dark-green
n-hexane fraction	CRA-1-03	3.1g	Sticky	Dark-green

Phytochemical investigation of the plant extract and fractions revealed the presence of bioactive secondary metabolites such as saponins, tannins and alkaloids. The ethanol crude and Chloroform fraction showed the presence of tannins, saponins and alkaloids. Saponin was also present in water, aqueous methanol and n-hexane fractions. Flavonoids and terpenoids were conspicuously absent in the ethanol crude extract and its fractions (Table 2).

Table 2 Results of Phytochemical Investigation of Ethanol Crude Extract and its Fractions

Phytochemical constituents	CRA-1	CRA-1-01	CRA-1-02	CRA-1-03	CRA-1-04	CRA-1-05
Tannins	+	+	-	-	-	-
Saponins	+	+	+	-	+	+
Alkaloids	+	+	-	-	-	-
Flavonoids	-	-	-	-	-	-
Terpenoids	-	-	-	-	-	-

Key: **+ =** present. - = absent

Table 3 Results of *In-vitro* Antiplasmodial Assessment of CRA-1 to CRA-1-05

Extract/Fraction	Parasitemia Initial Count Per Dose	Conc. (µg/ml)	Parasitemia Final Count Per Dose	No of Parasitemia Mortality After 72 hrs Per Dose	% Elimination of Parasites at the end of 72 hrs of Incubation Per Dose/Conc.
Artemether and Lumenfantrine (+ control)	98	5000	6	92	93.80
	98	2000	10	88	89.80
	98	1000	12	86	87.76
	98	500	16	82	83.67
CRA-1	98	5000	13	85	86.73
	98	2000	17	1	82.65
	98	1000	23	75	76.53
	98	500	27	71	72.45
CRA-1-01	98	5000	10	88	89.80
	98	2000	16	82	83.67
	98	1000	19	79	80.61
	98	500	27	71	72.45
CRA-1-02	98	5000	14	84	85.71
	98	2000	18	80	81.63
	98	1000	25	73	74.49
	98	500	37	61	62.24
CRA-1-03	98	5000	16	82	83.67
	98	2000	19	79	80.61
	98	1000	27	71	72.45
	98	500	34	64	65.31
CRA-1-04	98	5000	10	88	89.80
	98	2000	12	86	87.75
	98	1000	17	81	82.65
	98	500	21	77	78.57
CRA-1-05	98	5000	13	85	86.73
	98	2000	17	81	82.65
	98	1000	21	77	78.57
	98	500	25	73	74.49

Key: CRA-1: Ethanol Crude Extract; CRA-1-01: Chloroform fraction; CRA-1-02: Water fraction; CRA-1-03: Ethyl acetate fraction; CRA-1-04: Aqueous Methanol fraction and CRA-1-05: n-hexane fraction.

The *In-vitro* antiplasmodial evaluation of the ethanol crude extract and its fractions were carried out and the activities of the test solutions/samples were calculated as the percentage elimination of the malaria parasite after incubation periods of 24, 48 and 72 hours. The ethanol crude extract and each of its fractions showed certain degrees of antiplasmodial activity (Table 3). At concentration of 5000µg/ml, the ethanol crude extract exhibited highest parasitic elimination of 86.73%, followed by chloroform and methanol fractions indicating equipotency of 89.80%, n-hexane fraction (86.73%), water soluble fraction (85.71%) and ethyl acetate fraction (83.67%). At a dose of 2000µg/ml, methanol fraction had parasitic elimination of (87.75%), chloroform fraction (83.67%), water fraction (81.63%) and ethyl acetate fraction (80.61%) which reveals strong activity against multidrug resistant *Plasmodium falciparum* strain K1 (*In-vitro*). Considering the lowest dose of 500 µg/ml, methanol fraction eliminated (78.57%) of the parasites, which was the highest percentage suppression while water fraction with (62.24%) exhibited the lowest percentage antiplasmodial activity which implies that methanol fraction is the most bioactive fraction in this range. The positive control displayed antimalarial activity of 93.80% at 5000 µg/ml, and percentage inhibition of 83.67% at 500 µg/ml,

which is higher but not significant than the effects of test extract and each of its fractions on *Plasmodium falciparum* K1 strain tested. The percentage elimination of *Plasmodium falciparum* strain (K1) by positive control/reference standard (93.80%) was significantly higher than that of ethanol extract and its fractions at highest dose of 5000µg/ml. At the minimum concentration of 500 µg/ml, percentage reduction of *Plasmodium falciparum* strain (K1) by positive control/reference standard (83.67%) was found to be significantly greater than water fraction (62.24%) which exhibited the lowest antiplasmodial activity at the same concentration. The findings in this present study indicated that *In-vitro* antiplasmodial effect of ethanol extract and its fractions are concentration/dose dependent and aqueous methanol fraction was observed to be the most active fraction.

Considering the larvicidal (lethality) activities which were taken or considered as percentage mortalities, ethanol extract (86.67% and aqueous methanol fraction (83.33%) exerted relatively highest toxicity at 1000 µg/ml respectively while ethanol crude extract (0.03%) exhibited the lowest toxicity at 10 µg/ml. The cytotoxicity activities varied directly to the test dose concentration of crude extract and its fractions. Hence, the mortalities recorded for chloroform fraction were: 63.33% at concentration of 1000 µg/ml, 46.66% at 100 µg/ml and 30% at 10 µg/ml (Table 4). As for n – hexane, the order of severity was as thus: at 1000 µg/ml (56.67% mortality) > 100 µg/ml (43.33%) > 10 µg/ml (20% mortality). With regard to aqueous methanol fraction: at 1000 µg/ml (83.33%) > 100 µg/ml (30.33%) > 10 µg/ml (6.70%). As for ethanol crude extract: at 1000 µg/ml (87% deaths) > 100 µg/ml (10% deaths) > 10 µg/ml (0.03% deaths). With respect to ethyl acetate fraction: 1000 µg/ml (43.33 % deaths) > 100 µg/ml (20% deaths) > 10 µg/ml (23. 33% deaths). For water fraction: 1000 µg/ml (36.67% lethality) > 100 µg/ml (20% lethality) > 10 µg/ml (16.67% lethality). The mean percentage of mortality is in the order: chloroform fraction (46.67%) > aqueous methanol (40.12%) > n-hexane (40.00%) > ethanol (32.23%) > ethyl acetate (28.89%) > water fraction (24.45%). For positive control ($K_2Cr_2O_7$), at 10 µg/ml and 100 µg/ml. 100% mortalities was observed whereas at 10 µg/ml, 37% mortality was noticed. Throughout the laboratory investigation period, none of the brine shrimp larvae in the negative group consisting of 0 µg/ml of the extract or its fraction in only the salty sea water was shown to experience any mortality.

Table 4 *In-vitro* Cytotoxicity Test Results of Ethanol Crude Extract and Fractions

Concentration/Dose (µg/ml)	**No. of Live Brine Shrimp Larvae transferred into each test vial in triplicate of dose**			**No. of Survivals in each test vial in triplicate of dose**			**No. of Deaths in each test vial in triplicate of dose**			**Total No. of Deaths in each test vial in triplicate of dose**	**% Mortality Per Triplicate of dose**
Ethanol Crude Extract											
1000	10	10	10	2	1	1	8	9	9	26	86.67
100	10	10	10	10	8	9	0	2	1	3	10.00
10	10	10	10	10	10	9	0	0	1	1	0.03
Chloroform Fraction											
1000	10	10	10	4	1	6	6	9	4	19	63.33
100	10	10	10	4	8	4	6	2	6	14	46.67
10	10	10	10	7	7	7	3	3	3	9	30.00
Water Fraction											
1000	10	10	10	6	6	7	4	4	3	11	36.67
100	10	10	10	9	7	8	1	3	2	6	20.00
10	10	10	10	7	10	8	3	0	2	5	16.67
Ethylacetate Fraction											
1000	10	10	10	6	5	6	4	5	4	13	43.33
100	10	10	10	8	9	7	2	1	3	6	20.00
10	10	10	10	8	8	7	2	2	3	7	23.33
Aqueous Methanol Fraction											

1000	10	10	10	2	1	2	8	9	8	25	83.33
100	10	10	10	9	4	8	1	6	2	9	30.33
10	10	10	10	10	10	8	0	0	2	2	6.70
n-Hexane Fraction											
1000	10	10	10	3	4	6	7	6	4	17	56.67
100	10	10	10	5	6	6	5	4	4	13	43.33
10	10	10	10	8	8	8	2	2	2	6	20.00
Positive Control – Potassium heptaoxodichromate (VI)											
1000	10	10	10	0	0	0	10	10	10	30	100
100	10	10	10	0	0	0	10	10	10	30	100
10	10	10	10	6	8	5	4	2	5	11	37
Negative Control (0µg/ml of ethanol extract and fractions in Salty Sea Water)											
1000	10	10	10	10	10	10	0	0	0	0	0
100	10	10	10	10	10	10	0	0	0	0	0
10	10	10	10	10	10	10	0	0	0	0	0

4. Discussion

In this study, the results of phytochemical investigation revealed the presence of some secondary metabolites (tannins, saponins and alkaloids) in the ethanol extract and the fractions of the whole plant (Table 2), which is similar to the findings of Nuhu *et al* [35] who reported that alkaloids were present in the leaves of *Crotalaria miscrocarpa Hotchst* and *Crotalaria retusa* as well as in the aerial parts of *Crotaria naragulensis*. Treatment with increasing doses of the crude ethanol extract and its fractions resulted in relative reductions (*In-vitro*) of *Plasmodium falciparum* strain (K1) as indicated by the parasitaemia (malaria parasites) final count when compared to the initial count of parasitaemia (Table 3). Joanne *et al* in their report [36] disclosed that flavonoids, alkaloids, terpenoids, tannins and steroids possess anti-plasmodial activity. It was also reported that quinine; quinidine and cinchonine which were alkaloids exhibited antiplasmodial activity [37,38,39]. The antiplasmodial activities of *Crotolaria arenaria* whole plant extract and its fractions observed in this study could be accrued to the existence of bioactive secondary metabolites such as tannins, saponins and alkaloids. These findings further confirm and validate the report that the whole plant of *Crotalaria arenaria* has been effectively used in folklore medicine for the treatment of malaria [17]. The findings of this study are comparable with the results of Bello *et al* [40] who found out that methanol fraction of *Crotalaria mucronata*, a species in the Crotalaria genus displayed antiplasmodial activity even though the strain of *P. falciparum* which was tested against chloroquine sensitive (D6) strain with percentage elimination/suppression of 60%, 73% and 52% at different and lower concentrations and the most active antiplasmodial fraction was methanol extract of *Crotalaria mucronata* which was the same result obtained in this current study which shows that the most active antiplasmodial fraction was methanol fraction of *Crotalaria arenaria*. Also, some species of Crotalaria such as *C. medicagenia, C. orixensis* and *C. ramossimia* were investigated for their antimalarial activity [41] and found out that these three species exhibited antiplasmodial activity against NF-54 strain of *Plasmodium falciparum.*

The ethanol extract and its fractions demonstrated a certain degree of cytotoxicity as shown in the lethality of the test brine shrimp larvae at varying concentrations. The lethality indices were however concentration dependent. The crude ethanol extract and aqueous methanol fraction indicated significant toxicity at highest concentration of 1000 µg/ml on brine shrimp larvae. High cytotoxicity of ethanol crude extract at highest concentration of 1000 µg/ml observed in this study may be attributed to the presence of bioactive secondary metabolite such as alkaloid which has been found to be responsible for high cytotoxicity of majority of plant species in the genus of Crotolaria and this was consistent with some findings [40] which discovered that *Crotalaria spectabilis*, a species in the Crotalaria genus contains toxic alkaloid. The toxicity of the seed of *Crotalaria retusa* has been shown to be due to the presence of alkaloid monocrataline reported to be found in the seed of *Crotalaria retusa,* which was further substantiated by the result of Nuhu *et al* [35] who discovered that *Crotalaria retusa* was toxic to albino (Wistar) rats (*in-vivo*) as well as the lungs, kidneys and intestine of the albino rat were observed to have significant toxic features. It is essential to consider the extract and fractions obtained as a

result of partitioning of the crude extract for future toxicological and pharmacological investigation towards any important pharmacognostic agent such as antimalarial, anticancer, pesticidal and insecticidal agents. According to the findings in this study on the cytotoxicity of the crude extract and fractions obtained from it, they could be considered safe for oral consumption only at relatively low/moderate dose in treating malaria disease in traditional medicine in northern part of Nigeria. The determination of the LC_{50} value of the ethanol crude extract obtained from the plant and its fractions on *artermia salina larvae* is ongoing as this will give a prediction and further confirmation of its safety and usage in orthodox medicine.

5. Conclusion

The plant pythons are responsible for the potency and effectiveness of the ethanol crude extract and the fractions which have made it potent in the curing of malaria infection. The antiplasmodial activity of the extract and its various fractions indicated strongest activity at the highest dose of 5000 µg/ml as the bioassay is concentration dependent. The use of the whole plant of *C. arenaria* as an anti-malarial herb in traditional medicine in some parts of Nigeria has scientific backing based on the antiplasmodial (*In-vitro)* data obtained from this study. The larvicidal effects varied directly to the dose concentrations of the crude ethanol extract and its fractions and the degrees differ with the extract and each of its fractions. Precisely, the higher the concentration/dose, the higher the toxicity. Our finding suggests that ethanol crude extract of *Crotalaria arenaria* Benth (Whole plant) and its fraction indicated obvious high cytotoxicity in the *Artermia salina* Leach assay at higher concentration/dose. The finding of the present study do not agree with employment of the whole plant of *Crotalaria arenaria* by folklore healers in Otukpo in Benue State and in other part of Nigeria particularly at relatively unregulated high dose concentrations even though majority of the world's population depends on traditional medicine of herbal origin for their primary healthcare needs, thus the extract from the whole plant of *Crotalaria arenaria* could be used as therapeutic agent only in relatively low/moderate dose concentrations especially by less privileged and vulnerable people amidst urban and rural population who cannot afford modern drugs because of exorbitant cost. The active extract and its fractions reported in this study which exhibited strongest antiplasmodial activities and cytotoxicity is suggested for further pharmacological, toxicological and phytochemical investigations so as to define what sort of anticancer (antitumor) and anti-plasmodial activity they contain and to isolate the bioactive components which are responsible for the activities as potential antitumor, pesticidal, antiplasmodial and insecticidal agents via bioactive guided column chromatography fractionation. Investigations of this nature are necessary prior to general recommendation of phytotherapeutical agent for pharmaceutical use.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

References

[1] Pimenta, P. F. P., Alessandra, S. O., Ana, C. B., Ana, P. M. D., Claudia, M. R. , Fabrício, F. M., Felipe, A. C. P. et al. An Overview of Malaria Transmission from the Perspective of Amazon Anopheles Vectors. Mem Inst Oswaldo Cruz . 2015.110(1):23– 47.

[2] Adebayo, J.O, Krettli, A.U. Potential Antimalarials from Nigerian Plants: A Review. J Ethnopharmacol. 2011; 133:289–302.

[3] Saidu, K., Onah, J., Orisadipe, A,Olisola, A., Wambebe, C.and Gammel K. Antiplasmodial, Analgesic and Anti-inflammatory Activities of Aqueous Extract of Stem Bark of *Erythrina senegalensis*. J Ethnopharmacol. 2000; 71(1):273–280.

[4] Lothar, W., Casper, H.,Milena, P., Nikolai, K.,and Jorgen, A.K. Recombina Human Erythropoietin Increases Survival and Reduces Neuronal Apoptosis in a Murine Model of Cerebral Malaria. Malaria J. 2008; 7:3.

[5] Mohapatra, K.M. Profile of Severe falciparum Malaria in Diabetics. Int. J. Diab Dev Ctries. 2001; 2:156–161.

[6] Mishra, S.K. and Mohanty, S. (2003). Problems in the Management of Severe Malaria. J. Trop Med. 2003; 1:1– 10.

[7] Ogbodo, S.O., Okeke, A. C., Obu, H. A., Shu, E.N., Chukwurah, E.F. Nutritional Status of Parasitemic Children from Malaria Endemic Rural Communities in Eastern Nigeria. Curr Pediatr Res. 2010; 14:131–135.

[8] World Health Organization. Artemisinin Resistance and Artemisinin-based Combination Therapy Efficacy. Global Malaria Programme , Status Report 2018 .

[9] World Health Organization (2018). World malaria report.

[10] Batista, R., Ademir, D. S. J. and Bahia, U. E. S. Plant-Derived Antimalarial Agents: New Leads and Efficient Phytomedicines. Part II. Non-Alkaloidal Natural Products. Molecules. 2009; 14(8):3037-72.

[11] Akinniyi, J. A. and Sultanbawa, M.U.S. A Glossary of Kanuri Names of Plants with Botanical Names, Distribution and Uses. Annal of Borno. 1983; 1: 85 – 98.

[12] Muhammadu, H.J. African Traditional Medicine. A Case Study of Hausa Medicinal Plants and Therapy. Gaskiya Corporation Limited, Zaria, Nigeria.1990. Pp.26 – 93.

[13] Maydell, H. J. Trees and Shrubs of the Sahel: Their Characteristics and Uses. Gescitschaft furTechnichaZusammenarbeit (GT2). Verlag Josef Margraf, Wikershum. 1990. Pp. 164 – 370.

[14] Fatope, M.O. Phytocompounds: Their Bioassay and Diversity. Discov.Innov. 1995; 7(3):229 – 236.

[15] Fatope, M.O., Ibrahim, H.M. and Takeda, Y. Screening of Higher Plants Reputed as Pesticides using the Brine Shrimp Lethality Bioassay. International Journal of Pharmacognosy.1993; 31:250 – 256.

[16] Adoum, O.A, Dabo, N.T. and Fatope, M.O. Screening of Savannah Plants for Brine Shrimp and *In-vitro* Antimicrobial Bioactivity. International Journal of Pharmacognosy.1997; 35(5):334 – 337.

[17] Aba, T. L., Seck, D., Kindomihou, V., Agbanba, C. and Emile, S.M. Medicinal Plants Used In Some Rural Districts in Senegal (West Africa). American-Eurasian Journal of Sustainable Agriculture. 2012; 6(4):325-332.

[18] World Health Organization (W.H.O). Report on Pyrrolizidine Alkaloids. International Program on Chemical Safety: Environmental Health Criteria, 1998; Series No. 80.

[19] Arzt, J., and Mount, M. E. Hepatotoxicity Associated with Pyrrolizidine Alkaloid (Crotalaria spp) ingestion in a Horse on Easter Island. Veterinary and human toxicology.1999; 41(2):96-99.

[20] Mattocks, A.R. Chemistry and Toxicology of Pyrrolizidine Alkaloids. Orlando (FL): Academic Press. 1986; pp. 1–13, 130–57, 158–90.

[21] Hirono, I., Mori, H., Haga, M., Fujii, M., Yamada, K., Takanashi, H., Uchida, E., Hosaka, S., Ueno, I., Matsushima, I., Umezava, K., and Shirai, A. Edible Plants containing Pyrrolizidine Alkaloids in Japan. In: Miller, B.C.(Ed.), Naturally Occurring Carcinogens, Mutagens, and Modulators of Carcinogenesis. University Park Press, Baltimore, MD, USA, 1979; pp. 79–87.

[22] Hirono, I., Mori, H., and Haga, M. Carcinogenic Activity of Symphytum officinale. J. Natl. Cancer Inst. 1978; 61, 865–869.

[23] Green, C.R., Christie, G.S. Malformation of Rats Induced by the Pyrrolizidine Alkaloid, Heliotrine. Br. J. Exp. Pathol. 1961; 2, 369–378.

[24] Kovach, J.S., Moertel, C.G. and Hahn, R.G. A Phase 1 Study of Indicine N-oxide. Proc.Am. Assoc. Cancer Res. 1979; 20: 357–362.

[25] Schmeller, T., El-Shazly, A. and Wink, M. Allelochemical Activities of PyrrolizidineAlkaloids: Interactions with Neuroreceptors and Acetylcholine Related Enzymes. J. Chem. Ecol. 1997; 23: 399–416.

[26] Harborne, J.B. Phytochemical Methods: A Guide to Modern Technique of Plant Analysis, 3rd Eds. Chapman and Hall Int. Eds, 1998; New York.

[27] Kokate, C.K. Practical Pharmacognosy, 4th Eds. Vallabh Prakasan, Delhi, India. 1994; 107-111.

[28] Trease, G. E., and Evans, W.C. Textbook of Pharmacognosy (13th Edition). ELBS Baillers Tindal, London. 1989; 378-386.

[29] Lewis, S.M, Ban, B.J, Bates, I, Dacie, J.V and Dace, J.V. Dacie and Lewis Practical Haematology. Philadelphia. 15th ed. 2006; Churchill Livingstone/Elsevier.

[30] Hanne, I. Z, Dan, S., Jette, C., Lars, H., Henry, H., and Jorzy, W. J. *In-vitro* Plasmodium falciparum Sensitivity Assay. Antimicrobial Agent and Chemotheraphy. 2002; 42(6): 1441-1446.

[31] Devo A.A, Groary T.G, Davis N.L, Jonson J.B (1985). Nutritional Requirements of Plasmodium falciparum in Culture Exogenously Supplied with Dialyzable Components Necessary for Continuous Growth.; J Protozoal. 32: 59-64.

[32] Trager, W. Cultivation of Malaria Parasites. Pakistan Journal of Biological Science. 1982; 14(10):560–571.

[33] Mukhtar, M. D. and, Bashir, A. A. H. Comparative Invitro Studies on Antiplasmodial Activity ofsome Nigeria and Foreign Brands of Chloroquine Oral Formulation Marketed in Kano. Africa Journal of Biotechnology. 2006; 5(24):2464-2468.

[34] Adoum, O.A. Determination of Toxicity Levels of Some Savvanah Plants Using Brine Shrimp Test (BST). Bayero Journal of Pure and Applied Sciences. 2009; 2(1):135-138.

[35] Nuhu, H., Shok., M., Abdurahman, E. M., and Ibrahim, N. D. G. Alkaloidal Composition and Toxicity Studies of Three Nigerian Crotalaria Species. Nigerian Journal of Natural Products and Medicine. 2000; 4(1): 40-45

[36] Joanne, B., Michel, F, and Joelle, Q. Antimalarial Compounds Isolated from Plants used in Herbal Medicine. J. International Medicine. 2009; 61:1401–1433.

[37] Bloland, B. P and Ettling, M. Making Malaria-Treatment Policy in the face of Drug Resistances. Annals of Tropical Medicine and Parasitology. 1998; 93(1):5-23.

[38] Deloron et al. Efficacy of a 3-day Oral Regimen of a Quinine-quinidine-cinchonine Association (QuinimaxO) for Treatment of falciparum malaria in Madagascar. Transactions of the Royal Society of Tropical Medicine and Hygiene. 1989; 83:751-754.

[39] Qinghaosu, et al. Antimalaria Studies on Qinghaosu. Chinese Medical Journal. 1979; 92(12):811-816.

[40] Bello, O. M., Zaki, A. A., Khan, S. I., Fasinu, P. S., Ali, Z., Khan, I. A., Usman, L. A. and Oguntoye, O.S. Assessment of selected Medicinal Plant Indigenous to West Africa for Antiprotozoal Activity: South Africa Journal of Botany. 2017; (113); 200 – 211.

[41] Tadigoppula, N., Shweta, Khaliq, T., Rav, M.S., Srivastara, K. and Pur, S.K.S. Prenylated Chalones Isolated from Crotalaria genus Inhibited in vitro Malaria Parasite (Plasmodium falciparum). Biorganic and Medicinal Chemistry letters. 2005; 15(10): 2453 - 2455.

[42] Roger A. and Roger E. F.. The Structure of Monocrotaline, the Alkaloid in Crotalaria Spectabilis and Crotalaria Retusa. I. J. Am. Chem. Soc.1989; 61(10): 2815–2