Original Research Article

In vitro Cytotoxicity and Antiplasmodial activity of fractions from *Anthocleitsta djalonensis* A.Chev.

Abstract

AIM: To identify possible antiplasmodial agents from chromatographic root fractions of *Anthocleista djalonensis* of the Genatianceae family as well as evaluate their cytotoxicity against *HeLa* cells.

Place and Duration of study: The study was carried in department of organic chemistry, Rhodes University, Grahamstown, South Africa. The duration period was between March 2016 and July 2016.

Methodology: The *Anthocleista djalonensis* roots were collected from Arochukwu, Abia State, Nigeria. The concentration (0.01-100 ug/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 0.031, 75.214, 80.100, 0.013, and 60.012 µg/mL respectively. CC_{50} values of 95.12, 100.02,135.46, 78.51, 80.21 µg mL^{-1} were recorded for fractions A1, A2, A3, A4 and A5 respectively. Fractions were classified as marginally active (A2, A3, A5) showing SI of 1.33, 1.68 and 1.30 and as active (A1, A4,) exhibiting SI of 441.25,1560.03 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 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 and anticancer drugs.

Keywords: Antiplasmodial activity; Cytotoxicity; IC50; Fractionation; Anthocleista djalonensis

1. INTRODUCTION

Medicinal plants contain chemical substances or constituents that have pharmacological activities[1]. These activities include anti-cancer, anti-tumor, anti-oxidant and anti-microbial activities[2,3]. 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 due to which man learned to pursue drugs in barks, seeds, fruit bodies, and other parts of the plants[4]. 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[4].

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[5]. Traditionally, the plant is used to treat wound, malaria, constipation, dysentery, diarrhoea, hepatitis, skin infection, and inflammation[6]. Recently, Ethnobotanical investigation revealed the use of *Anthocleista djalonensis* for the treatment of cancer[7].

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[8]. One of the greatest obstacles to the control of malaria has been the spread of resistance to drugs used on a large scale[9]. Chloroquine, though effective as a blood schizontocidal drug, is ineffective or partially effective in resistant cases[10].The emergence of strains of *Plasmodium falciparum* resistant to chloroquine and many other drugs in succession has stimulated efforts to identify new antimalarial agents[10].

Previous pharmacological and bioactivity study of Anthocleista djalonensis extracts has necessitated this investigation. Therefore the present study was undertaken to evaluate the cytotoxicity and antiplasmodial potential of chromatographic fractions from the acetone root extract.

2. MATERIAL AND METHODS

2.1. Plant materials

The root of *Anthocleista djalonensis* was obtained from Arochukwu, Abia state, Nigeria.The plant taxanomic identification was established by Mr Ibe of the 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 a 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^{o}C to give AF01, AF02, AF03, respectively with AF00 as the crude extract.

2.3. Fractionation of Acetone extract

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. Eluate was collected in several bottles of 50 mL, each of which was given a number then analysed by TLC. The spots separated in TLC was observed with 254 nm UV light. The Rf and eluate which have the same pattern spots appearance on TLC were combined as one fraction then concentrated. Percentage 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

Human cervix adenocarcinoma cell (*HeLa*) obtained (from ATCC CCL-2 LGC standard Wesel, Germany) were cultured in a 5%CO_2 incubator at 37^{o}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): the cells 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 was regularly assessed using an inverted light microscope.Cells was cryopreserved by detaching the cells from the culture flask in trypsin/EDTA, pelleting the cells, transferring them to cryotubes 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 CC_{50}.

2.5. Invitro Cytotoxicity assay

Cytotoxic activity was determined by resazurin reduction based assay[11]. *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 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*, respectively, obtained from Rhodes University Gramhamstown, South Africa were kept in continuous *in vitro* culture according to the modified candle-jar method of Trager and Jensen[12]. 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. Invitro antiplasmodial assay

Antimalarial test was carried out using the Immunocapture parasite lactate dehydrogenase (IcpLDH) assay method as described by Makler et al.,[13]. 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. For each concentration, % parasite viability – the PLDH activity in treated wells relative to untreated controls was calculated.

2.8. Data analysis

Data represent the mean±standard error (SEM) of the indicated number of experiments. Graphs were prepared by Prism software. Statistical analysis of the data was carried out by one way ANOVA (Graph Pad Prism 5.02 Software). A value of $p< 0.05$, $p<0.01$, $p<0.0001$ 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,[14]. 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 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**

3.2. Invitro 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 vassal. 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}) ug/mL	HeLa Cells(CC50) ug/mL	SI	Classification
A1	**0.031**	**95.12**	**441.25**	**Active**
A2	**75.214**	**100.03**	**1.33**	**Marginally Active**
A3	**80.100**	**135.46**	**1.68**	**Marginally Active**
A4	**0.013**	**78.51**	**1560.03**	**Active**
A5	**60.020**	**80.21**	**1.30**	**Marginally Active**

3.2.1. Invitro Cytotoxicity assay

The test results on *HeLa* cell indicated growth inhibition by the fractions of acetone root extract from *A.djalonensis*. Fractions A1, A2, A4 and A5 had $CC_{50} \leq 100$, while $CC_{50} > 100$ was observed for fraction A3. The highest cytotoxicity activity was demonstrated by A4 with CC_{50} value of 78.51 ug/mL followed by A5, A1, and A2 with CC_{50} value of 80.21 ug/mL, 95.12 ug/mL and 100.02 ug/mL respectively. Fraction A3 showed the lowest cytotoxicity with CC_{50} value of 135.46 ug/mL (fig1,2,3,4,5,6).

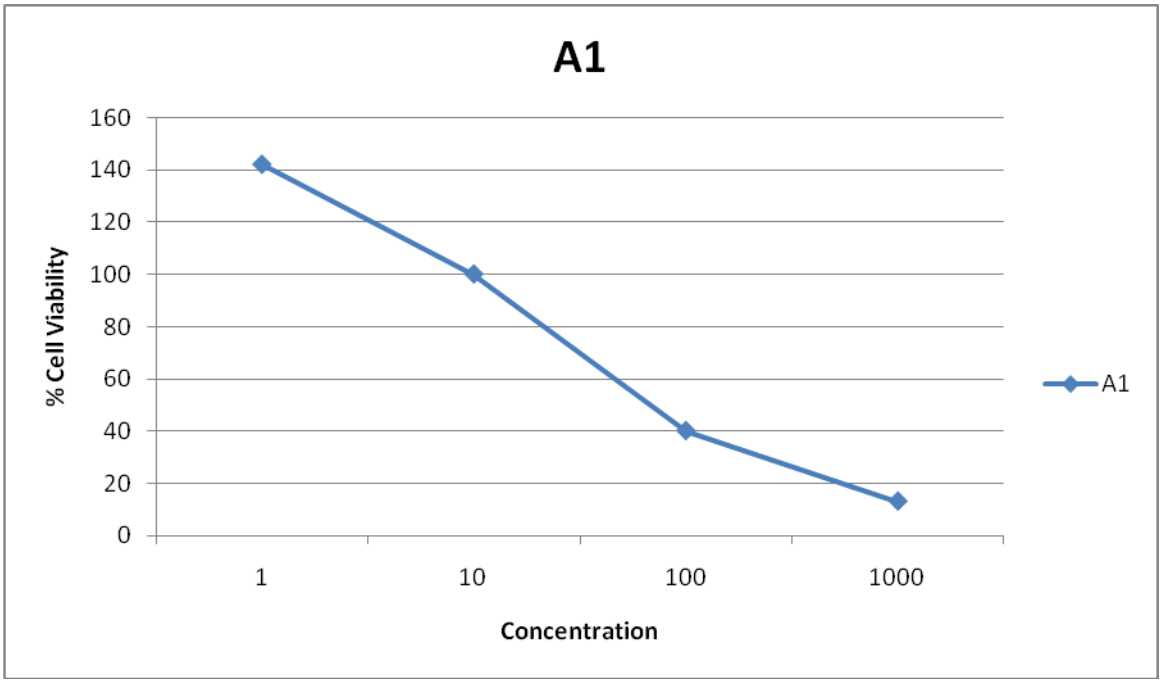

Fig 1:Cytotoxicity of fraction A1 against *HeLa* cells.

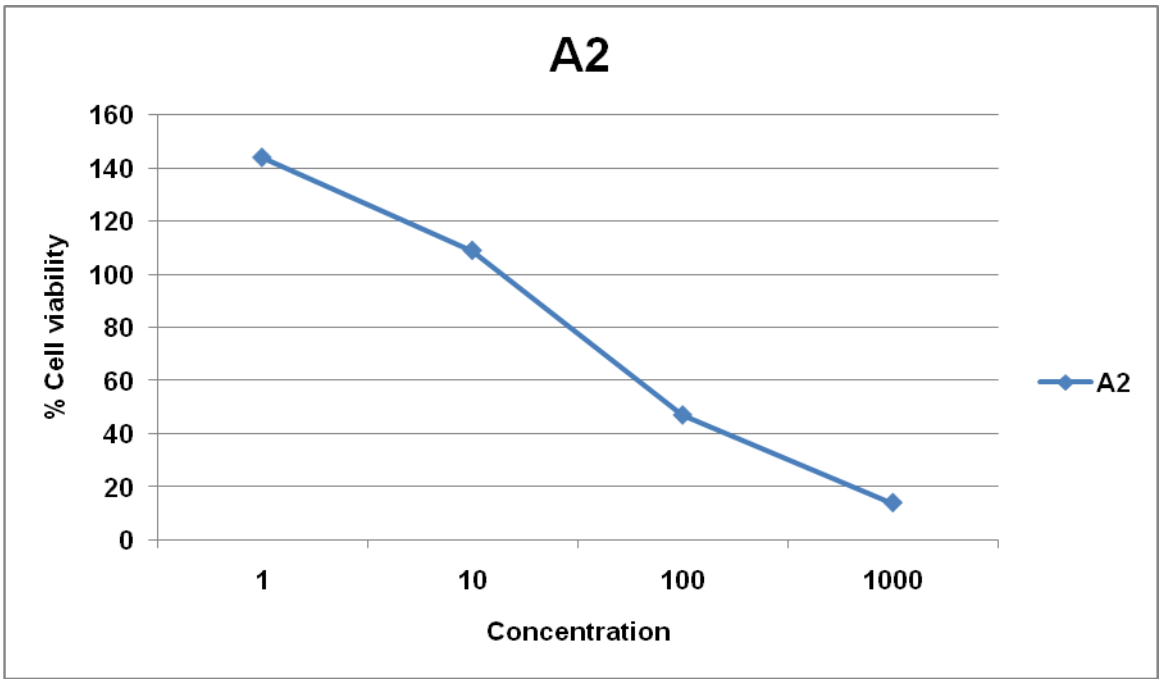

Fig 2: Cytotoxicity of fraction A2 against *HeLa* cells.

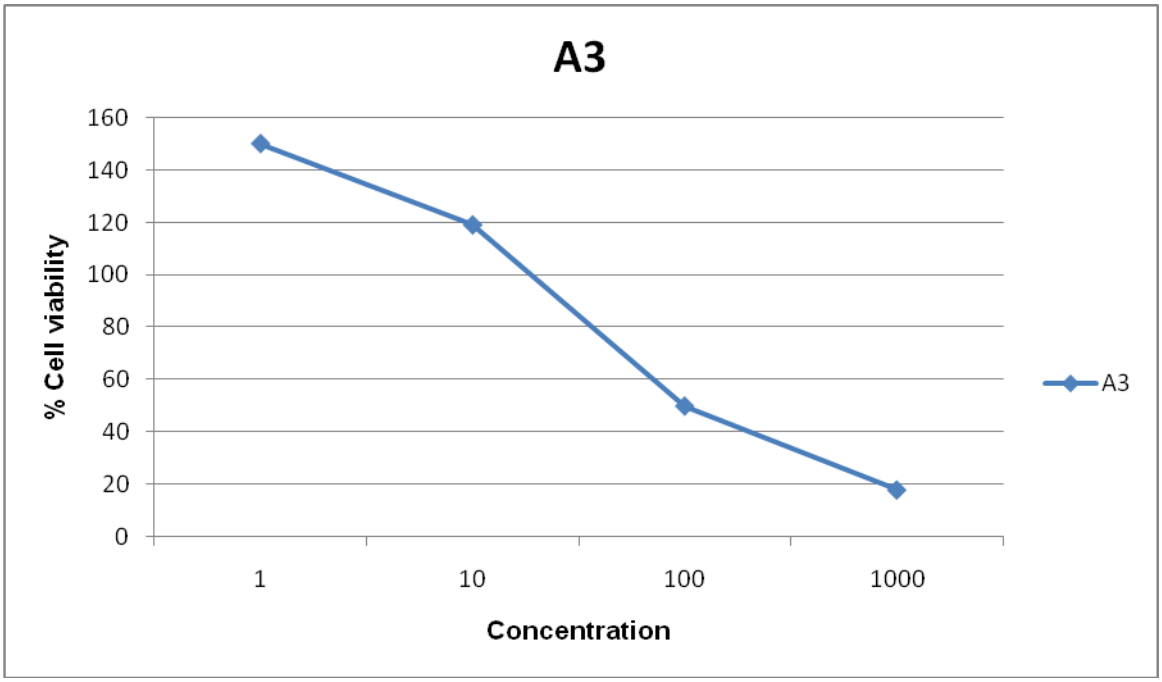

Fig 3: Cytotoxicity of fraction A3 against *HeLa* cells.

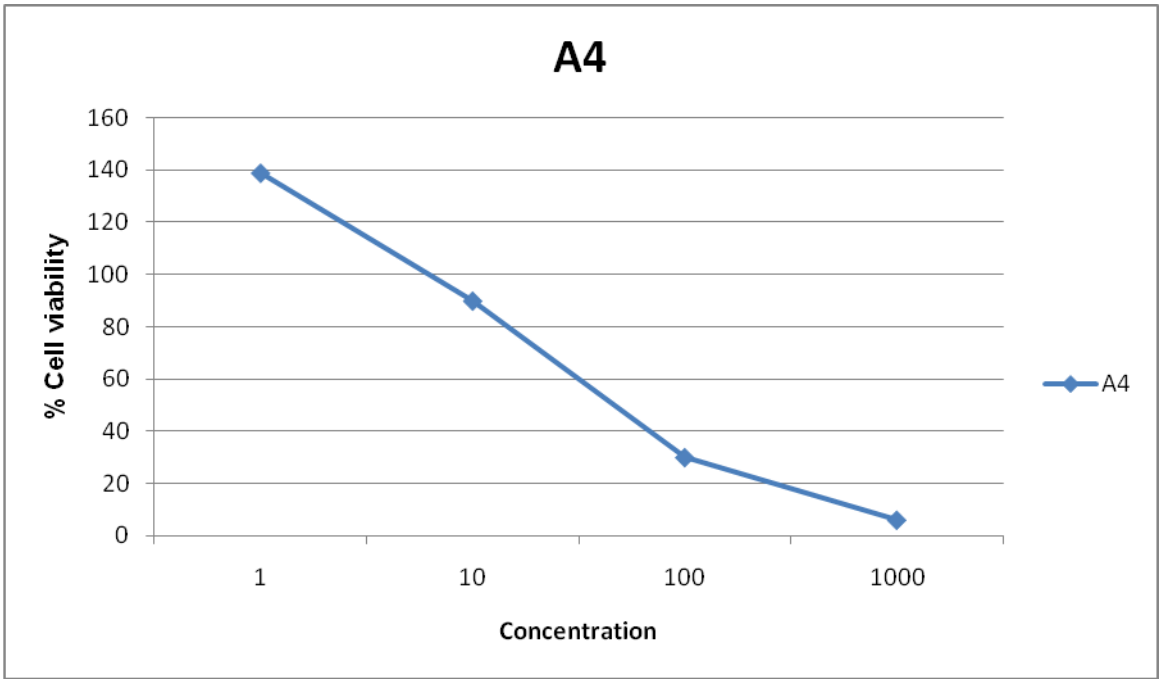

Fig 4: Cytotoxicity of fraction A4 against *HeLa* cells.

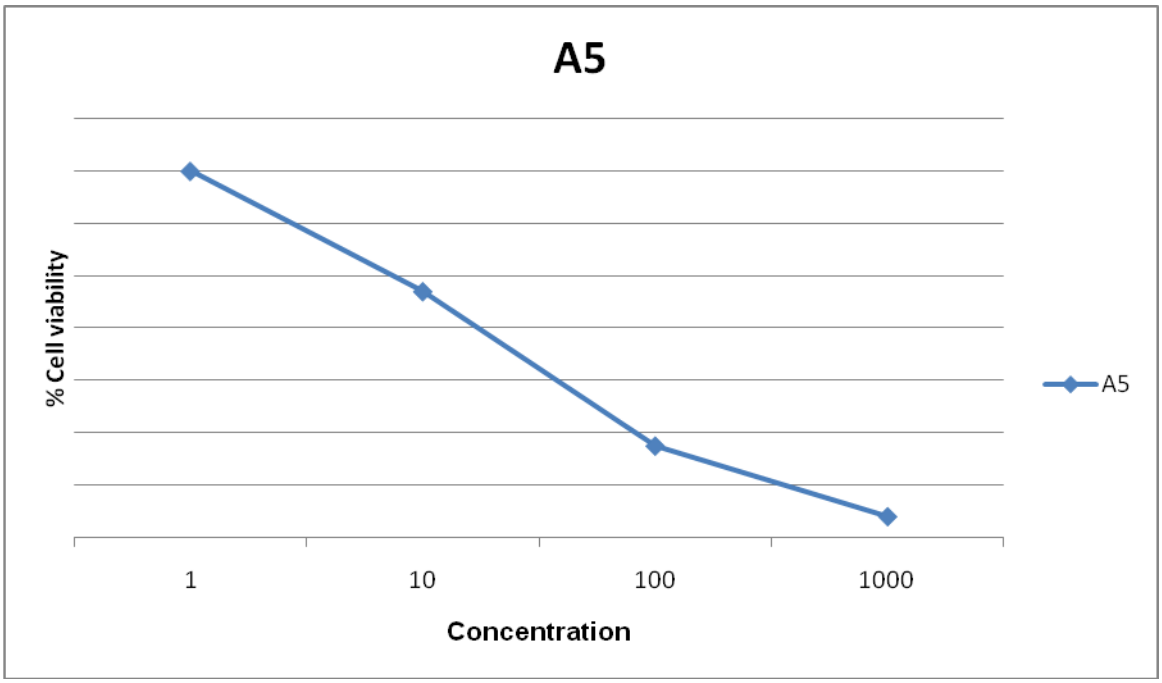

Fig 5: Cytotoxicity of fraction A5 against *HeLa* cells.

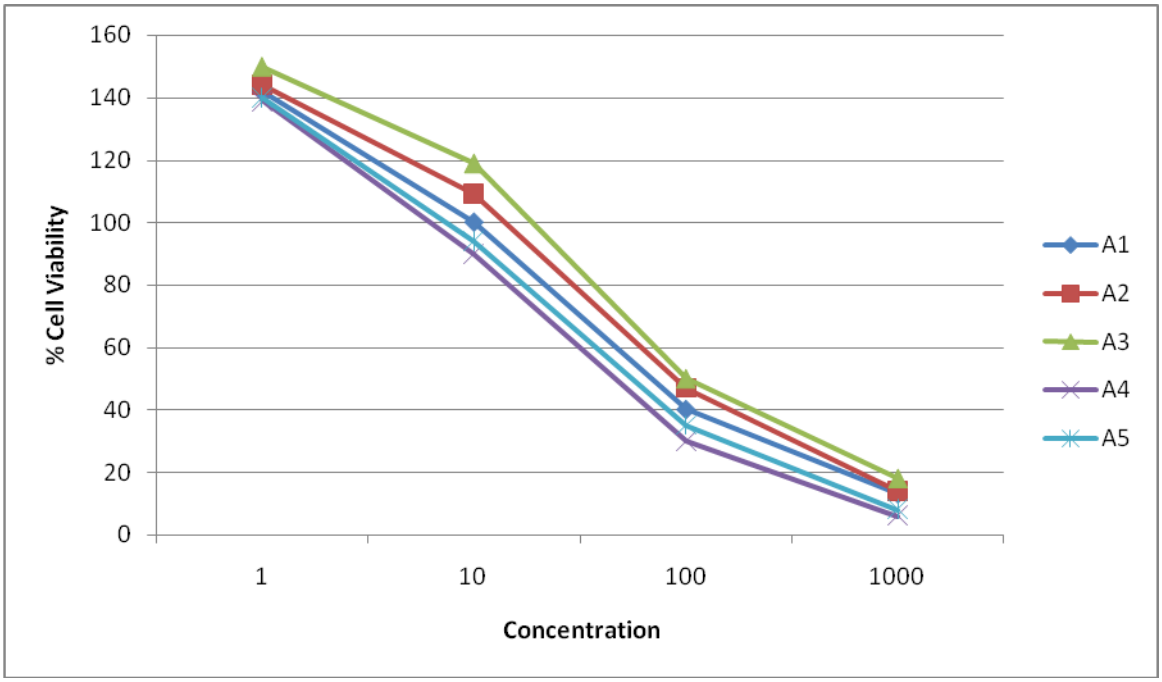

Fig 6: Cytotoxicity of all five fractions against *HeLa* cells.

3.2.2. In vitro antimalarial activity

The test results of *in vitro* antimalarial activity showed that all fractions of *A.djalonensis* acetone extract had the ability to inhibit the growth of *P. falciparum* (fig 7,8,9,10,11,12). The IC_{50} values and selectivity indices (SI = ratio of cytotoxicity to antimalarial activity) of extracts are shown in Table 2. The IC_{50} values for fractions A1, A2, A3, A4, and A5 were 0.031 ug/mL, 75.214 ug/mL, 80.100 ug/mL, 0.013 ug/mL and 60.020 ug/mL respectively. Fractions were classified as marginally active (A2, A3 and A5) showing SI of 1.33, 1.68 and 1.30 and as active (A1and A4) exhibiting SI of 441.25 and 1560.03 respectively. Furthermore, A1 and A4 showed SI > 10 and IC_{50} < 10 ug/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:

0.031ugm/L and 0.013 ug/mL). This showed A1 and A4 as being very prospective fractions to be developed as an anti-malarial agents.

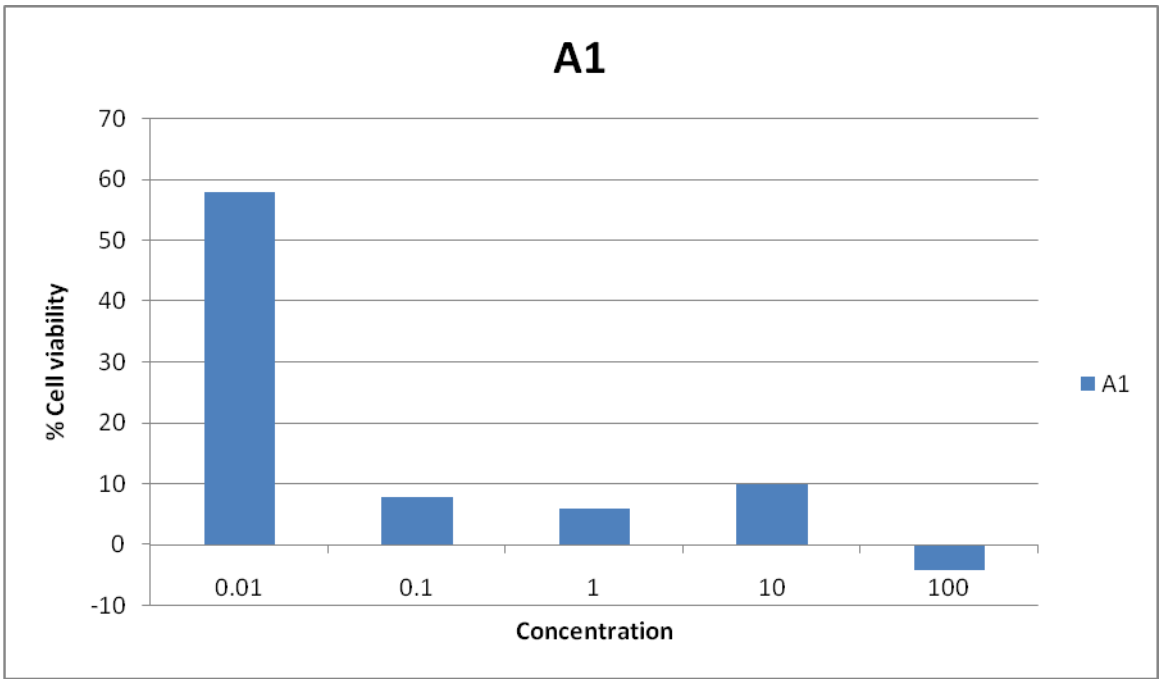

Fig 7: Activity of fraction A1 against *P.falciparum*.

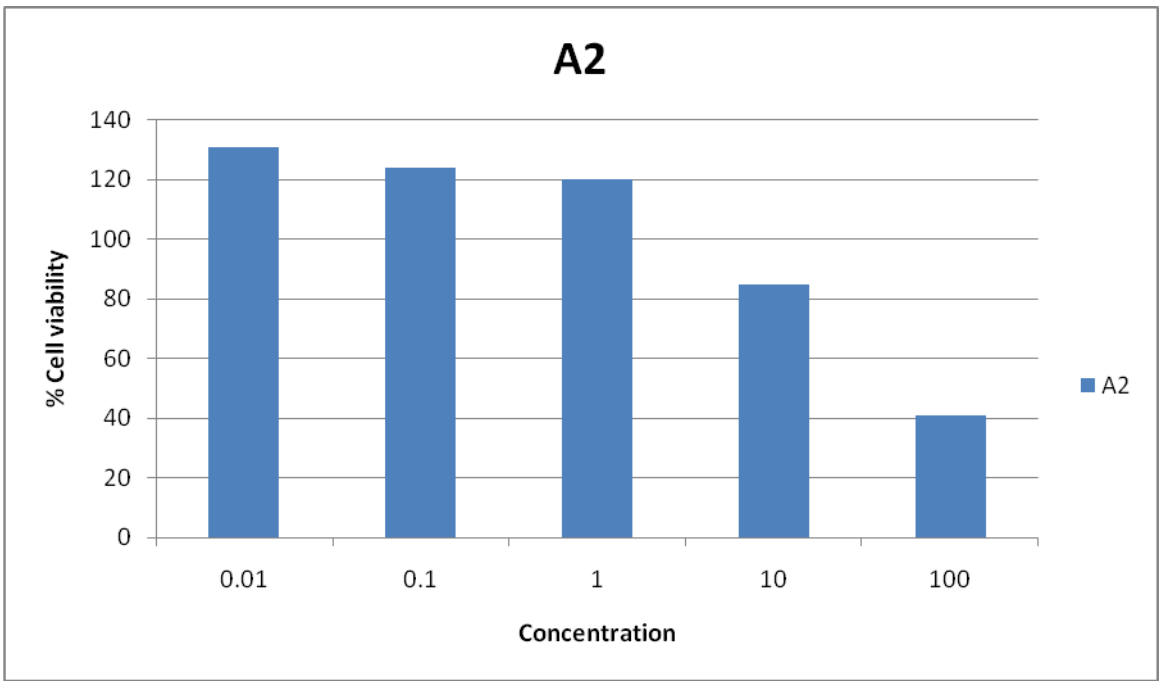

Fig 8: Activity of fraction A2 against *P.falciparum*

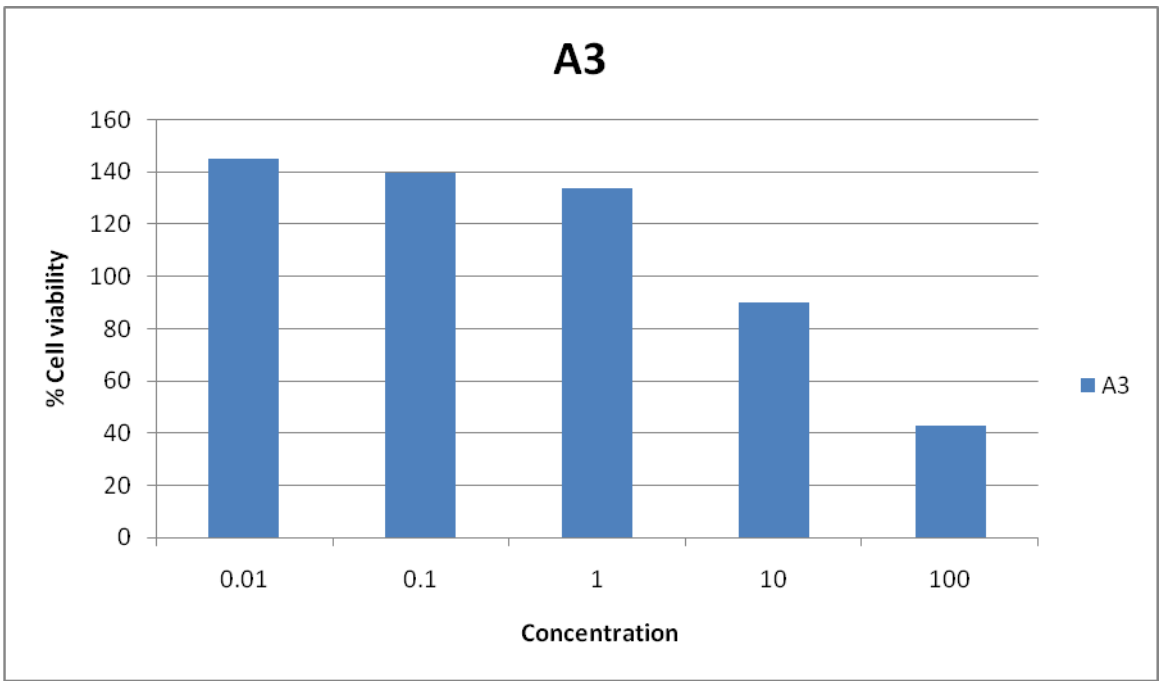

Fig 9: Activity of fraction A3 against *P.falciparum*

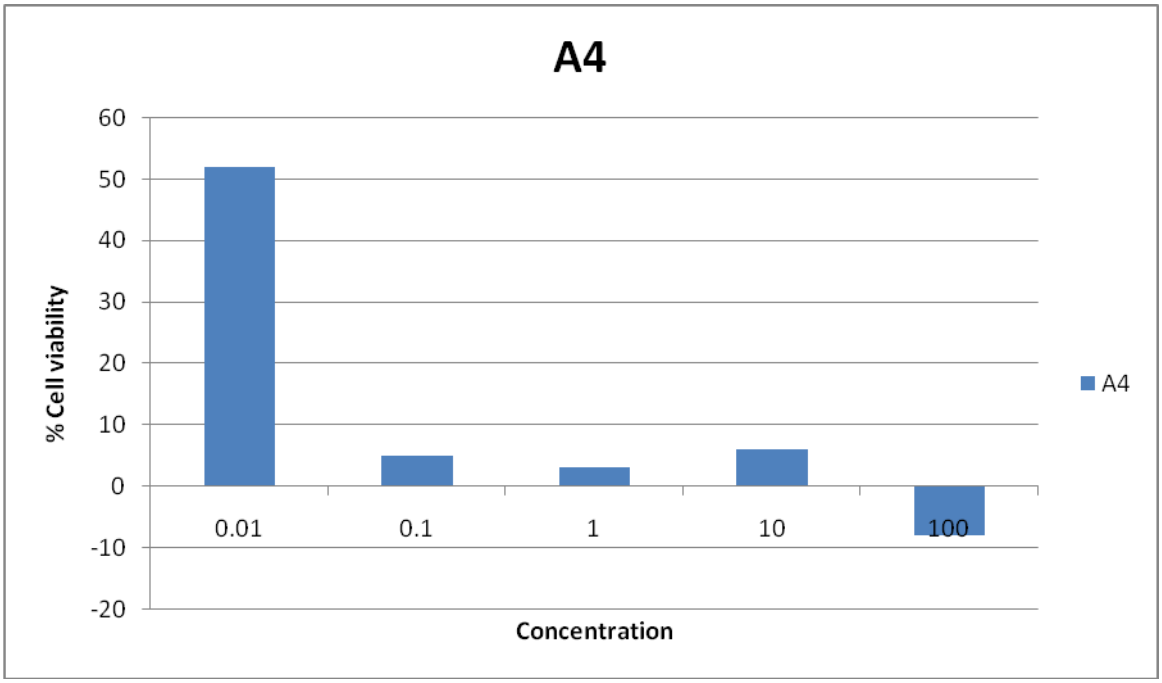

Fig 10: Activity of fraction A4 against *P.falciparum*

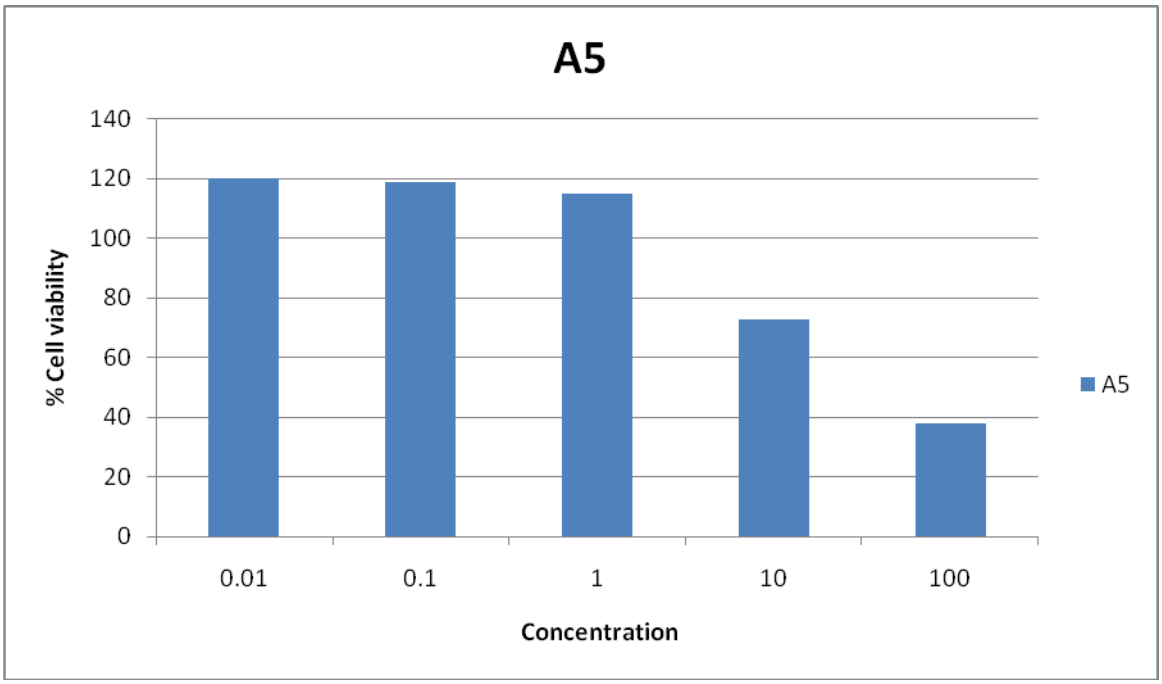

Fig 11: Activity of fraction A5 against *P.falciparum*

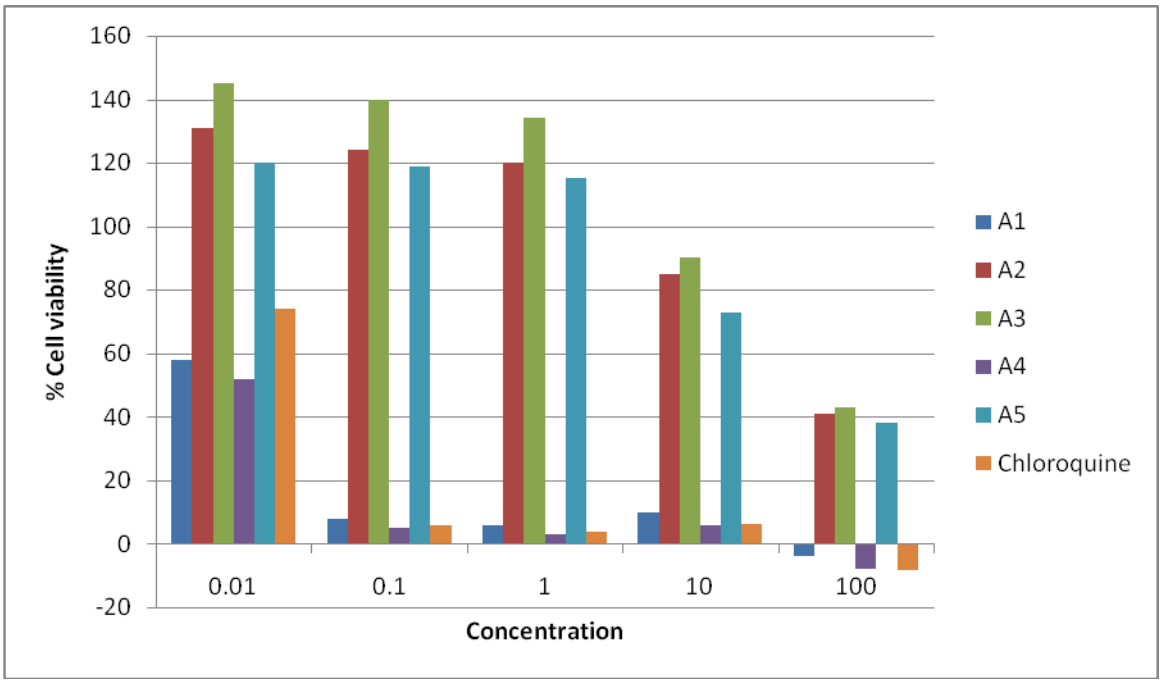

Fig 12: Activity of all fractions and Chloroquine (Positive control) against *P.falciparum*

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[15]. Anti-malarial drug resistance develops when spontaneously occurring parasite mutants with reduced susceptibility are selected, and are then transmitted[16]. Chloroquine resistance is associated with mutations in the polymorphic gene encoding aputative chloroquine transporter and located on chromosome "7" [15]. 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[17]. Increasing drug resistance in *plasmodium falciparum* and a resurgence of malaria in tropical areas have effected a change in treatment of malaria[17]. 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[18]. 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[19]. Anthocleista djalonensis of Gentianceae family is one plant with diverse medicinal uses. Several activity of the parts of the plant against malaria has 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[20]. Akpan et alal.,[21] investigated the antimalarial activities of ethanolic root extract/fractions of Anthocleista djalonensis in *Plasmodium berghei* in infected mice. The extract and its fractions dose-dependently reduced parasitaemia induced by chloroquine sensitive *Plasmodium berghei* infection in prophylactic, suppressive and curative models in mice. Ethanolic leaf and stem extracts exhibited a significant activity in mice infected with *Plasmodial berghei* with a considerable mean survival time, which was incomparable to that of the standard drug, chloroquine[22].

This is the first scientific study of the root fractions of *Anthocleista djalonensis* on *Plasmodium falciparum.* In this study we investigated the cytotoxicity and antimalarial 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 antimalarial 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,[23], 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 below10 ug/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[24].

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 the fractions of root extract of *A.djalonensis* suggests the presence of terpenoids, flavonoids and anthraquinones as chemical classes with widely demonstrated effective antimalarial activity[25]. The high activity of fraction A1 and A4 is an indication that these fractions are promising sources as antimalarial agents.

4. CONCLUSION

The chromatographic root fractions of *Anthocleista djalonensis* showed potent antimalarial and cytotoxic activities. The results highlighted that the plant can be used as a source of natural antiplasmodial and anticancer compound. The antimalarial use of *A.djalonensis* was validated The remarkable antimalarial activity of *A.djalonensis* encourages the investigation of native and naturalized African plants to explore as a potential source of antimalarial 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.

REFERENCES

1. Tyler V, Herb of Choice:The Therapeutic Use of Phytomedicinals, Pharmaceutical Products Press: New York, NY,1994; p.119.

2. Van Wyke BE, Wink C, Wink M, Phytomedicines, Herbal Drugs and Plant Poisons of the World. Royal Botanic Gardens, Kew, UK; University of Chicago Press, USA,2015; pp 52-80

3. Wink M1, Ashour ML, El-Readi MZ, Secondary Metabolites from Plants Inhibiting ABC Transporters and Reversing Resistance of Cancer Cells and Microbes to Cytotoxic and Antimicrobial Agents. Front. Microbiol., 2012; 3:130. Doi: 10.3389/fmicb.2012.00130;

4. Petrovska BB, Historical review of medicinal plants usage.Pharmacogn Rev, 2012; 6(11): 1–5 Doi: 10.4103/0973-7847.95849; PMCID: PMC3358962

5. Jensen SR, Schripsema J, Chemotaxonomy and pharmacology of Gentianaceae. In book: Gentianaceae - Systematics and Natural History, Chapter: 6, Publisher: Cambridge Univ. Press.2002; pp.573-631

6. Okoli AS, Iroegbu CU, Evaluation of extracts of Anthocleista djalonensis, Nauclea latifolia and Uvaria afzalii for activity against bacterial isolates from cases of non-

gonococcal urethritis. Journal of Ethnopharmacology, 2004; 92(1):135-44 . DOI: 10.1016/j.jep.2003.12.015;

7. Gbadamosi, Idayat Titilayo and Erinoso, Sakiru Morenikeji, A review of twenty ethnobotanicals used in the management of breast cancer in Abeokuta, Ogun State,Nigeria. African Journal of Pharmacy and Pharmacology, 2016;Vol. 10(27), pp. 546-564, DOI: 10.5897/AJPP2015.4327;http://www.academicjournals.org/AJPP;

8. White NJ, Antimalarial drug resistance J Clin Invest. 2004 Apr 15; 113(8): 1084–1092. Doi: 10.1172/JCI200421682;PMCID: PMC385418;

9. Travassos MA and Miriam K. Laufer, Resistance to antimalarial drugs: molecular, pharmacological and clinical considerations Pediatr Res. 2009 ; 65(5 Pt 2): 64R–70R. Doi: 10.1203/PDR.0b013e3181a0977e; PMCID: PMC2837525;NIHMSID; NIHMS108692;

10. Bickii J, Njifutie N, Foyere JA, Basco LK, Ringwald P, *In vitro* antimalarial activity of limonoids from *Khaya grandifoliola* C.D.C. (Meliaceae) J. Ethnopharmacol, 2000; 69: 27-33. https://doi.org/10.1016/S0378-8741(99)00117-8;

11. *Pesch, K. L., U.Simmert, "Combined assays for lactose and galactose by enzymatic reactions". Milchw. Forsch., 1929;* ***8****: 551.*

12. Trager W, Jensen JB. Human parasites in continuous culture. Sciences1976; 193: 673- 5.

13. Makler MT, Piper RC, Milhous WK, Lactate Dehydrogenase and the Diagnosis of Malaria. Trends in parasitology,1998;Volume 14, Issue 9, p376–377, 1. Doi: http://dx.doi.org/10.1016/S0169-4758(98)01284-8;

14. Valdés AF, Martínez JM, Lizama RS, Gaitén YG, Rodríguez DA, Juan Abreu Payrol JA, *In vitro* antimalarial activity and cytotoxicity of some selected cuban medicinal plants, Rev. Inst. Med. trop. S. Paulo, 2010;vol.52 no.4. http://dx.doi.org/10.1590/S0036-46652010000400006;

15. Petersen I, Eastman R and Michael lanzer, Drug-resistant malaria: Molecular mechanisms and implications for public health. FEBS Letters 2011; Volume 585, Issue 11, Pages 1551-1562 https://doi.org/10.1016/j.febslet.2011.04.042;

16. White N, Antimalarial drug resistance and combination chemotherapy. Philos Trans R Soc Lond B Biol Sci.1999; 354:739-749. PMID:10365399; PMCID:PMC1692562; DOI:10.1098/rstb.1999.0426;

17. White NJ, The treatment of malaria. N Engl J Med., 1996 ;335(11):800-6Doi:10.1056/NEJM199609123351107;PMID:8703186;

18. WHO. WHO briefing on Malaria Treatment Guidelines and artemisinin monotherapies.Geneva: WHO, 2009 [online] Availablefrom:http:/www.who.int/malaria/publications/atoz/meetingbriefing19april.pdf

19. Wright CW, Plant derived antimalarial agents: New leads and challenges Phytochemistry Reviews (2005) 4: 55–61Springer 2005.DOI: 10.1007/s11101-005-3261-7

20. Leke L, Onaji RA, Ahmad G, Uchenna OM, Phytochemical Screening and Anti-Microbial Activity Studies of the Root Extract of *Anthocleista Djalonensis* (Cabbage Tree) International Journal of Chemistry, 2012; Vol. 4, No. 4. doi:10.5539/ijc.v4n4p37;

21. Akpan EJ, Okokon JE, and Etuk IC, Antiplasmodial and antipyretic studies on root extracts of Anthocleista djalonensis against Plasmodium berghei. Asian Pacific Journal of Tropical Disease, 2012; Doi:10.1016/S2222-1808(12)60009-7;

22. Bassey AS, Okokon JE, Etim EI, Umoh FU, Bassey E, Evaluation of the *in vivo* antimalarial activity of ethanolic leaf and stembark extracts of *Anthocleista* djalonensis Indian J Pharmacol. 2009 ; 41(6): 258–261. Doi: 10.4103/0253-7613.59924 PMCID: PMC2846499;

23. Soh PN, Benoit-Vical F, Are West African plants a source of future antimalarial drugs? J Ethnopharmacol.2007;114:130-40. https://doi.org/10.1016/j.jep.2007.08.012

24. Benoit-Vical F. Ethnomedicine in malaria treatment. I Drugs. 2005;8:45-52 Krettli AU.

25. Onocha, P. A., Oorie, D. A., Conolly, J., & Joy Croft, D. S. (1995). Monoterpene diol, iridoid, glucoside and dibenzoaldpha-pyrone from Anthocleeista djalonensis. *Photochemistry, 40*(4), 1183-1189.