Original Research Article

In vitro Cytotoxicity and Antiplasmodial activity of fractions from *Anthocleista djalonensis* A. Chev. Acetone root extract

Abstract

AIM: Malaria caused by plasmodium falciparum is one of the killer diseases in Africa today and the uncontrollable spread of drug resistance and limited drugs with therapeutic efficacy makes it necessary to discover agents against this deadly parasite.Traditionally ***Anthocleista djalonensis*** **root extract is used in the treatment of Malaria in many parts of Africa and has demonstrated to be a source of antiplasmodial agents.** This **study aims at** identifying possible antiplasmodial agents from chromatographic root fractions of *Anthocleista djalonensis* of the Genatianceae family as well as to evaluate their cytotoxicity against *HeLa* cells.

Place and Duration of study: The study was undertaken in the Department of Organic Chemistry, Rhodes University, Grahamstown, South Africa. The duration period was between March and July 2016.

Methodology: The *Anthocleista djalonensis* roots were collected from Arochukwu, Abia State, Nigeria. The acetone extract was obtained from successive maceration of the methanolic crude extract with hexane, ethyl acetate and acetone. The concentration (0.01-100 µg/mL range) of the chromatographic fractions from acetone root extract of *Anthocleista djalonensis* were tested for antimalarial activity against *Plasmodium falciparium* (*P.falciparum*). Cytotoxicity against *HeLa* cells was also evaluated using resazurin based assay.

Results: The Five fractions obtained from the chromatographic fractionation of acetone extract labelled A1, A2, A3, A4, and A5 with percentage yield (13.02, 26.66, 24.70, 0.05 and 26.66 % respectively) showed excellent antiplasmodial activity. The antimalarial bioassay test showed fractions A1, A2, A3, A4 and A5 with IC_{50} value of 0.0360 ± 0.0100, 8.1299 ± 2.0358, 46.2482 ± 1.2720, 0.0151 ± 0.0010, and 9.8013 ± 0.8171 µg/mL respectively. CC_{50} values of 44.2010 ± 8.6790, 50.0000 ± 5.6412, 71.6221 ± 2.9600, 36.7212 ± 5.8900 and 0.5132 ± 3.770 µg mL^{-1} were recorded for fractions A1, A2, A3, A4 and A5 respectively. Fractions were classified as marginally active (A3) showing SI of 1.540 ± 0.0091, partially active (A2 and A5) with SI 6.150 ± 0.0200 and 4.133 ± 0.015 and as active (A1, A4,) exhibiting SI of 1227.805 ± 8.210 and 2431.867 ± 1.589 respectively. A1 and A4 showed SI > 10 and IC_{50} < 10 ug/mL. Chloroquine, used as a reference antimalarial drug, tested in parallel had an IC_{50} of 0.0125 ± 0.0001 µM and was comparable with A1 and A4.

Conclusion: The chromatographic fractions from acetone root extract of *Anthocleista djalonensis* are potential sources for antimalarial agents of lead compounds for the development of antiplasmodial drugs.

Keywords: **Antiplasmodial activity; Cytotoxicity; IC_{50}; Fractionation; *Anthocleista djalonensis***

1. INTRODUCTION

Medicinal plants contain chemical substances or constituents that have pharmacological activities [1-7]. These activities include anti-cancer [8,9] anti-tumor[10]anti-oxidant[11] and anti-microbial activities[12, 2]. Healing with medicinal plants is as old as mankind itself. The connection between man and his search for drugs in nature dates from the far past. Awareness of medicinal plants usage is a result of the many years of struggles against illnesses by which man learned to pursue drugs in barks, seeds, fruit bodies, and other parts of the plants [13]. Contemporary science has acknowledged their active action, and it has included in modern pharmacotherapy a range of drugs of plant origin, known by ancient civilizations and used throughout the millennia. The knowledge of the development of ideas related to the usage of medicinal plants as well as the evolution of awareness has increased the ability of pharmacists and physicians to respond to the challenges that have emerged with the spreading of professional services in facilitation of man's life [13].

The medicinal plant *Anthocleista djalonensis*, A. Chev –Gentianaceae is a large tree which grows up to 20 feet; bole up to 4cm in diameter, stilt-rooted, twig sometimes erect, spines above the leaf axils and with white flowers that are scented [14]. Traditionally, the plant is used to treat wound, malaria, constipation, dysentery, diarrhea, hepatitis, skin infection, and inflammation [15]. Ethnobotanical investigation revealed the use of *Anthocleista djalonensis* for the treatment of cancer [16]. Three compounds (monoterpene diol, djalonenol and iridoid glucoside djalonenoside) were isolated from *Anthocleista djalonensis* [17]. These compounds when tested in addition with six semisynthetic derivatives for cytotoxicity of constituents, djalonenol and iridoid glucoside demonstrated cytotoxic effect on the brain tumor fibroblasts (18). The root of *A. djalonensis* are used traditionally to treat malaria jaundice, diabetes and abscesses [15] Reports of

antibacterial and wound healing activity [19,15], *in vitro* anthelmintic [20] and antimalarial activity[21, 22] of the plant have been published.

Malaria, the most prevalent and most pernicious parasitic disease of humans, is estimated to kill between one and two million people (mainly children) each year [23]. One of the greatest obstacles to the control of malaria has been the spread of resistance to drugs used on a large scale [24]. Chloroquine, though effective as a blood schizontocidal drug, is ineffective or partially effective in resistant cases [25].The emergence of strains of *Plasmodium falciparum* resistant to chloroquine and many other drugs in succession have stimulated efforts to identify new anti- malarial agents [25].

Previous pharmacological and bioactivity study of *Anthocleista djalonensis* root extracts has necessitated this investigation. In this study, the activity of chromatographic fractions from the acetone root extract against *P. falciparum* was assayed and the cytotoxicity on HeLa cells was also evaluated in order to determine the selectivity of anti-malarial action. *Plasmodium falciparum* responsible for malaria in human was quite suitable for this study.

2. MATERIAL AND METHODS

2.1. Plant materials

The roots of *Anthocleista djalonensis* were obtained from Arochukwu, Abia state, Nigeria.The taxanomic classification of the selected plant was established by Mr Ibe Ndukwe of the Taxonomy Section, Forestry Department, Michael Okpara University of Agriculture Umudike, Abia State, Nigeria. Voucher samples of the plants are deposited in the Herbarium of Michael Okpara University of Agriculture Umudike, Abia State, Nigeria. The roots were dried under shade for three weeks.

2.2. Acetone extract preparation

The dried plant samples were pulverized to coarse powder using a laboratory mill (Model 4 Arthur Thomas, USA). The 93 g (AF00) was extracted successively with Hexane (4 x 100 mL), Ethyl acetate (4 x 100 mL), acetone (4 x 100 mL) by maceration. The extracts were

concentrated individually with Rota vapor at 35°C to give AF01, AF02, AF03, respectively with AF00 as the crude extract.

2.3. Fractionation of Acetone extract

Acetone extract being the most active of all the extracts was made candidate for column chromatography (The activity of this extract will be Published elsewhere). About 30 g of concentrated acetone extract was fractionated using chromatography column. Packaging was done using an 80 g column of silica gel. Best eluent (hexane, dichloromethane and methanol) were used as mobile phase with a gradient system. Eluates were collected in several bottles of 50 mL, each of which was given a number then analyzed by TLC. The spots separated in TLC were observed with 254 nm UV light. The Rf and eluates which had the same pattern spots appearance on TLC were combined as one fraction then concentrated. Percentages of fractions were calculated using the formula:

% Fraction = Weight of Fraction (g)/Weight of plant extract x 100

2.4. *HeLa* cell culture and treatment [26]

Human cervix adenocarcinoma cells (*HeLa*) obtained (from ATCC CCL-2 LGC standard Wesel, Germany) were cultured in a 5%CO_2 incubator at 37°C in DMEM medium supplemented with 10% fetal bovine serum and antibiotics (penicillin/streptomycin/fungizone).The cells were split every 3-5 days (when the cells have reached close to full confluency), and were detached from the culture flask surface using trypsin/EDTA, and the majority aspirated off. Medium was added to the flask and the remainder of the cells, and the flask returned to incubation. The confluency and state of the cells were regularly assessed using an inverted light microscope. Cells were cryo-preserved by detaching the cells from the culture flask in trypsin/EDTA, pelleting the cells, transferring them to cryo-tubes in 10% DMSO in fetal bovine serum, and placing the tubes in a -80

freezer. For the cytotoxicity assay a range of concentrations of extract (1-1000 µg mL^{-1}) was used for 24 h treatment for the determination of 50% cytotoxic concentration (CC_{50}).

2.5. *In vitro* Cytotoxicity assay

Cytotoxic activity was determined by resazurin reduction based assay [27]. *HeLa* cells were used for the determination of the CC_{50} value of the cytotoxicity of chromatographic fractions from *Anthocleista djalonensis*. To assess the overt cytotoxicity of the compounds, extracts were incubated at various concentrations in 96-well plates containing *HeLa* (human cervix adenocarcinoma) cells for 24 hours. The numbers of cells surviving on drug exposure were also determined by using the resazurin based reagent and reading resorufin fluorescence in a multiwell plate reader. Reagent was prepared by dissolving high purity resazurin in DPBS (pH 7.4) to 0.15 mg/mL. The resazurin solution was filtered and sterilized through a 0.2 µm filter into a sterile, light protected container. The resazurin solution was stored and protected from light at 4 °C for frequent use or at -20 °C for long term storage. Cells and test compounds were prepared in opaque-walled 96-well plates containing a final volume of 100 µL/well. An optional set of wells were prepared with medium only for background subtraction and instrument gain adjustment. This was incubated for desired period of exposure. 20 µl resazurin solution was added to each well. This was incubated for 1 to 4 hours at 37 °C. The fluorescence was recorded using a 560 nm excitation / 590 nm emission filter set.

2.6. *Plasmodium falciparum* cultivation

Chloroquine sensitive and resistant strains of *P. falciparum* 3D7, respectively, obtained from Rhodes University Gramhamstown, South Africa was kept in continuous *in vitro* culture

according to the modified candle-jar method of Trager and Jensen [28]. Human red blood cells (blood type A+) in RPMI 1640 medium (Sigma Chemical Co., USA) supplemented with 25 mM HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (Sigma), 0.2 per cent sodium bicarbonate (Sigma), and 15 per cent complement inactivated human AB+ serum were used for parasite culture.

2.7. *In vitro* antiplasmodial assay

Anti-malarial test was carried out using the Immunocapture parasite lactate dehydrogenase (IcpLDH) assay method as described by Makler et al., [29]. Extracts with concentration (0.01-100 ug/mL) were added to parasite cultures in 96-well plates and incubated for 48 h in a 37 ^{o}C CO_2 incubator. After 48 h the plates were removed from the incubator. Twenty µL of culture was removed from each well and mixed with 125 µL of a mixture of Malstat solution and NBT/PES solution in a fresh 96-well plate. These solutions measure the activity of the parasite lactate dehydrogenase (pLDH) enzyme in the cultures. A purple product was formed when pLDH was present, and this product was quantified in a 96-well plate reader by absorbance at 620 nm (Abs_{620}). The Abs_{620} reading in each well is thus an indication of the pLDH activity in that well and also the number of parasites in that well. Each sample was tested in duplicates. For each concentration, % parasite viability – the PLDH activity in treated wells relative to untreated controls was calculated.

2.8. Data analysis

All experiments were performed in duplicates and presented as the Mean ± SD. Statistical analysis of the data was carried out by one way ANOVA (Graph Pad Prism 5.02 Software). A value of $p< 0.05$, $p<0.01$,$p<0.001$ and $p<0.0001$ were considered to be significant, very significant and highly significant, respectively. Linear regression analysis was used to calculate CC_{50} and IC_{50}.The antiplasmodial activities of fractions were expressed by the inhibitory concentrations (IC_{50}) of the drug that induced 50% reduction in parasitaemia

compared to the control (100 %) parasitaemia.The activity was analysed according to the classification for antiplasmodial activity by Valdes [30]. Therefore extract exhibiting $IC_{50\ P.falciparum}$ > 100 µg/mL was considered inactive. Extract showing $IC_{50\ P.falciparum}$ < 100 µg/mL was classified as follows: - Marginally active at SI < 4, partially active at SI 4-10 and active at SI > 10. Active extract showing $IC_{50\ P.falciparum}$ < 10 µg/mL was to be selected for further bioassay-guided fractionation. Selectivity index was calculated as the ratio of cytotoxicity of extract on *HeLa* cell line (cytotoxicity) to the IC_{50} of the extract against *P. falciparum* (antiplasmodial activity) strains.

3. RESULTS AND DISCUSSION

3.1. Fractionation of acetone extract

Fractionation of the acetone extract using column chromatography produced five fractions (Table 1). Fraction grouping was based on the TLC analysis using hexane, dichloromethane and methanol as eluents. Based on Table 1, the yield of fractions obtained ranged from 0.05-26.66%. Fraction number A2 and A5 had the highest yield of 26.66% followed by fraction A3, A1 and A4. The smallest yield was fraction A4 (0.05%). From the TLC analysis all fractions had many number of spots and none with a single spot. The fractions were observed to have different colours visually. This may be due to different types of constituents found in each fraction.

Table 1: Yield of Fractions of *A.djalonensis* Acetone root extract

Fractions	Weight(g)	% Yield	Colour of Fraction	Rf
A1	3.90	13.02	White	0.66
A2	8.00	26.66	light yellow	0.56
A3	7.43	24.70	Yellow	0.62
A4	1.50	0.05	Brown	0.70
A5	8.00	26.66	Dark brown	0.61

Rf= Retardation factor

3.2. *In vitro* assays

Cytotoxicity and antimalarial activity was determined from CC_{50} and IC_{50} value of the fractions. The CC_{50} and IC_{50} value is always inversely proportional to the cytotoxicity and anti plasmodial activity respectively. This meant the higher the CC_{50} and IC_{50} values, the lower the activities and vice versa. The results showed that there was a positive correlation between the concentrations of fractions with the percentage of *HeLa* cells and *P. falciparum* parasite cell viability (Fig. 6 and 12).

Table 2 shows the CC_{50} and IC_{50} values of all five chromatographic fractions of *A.djalonensis* acetone extract.

Fractions	*P.falciparum* (IC_{50}) µg/mL	HeLa Cells(CC_{50}) µg/mL	SI	Classification
A1	0.0360 ± 0.0100	44.2010 ± 8.6790	1227.8055 ± 8.2100**	Active
A2	8.1299 ± 2.0358*	50.0000 ± 5.6412**	6.150 ± 0.0200****	Partially active
A3	46.2482 ± 1.2720**	71.6221 ± 2.9600**	1.540 ± 0.0091****	Marginally active
A4	0.0151 ± 0.001	36.7212 ± 5.8900	2431.8675 ± 1.5890****	Active
A5	9.8013 ± 0.8171**	40.5132 ± 3.7700*	4.133 ± 0.0150****	Partially active
CON	0.0125 ± 0.0001	18.6430 ± 1.6969	1491.2000 ± 3.009	Active

A1-A5 = fractions from acetone root extract of *Anthocleista djalonensis*.CON = control, IC_{50} = The half maximal inhibitory concentration, CC_{50} = The 50% Cytotoxic concentration, SI = selective index. Results were expressed as mean ± STD.*p<0.05, **p<0.01, ***p<0.001and ****p<0.0001 compared to positive control. Marginally active at SI < 4, partially active at SI 4-10 and active at SI > 10

3.2.1. *In vitro* Cytotoxicity assay

The test results on *HeLa* cells indicated growth inhibition by the fractions of acetone root extract from *A.djalonensis*. All Fractions A1, A2, A3,A4 and A5 had $CC_{50} \leq 100$. The highest cytotoxicity activity was demonstrated by A4 with CC_{50} value of 36.72 µg/mL followed by A5, A1and A2 with CC_{50} value of 40.51 µg/mL, 44.20 µg/mL and 50.00 µg/mL respectively. Fraction A3 showed the lowest cytotoxicity with CC_{50} value of 71.62 µg/mL (Fig1-6).

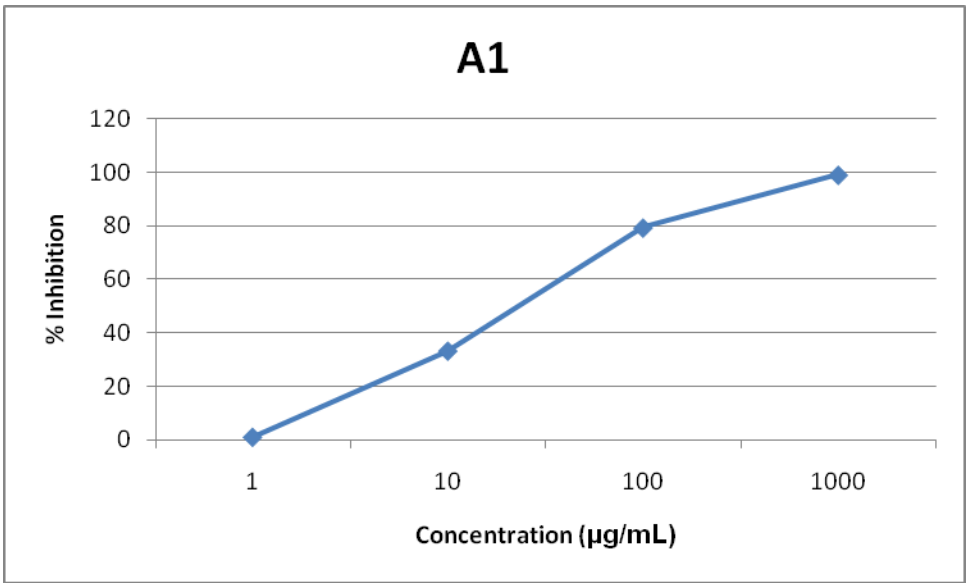

Fig 1:Cytotoxicity of fraction A1 against *HeLa* cells at different concentration (µg/mL)

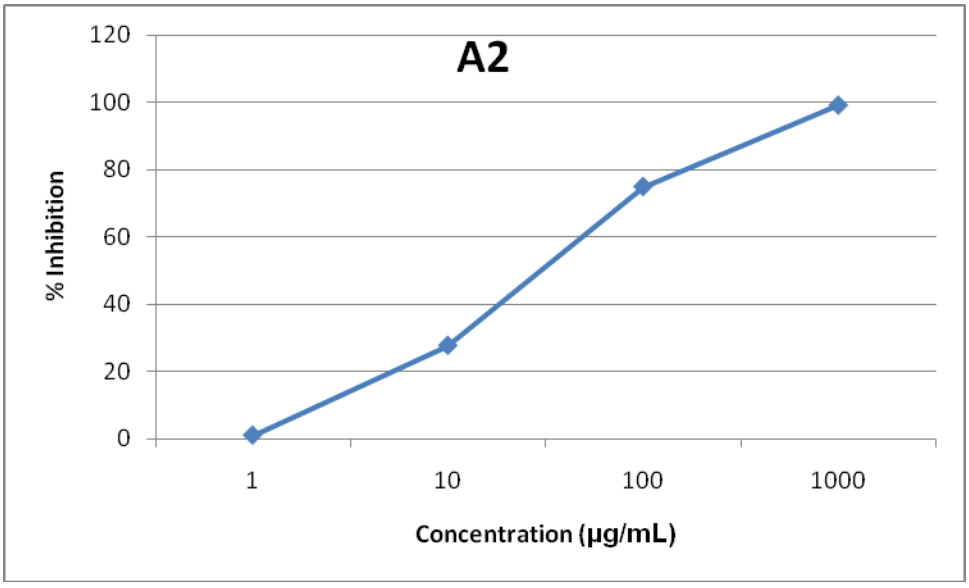

Fig 2: Cytotoxicity of fraction A2 against *HeLa* cells at different concentration (µg/mL)

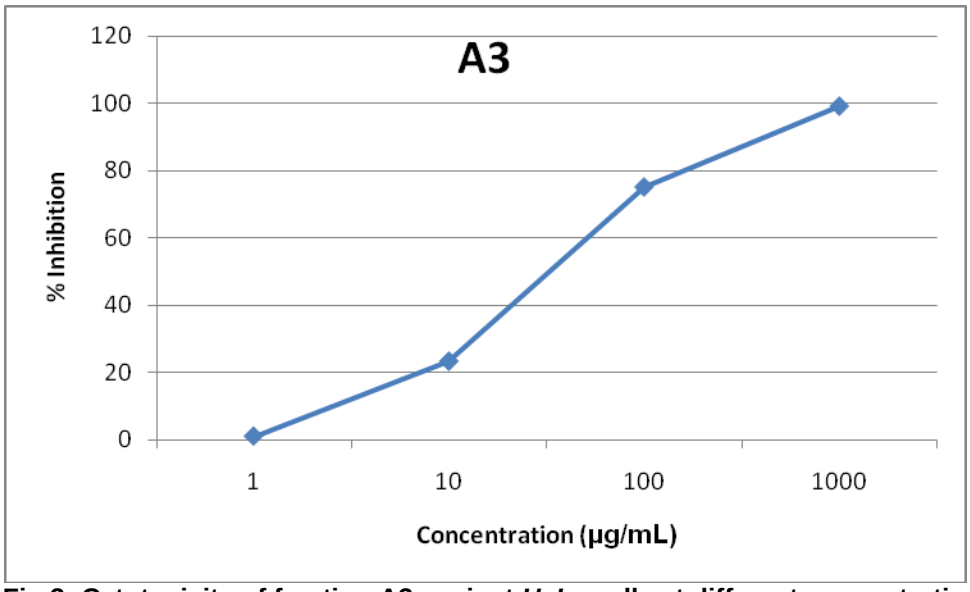

Fig 3: Cytotoxicity of fraction A3 against *HeLa* cells at different concentration (µg/mL)

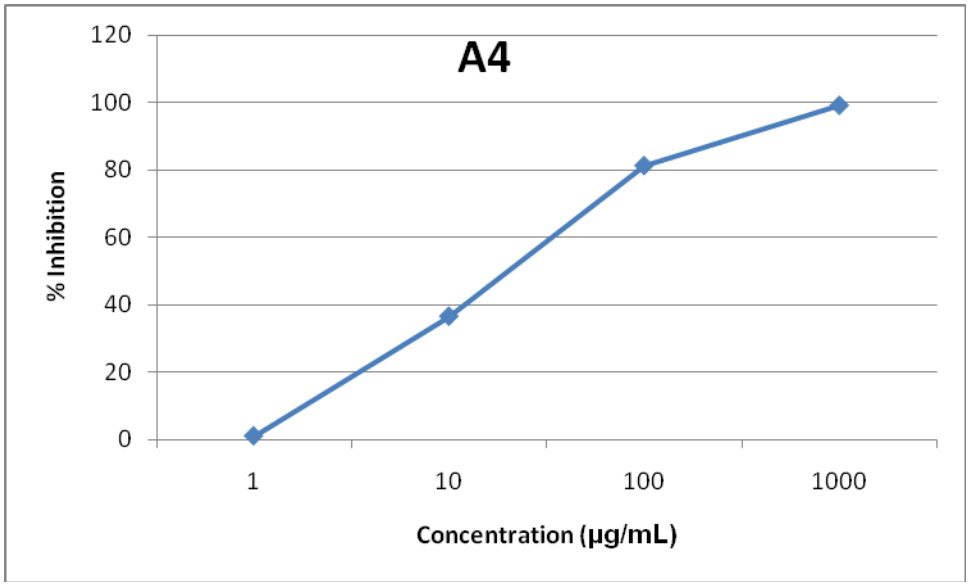

Fig 4: Cytotoxicity of fraction A4 against *HeLa* cells at different concentration (µg/mL)

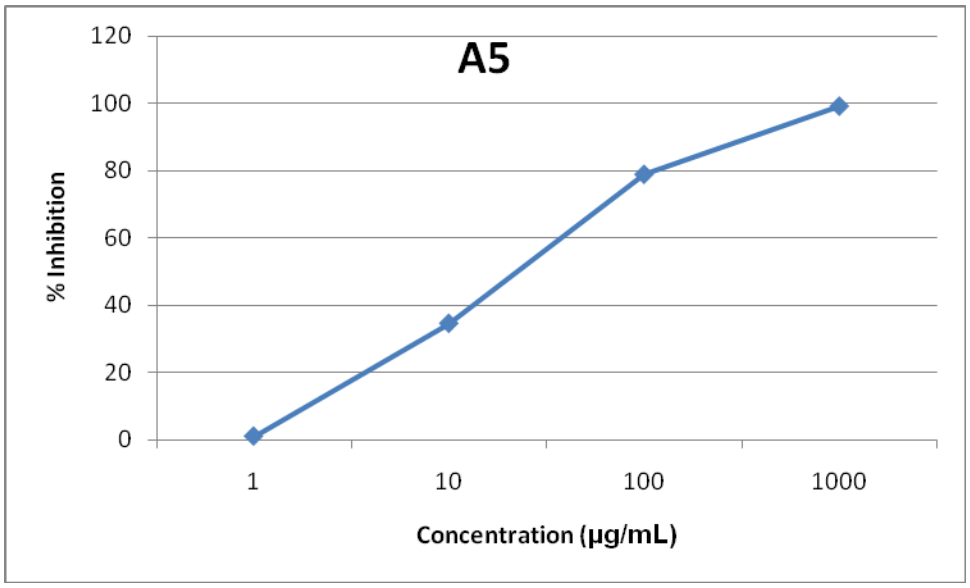

Fig 5: Cytotoxicity of fraction A5 against *HeLa* cells at different concentration (µg/mL)

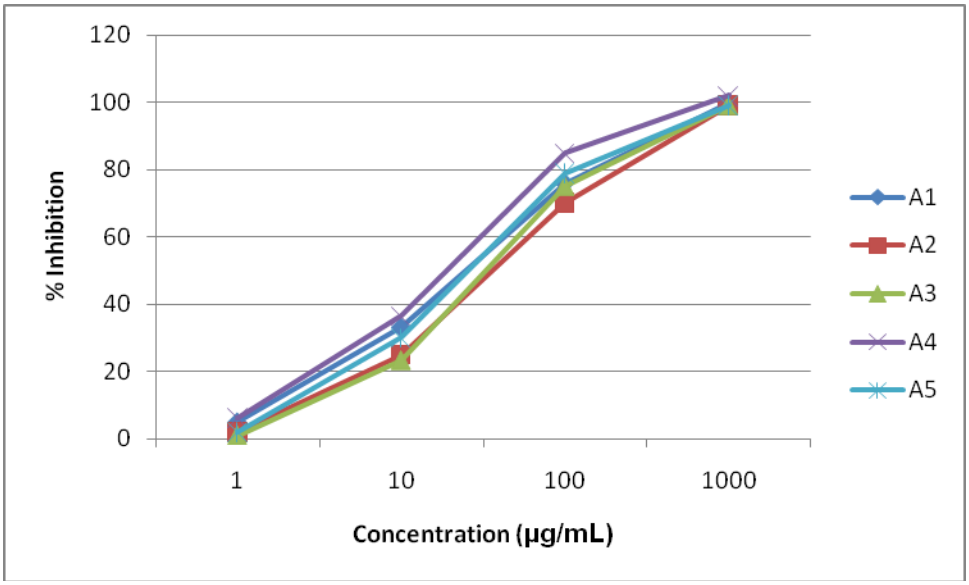

Fig 6: Cytotoxicity of all five fractions against *HeLa* cells at different concentration (µg/mL)

3.2.2. *In vitro* anti-malarial activity

The test results of *in vitro* anti-malarial activity showed that all fractions of *A.djalonensis* acetone extract had the ability to inhibit the growth of *P. falciparum* (Fig 7-11). The IC_{50} values and selectivity indices (SI = ratio of cytotoxicity to anti-malarial activity) of extracts are shown in Table 2. The IC_{50} values for fractions A1, A2, A3, A4, and A5 were 0.036 µg/mL, 8.129 µg/mL, 46.248 µg/mL, 0.0151 µg/mL and 9.801 µg/mL respectively. Fractions were classified as marginally active (A3) showing SI of 1.540, partially active (A2 and A5) with SI of 6.150 and 4.133 respectively and as active (A1and A4) exhibiting SI of 1227.805 and 2431.867 respectively. Furthermore, A1 and A4 showed SI > 10 and IC_{50} < 10 µg/mL. Chloroquine, used as a reference anti-malarial drug, tested in parallel had an IC_{50} of 0.0125 µM and was comparable with A1 and A4 (IC50: 0.0360µgm/L and 0.0151 µg/mL). This showed A1 and A4 as being very prospective fractions to be developed as anti-malarial agents.

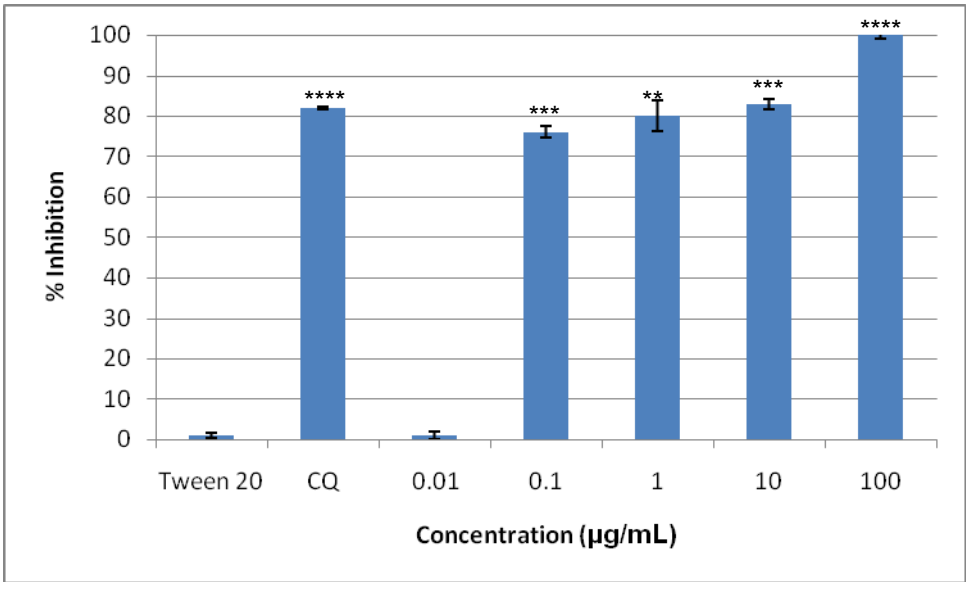

Fig 7: **Activity of fraction A1 against *P.falciparum* at different concentration of 0.01,0.1,1,10,100 µg/mL.** CQ (0.1ug/mL) was used as positive control and negative control was treated with tween 20. Results were expressed as mean ± STD. **p<0.01, ***p<0.001and ****p<0.0001 compared to negative control

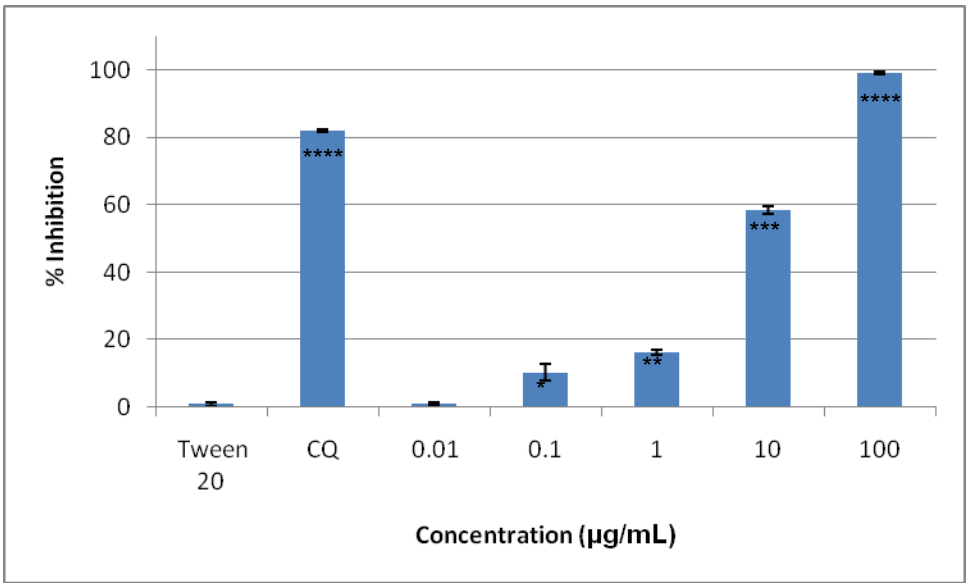

Fig 8: **Activity of fraction A2 against *P.falciparum* at different concentration of 0.01,0.1,1,10,100 µg/mL.** CQ (0.1ug/mL) was used as positive control and negative control was treated with Tween 20. Results were expressed as mean ± STD. *p<0.05, **p<0.01, ***p<0.001and ****P<0.0001 compared to negative control.

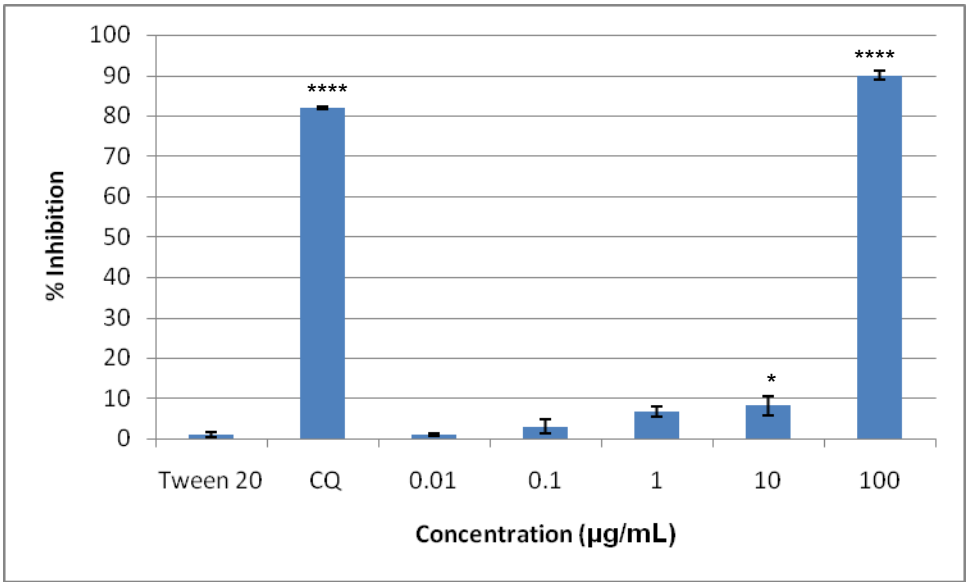

Fig 9: **Activity of fraction A3 against *P.falciparum* at different concentration of 0.01,0.1,1,10,100 µg/mL.** CQ (0.1ug/mL) was used as positive control and negative control was treated with tween 20. Results were expressed as mean ± STD. **p<0.05,****p<0.0001 compared to negative control.

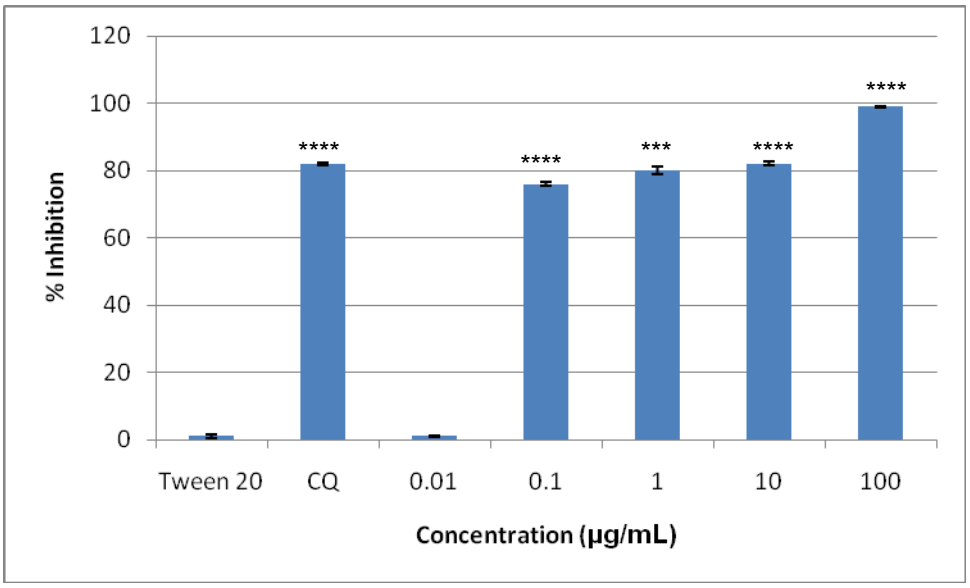

Fig 10: **Activity of fraction A4 against *P.falciparum* at different concentration of 0.01,0.1,1,10,100 µg/mL.** CQ (0.1ug/mL) was used as positive control and negative control was treated with tween 20. Results were expressed as mean ± STD. ***p < 0.01 and ****p < 0.0001compared to negative control.

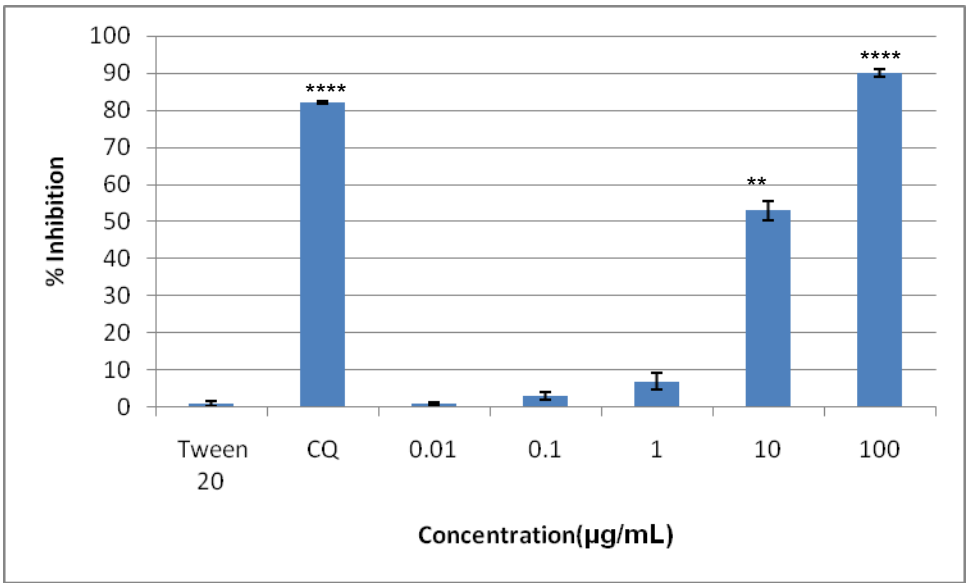

Fig 11: **Activity of fraction A5 against *P.falciparum* at different concentration of 0.01,0.1,1,10,100 µg/mL.** CQ (0.1ug/mL) was used as positive control and negative control was treated with tween 20. Results were expressed as mean ± STD. **p < 0.01 and ****p<0.0001 compared to negative control

3.3 Discussion

Resistance to anti-malarial drugs has often threatened malaria elimination efforts and historically has led to the short-term resurgence of malaria incidences and deaths [31]. Anti-malarial drug resistance develops when spontaneously occurring parasite mutants with reduced susceptibility are selected, and are then transmitted [32]. Chloroquine resistance is associated with mutations in the polymorphic gene encoding aputative chloroquine transporter and located on chromosome “7” [31]. Chloroquine-resistant *Plasmodium falciparum* malaria is a major health problem. *P. falciparum* infections acquired in most of Africa, and some parts of Asia and South America cannot be treated with chloroquine [33]. Increasing drug resistance in *plasmodium falciparum* and a resurgence of malaria in tropical areas have effected a change in treatment of malaria [34]. A combination of antimalarial drugs is responsive to *P.falciparum* with high grade resistance to chloroquine. Artemisinin and its semi-synthetic derivatives are anti-malarial drugs effective against CQ-resistant *P. falciparum* as single therapeutic agents. However, to minimize the risks of recrudescence and the development of resistance, a combination treatment with a second antimalarial drug is recommended [35]. Newer drug combination of compounds from biodiversity to combat malarial disease and drug resistant strand are urgently needed. The long-established use of quinine and the more recent introduction of artemisinin and its derivatives as highly effective antimalarials demonstrates that plant species are an important resource for the discovery of new antimalarial agents[36]. *Anthocleista djalonensis* of Gentianceae family is one plant with diverse medicinal uses. Some activities of the leaf stem [22] and roots [15, 21] parts of the plant against malaria have been reported. The phytochemical screening of the root extract of *Anthocleista djalonensis* carried out indicated the presence of saponins, flavonoids, tannins, reducing sugar, steroids, phlobatanins, volatile oils and alkaloids which are active components present in the plant that makes it medicinal [36].

In vivo activity of the root extracts and fractions against P. berghei may have been reported [21], yet this is the first scientific study of the fractions from the root of *Anthocleista djalonensis* on *Plasmodium falciparum* (the parasite responsible for human malaria). In this study we investigated the anti-malarial and cytotoxicity activity of acetone chromatographic fractions of *A.djalonensis*. Fractions A2, A3, and A5 with low SI (1.33, 1.68 and 1.30) revealed that the anti-malarial activity was dependent on the cytotoxicity and independent on the activity against the parasites. While A1and A4 with high SI (441.25 and 1560.03) meant that activity against the parasites was attributed to the parasites themselves and not cytotoxicity. According to Soh [37], high selective index means safer therapy. Hence a cutoff point of 4 certified safe anti-malarial use. Whereas, SI greater than 10 and IC_{50} values below 10 μg/mL should be promising sources of anti-malarial molecules. Activity against *HeLa* cells and *P. falciparum* in a high percentage of tested fractions were observed. Activity was directly proportional to the concentration. Obviously, *in vitro* study presumes a direct action on the parasite [38].

Although, anti-malarial activity has been detected in some parts of *A.djalonensis* plants like the stem and leaves [22], this is the first report for the chromatographic fractions of acetone root of *A.djalonensis plant*. Phytochemical analysis of these fractions from acetone root extract of *A.djalonensis* suggests the presence of triterpenoids, flavonoids and anthraquinones [17] as chemical classes with widely demonstrated effective antimalarial activity [39]. Flavonoids act by inhibiting the fatty acid biosynthesis (FAS II) of the parasite [40,41] as well as inhibiting the influx of *L*-glutamine and myoinositol into infected erythrocytes [43]. Antimalarial activity of anthoquinone could be due to nitric oxide generation from macrophages using polysaccharide. In combination of high concentration of this oxide with sub-optimal doses of chloroquine, the parasitaemia in chloroquine resistant malarial infection was suppressed [43]. While triterpenoids mechanism of action is in the arresting of parasite development, inhibition of the hemozoin polymerization on the parasite

[44, 45] and the lactase dehydrogenase of the *Plasmodium falciparium* (an essential enzyme used to generate energy within the parasite) [46, 47].

The high activity of fraction A1 and A4 is an indication that these fractions are promising sources as anti-malarial agents.

4. CONCLUSION

The chromatographic root fractions of *Anthocleista djalonensis* showed potent anti-malarial and cytotoxic activities. The results highlighted the safety in the use of the plant of which can become a source of lead compounds of high therapeutic efficacy for malarial diseases. The remarkable anti malarial activity of *A.djalonensis* encourages the investigation of native and naturalized African plants to explore as a potential source of anti malarial drugs. However, fractions were selected for further purification, isolation and identification of active chemical classes with proved efficacy against *P. falciparum*.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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