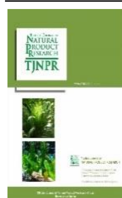

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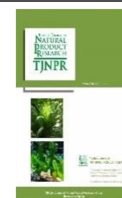

Antiprotozoal and Cytotoxicity Studies of Fractions and Compounds from *Enantia chlorantha*

Vincent Imieje[1,2]*, Ahmed A. Zaki[2,3], Pius S. Fasinu[4], Zulfiqar Ali[2], Ikhlas A. Khan[2], Babu Tekwani [2,5], Shabana I. Khan [2], Egiebor O. Nosa[6], Abiodun Falodun[1]

[1]*Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Benin, Benin City, 300001, Nigeria.*

[2]*National Center for Natural Products Research, Institute of Pharmaceutical Sciences, University of Mississippi, 38677, USA.*

[3]*Pharmacognosy Department, Faculty of Pharmacy, Mansoura University, Mansoura 35516, Egypt.*

[4]*Department of Pharmaceutical Sciences, College of Pharmacy and Health Sciences, Campbell University, Buies Creek, NC 27506, USA.*

[5]*Department of Bio-Molecular Sciences, School of Pharmacy, University of Mississippi, 38677, USA.*

[6]*Office of Global engagement, University of Mississippi, 38677, USA.*

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ABSTRACT

Enantia chlorantha Oliv. (Annonaceae) has been employed in Nigerian ethnomedicine for the treatment of various ailments such as malaria, ulcer, fever, wounds and inflammation. The methanol extract of the stem bark was re-extracted with hexane to obtain HF, ethyl acetate EF, butanol BF and the aqueous phase extract AF. The antiprotozoal screening of all fractions against *P. falciparum* (D6 and W2 strains), *L. donovani* and *T. brucei* revealed a significant antiplasmodial activity with IC_{50} values < 5.28 µg/mL at concentration ranges of 47.600 - 5.288 µg/mL. The hexane fractions exhibited the least activity with IC_{50} of 14.57 and 11.23 µg/mL for D6 and W2 strains, respectively. The crude extract also exhibited selective and significant leishmanicidal and trypanosomicidal activities against *L. donovani* and *T. brucei* with IC_{50} <0.8 and 15.2 µg/mL, respectively. Chromatographic analysis of the butanol and EtOAc fractions led to the isolation of the isoquinoline alkaloids; jatrorrhizine (**1**), palmatine (**2**), columbamine (**3**) together with *β*-sitosterol (**4**). The isolated compounds where significantly active in inhibiting *P. falciparum* parasites with IC_{50} values ranging between 1.7464 and >11.487 µM against D6 and W2 strains. Compounds **1** and **2** showed significant inhibition of *T. brucei* with IC_{50} and IC_{90} values 18.31 µM and 27.71 µM and 15.19 µM and 23.69 µM, respectively. The findings from this study justify the antiprotozoal usage of the plant extracts in Nigeria ethnomedicine.

Introduction

Malaria still remains a major public health problem with high mortality rate having a significant impact in developing countries and affecting several hundred millions of people worldwide. It is estimated that 3.3 billion people all over the world are at risk of malaria, of which 1.2 billion are at high risk [1]. There is an estimated 198 million cases of malaria recorded globally with resultant 584,000 deaths of which 90% occur in Africa especially among children under five years [1]. Malaria is an infectious disease caused by highly adaptable protozoan parasites of the genus *Plasmodium*, transmitted by a bite from an infected female anopheles mosquito. Four species of plasmodium are known to infect humans; however, it is *Plasmodium falciparum* that causes the majority of illnesses and deaths [1]. The development of resistance by *Plasmodium* parasite to known antimalarial drugs and resistant of the vector (mosquitoes) to insecticides necessitates an urgent need for the discovery of new, safe and affordable antimalarial agents. On the other hand, leishmaniasis is amongst the world's most neglected diseases affecting millions of people especially the poor from developing countries, caused by the protozoan *Leishmania* parasites which are transmitted by the bite of infected female phlebotomine sandflies.

*Corresponding author. E mail: vincent.imieje@uniben.edu
Tel: +23480802 411 8853

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Reports of WHO 2015, showed the presence of the disease in 98 countries with an estimated 0.9 - 1.3 million new cases annually, of which 1.5 million cases are due to cutaneous leishmaniasis (CL) and 0.5 million of visceral leishmaniasis (VL), with about 12 million people currently infected, resulting in annual deaths of 20,000 to 30,000 [2, 3]. Different studies [4, 5] established the existence and prevalence of CL mostly in the Northern and middle belt regions of Nigeria. The disease is said to affect mostly agrarian communities [5, 6] with higher point prevalence rate in males in the Muslim dominated north whilst higher amongst females in the Central, Eastern and Southern areas, where there is less seclusion [5].
Different species of the parasite cause disease in humans, the most severe and fatal being VL caused by *leishmania donovani* species complex, and is characterized by disseminated visceral infection of the reticuloendothelial system. Current drugs used in the treatment of leishmaniasis are not only relatively toxic and expensive but are of limited efficacy and their usage is also hampered by the development of resistance [7, 8]. There have been renewed interests in the search for medicinal plants for new and novel drug leads with better activity, less adverse effects to replete the rather depleted current pipeline of leishmanicidal drugs.
Human African trypanosomiasis (HAT), also known as sleeping sickness, is caused by extracellular hemoflagellates of the genus, *Trypanosoma.* The vector borne parasitic disease (HAT), is transmitted through the bite of an infected tsetse fly (genus *Glossina*). Two pathogenic subspecies (*Trypanosoma brucei* gambiense and *Trypanosoma brucei* rhodesiense) are implicated in human trypanosomiasis. These species though occur under the general term HAT, should be considered as separate diseases as

they are known to cause distinct disease with different epidemiological and clinical patterns as well as different patient management [1].

Availability, paucity of information on toxicity, pharmacokinetics and pharmacodynamics of currently used trypanocides such as; melarsoprol, pentamidine and D,L-α-difluoromethylornithine (DFMO), demand that alternatives, especially less toxic, affordable and available agents be introduced into the clinicians armamentarium in the fight against this disease, considered to have a case fatality rate close to 100% [1, 9].

Enantia chlorantha Oliv. (African yellow wood), family Annonaceae, is known for several medicinal uses. Decoctions, concoctions and infusions of the stem bark of *E. chlorantha* are used in traditional health systems of Nigeria, Cameroon and other west African countries for the treatment of various ailments such as stomach problems, rickettsia, typhoid fever and infective hepatitis, jaundice, urinary tract infections, fevers, malaria, tuberculosis, some forms of ulcer [10-14], and uterine stimulant [15]. Previous phytochemical studies of the stem bark of *E. chloranta* resulted in the isolation of berberine and protoberberine alkaloids possessing antimalarial [14, 16], antibacterial [17, 18], trypanosomicidal [19], anti HIV [20], hepatoprotective [21], cytoprotective and ulcer healing [22] properties. Similarly, Fasola *et al.*, reported significant activity of water extract of the stem bark of *E. chlorantha* against the African yellow fever virus [23]. Other phytochemical investigations on this plant also revealed the presence of isoquinoline, aporphine and phenanthrene alkaloids and sesquiterpenes [24].

Enantia chlorantha is locally known as *Awogba, Oso pupa* or *Dokita-igbo* (Yoruba), *Osomolu* (Ikale), *Kakerim* (Boki) and *Erenba-vbogo* (Bini). It is widely distributed along the coasts of West and Central Africa and very common in the forest regions of Nigeria [10]. This study aims to evaluate the anti-malarial, antileishmanial, and antitrypanosomal potentials of crude extract, fractions and isolated compounds from the stem bark of *E. chlorantha,* through *in vitro* and *ex vivo* primary, secondary and tertiary screening and also determine their cytotoxicity using VERO cell lines, in order to validate the ethnomedicinal use of different parts of the plant alone or in combination with other plants in the treatment of protozoal related diseases.

Materials and Methods

General experimental

All NMR experiments were carried out on a Bruker Avance III 400 MHz NMR spectrometer. All spectra were run at 27 °C and samples were dissolved in DMSO-d_6, CD_3OD or CD_3Cl. Chemical shifts are expressed in ppm relative to the solvent peaks serving as an internal standard. High resolution mass spectra were measured on Agilent Technologies 6200 series mass spectrometer. Gravity column chromatography (CC) was performed using silica gel (40-63µm, Sorbitech, USA). Thin layer chromatography (TLC) was carried out on pre-coated aluminum sheets silica 60 F_{254} plates (Fluka, Germany).

Plant collection

Fresh stem bark of *Enantia chlorantha w*ere collected from Ore, Ondo state, Nigeria in Jan. 2015 by Mr. Sunny Nweke of the Pharmacognosy Department, Faculty of Pharmacy, University of Benin, Benin City. The plant was identified by Ugbogu O.A. and Shasanya O.S. of the Forestry Research Institute of Nigeria (FRIN) Ibadan, were also a voucher specimen numbered FHI102003 is deposited

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Preparation of Extracts and Isolation of compounds

The plant stem bark was cut into pieces with the aid of a sterile knife, dried under shade for two weeks and pulverized with an electric blender (Blender/Miller III, model MS-223, China). The ground air-dried stem bark (1.6 kg) was extracted exhaustively with methanol in a Soxhlet apparatus for 16 h. The extract was concentrated *in vacuo* in a rotary evaporator at 40ºC to yield 170 g of a dark-solid. This solid (120 g) was suspended in 1 L of water and extracted with hexane to yield HF. The mother liquor was further extracted with ethyl acetate to give EF and then with butanol to obtain BF. The fractions were concentrated and dried under vacuum to yield 1.3 g, 27.20 g and 34.33 g for HF, EF and BF, respectively. The aqueous phase (AF) was lyophilized (26.6 g). The BF (30 g), was chromatographed over silica gel column (4.5 x 90 cm), eluted gradient wise with methanol (10 %) in chloroform to obtain 108 (F1-F108) fractions. The column eluates were monitored with TLC and visualized under UV 254 and 365 nm. Vanillin/sulfuric acid and Dragendorff's reagent were used to spray the TLC plates. Fractions F12-F22 (3.4 g) were combined on the basis of similar TLC profile. Repeated CC of this fraction eluting with $CHCl_3$: MeOH (10: 1) afforded compound **1** (180.8 mg) as yellow pellets. Combined fractions F33-F48 (4.56 g) was subjected to CC (4 cm x 60 cm), and eluted with $CHCl_3$: MeOH (9:1; 8.5:1.5; 8.0:2.0 v/v) to afford 17 sub-fractions (EA1-17). Fraction EA10 (150 mg) was further chromatographed over silica gel CC (2 cm x 50 cm) eluting with EtOAc: $CHCl_3$: MeOH (6: 4 : 4, v/v/v) to give compound **2** (83.7 mg), an amorphous orange color solid. Fractions F51-F58 (860 mg) were further chromatographed on normal silica gel column in $CHCl_3$: MeOH with increasing polarity to afford 20 sub fractions (EA1A-EA20A). Repeated chromatography of EA8A-EA15A gave compound **3,** a dark brown solid (32mg). The EF (15 g) fraction was subjected to CC eluted with Hexane/EtOAc (100, 90, to 10%) gradient wise to afford 158 fractions pooled into EC1-EC23 fractions based on TLC analysis. Sub-fractions EC8 (823mg) was subjected to normal silica gel column and eluted with $CHCl_3$/MeOH (20:1) to afford 32 fractions EC8A1-EC8A32. Compound **4** was purified from fraction EC8A24 as a white fluffy solid (22 mg).

Jatrorrhizine (**1**): yellow pellets,^{1}H NMR (400 MHz, DMSO-d$_6$) δ_H 9.85 (1H, *s*, H-8), 9.0 (1H, *s*, H-13), 8.2 (1H, *d*, *J* = 9.1 Hz, H-11), 8.02 (1H, *d*, *J* = 9.2 Hz, H-12), 7.70 (1H, *s*, H-1), 6.87 (1H, *s*, H-4), 4.91 (2H, *t*, *J* = 6.4 Hz, H-6), 4.07 (3H, *s*, 10-OCH_3), 3.94 (3H, *s*, 2-OCH_3), 4.11 (3H, *s*, 9-OCH_3), 3.2 (2H, *t*, *J* = 6.4 Hz, H-5). ^{13}C NMR (100 MHz, DMSO-d_6) δ_C 109.8 (*d*, C-1), 148.1 (*s*, C-2), 150.2 (*s*, C-3), 115.4 (*d*, C-4), 128.9 (*s*, C-4a), 26.6 (*t*, C-5), 55.5 (*t*, C-6), 145.5 (*d*, C-8), 121.4 (*s*, C-8a), 143.6 (*s*, C-9), 150.3 (*d*, C-10), 126.9 (*d*, C-11), 123.5 (*d*, C-12), 133.4 (*s*, C-12a), 119.5 (*d*, C-13), 138.4 (*s*, C-13a), 117.7 (*s*, C-13b), 56.5 (*q*, 2-OCH3), 62.2 (*q*, 9-OCH_3), 57.4 (*q*, 10-OCH_3). The HRESI-MS of compound **1** exhibited a molecular ion peak at *m/z* 338.1399 consistent with the formula $C_{20}H_{20}NO_4$. Comparison with literature data, compound **1** was found to be jatrorrhizine [20, 25, 26].

Palmatine (**2**): An amorphous orange color solid,^{1}H NMR (400 MHz, CD_3OD) δ_H 9.78 (1H, *s*, H-8), 8.82 (1H, *s*, H-13), 8.13 (1H, *d*, *J* = 9.1 Hz, H-11), 8.05 (1H, *d*, *J* = 9.1 Hz, H-12), 7.69 (1H, *s*, H-1), 7.08 (1H, *s*, H-4), 3.31 (2H, *t*, *J* = 6.4 Hz, H-5), 4.96 (2H, *t*, *J* = 6.4 Hz, H-6), 4.13 (3H, *s*, 10-OCH_3), 4.02 (3H, *s*, 2-OCH_3), 3.95 (3H, *s*, 3-OCH_3), 4.23 (3H, *s*, 9-OCH_3). ^{13}C NMR (100 MHz, CD_3OD) δ_C 109.8 (*d*, C-1), 150.7 (*s*, C-2), 153.6 (*s*, C-3), 112.0 (*d*, C-4), 129.9 (*s*, C-4a), 27.6 (*t*, C-5), 57.1 (*t*, C-6), 145.5 (*d*, C-8), 123.1 (*s*, C-8a), 146.2 (*s*, C-9), 151.7 (*d*, C-10), 127.9 (*d*, C-11), 124.2 (*d*, C-12), 135.1 (*s*, C-12a), 121.1 (*d*, C-13), 139.6 (*s*, C-13a), 120.3 (*s*, C-13b), 56.8 (*q*, 2-OCH_3), 56.5 (*q*, 3-OCH_3), 62.3 (*q*, 9-OCH_3), 57.5 (*q*, 10-OCH_3). The HRESI-MS spectrum exhibited a molecular ion peak at *m/z* 352.1571 (Cal. 352.1543), which is consistent with the formula $C_{21}H_{22}NO_4$. The combination of the different spectroscopic 1D, 2D NMR, and HR-ESI-MS data and comparison with reported literature data, compound **2** was found to be palmatine [20, 26, 27].

Columbamine (**3**): dark brown solid,^{1}H NMR (400 MHz, CD_3OD) δ_H 9.49 (1H, *s*, H-8), 8.41 (1H, *s*, H-13), 7.94 (1H, *d*, *J* = 9.1 Hz, H-11), 7.84 (1H, *d*, *J* = 9.1 Hz, H-12), 7.32 (1H, *s*, H-1), 6.51 (1H, *s*, H-4), 4.77 (2H, *t*, *J* = 6.4 Hz, H-6), 4.16 (3H, *s*, 9-OCH_3), 3.90 (3H, *s*, 3-OCH_3), 4.04 (3H, *s*, 10-OCH_3), 3.07 (2H, *t*, *J* = 6.4 Hz, H-5). ^{13}C NMR (100 MHz, CD_3OD) δ 108.9 (*d*, C-1), 145.6 (*s*, C-2), 151.7 (*s*, C-3), 111.9 (*d*, C-4), 129.8 (*s*, C-4a), 27.8 (*t*, C-5), 57.2 (*t*, C-6), 144.8 (*d*, C-8), 122.4 (*s*, C-8a), 145.5 (*s*, C-9), 151.03 (*d*, C-10), 128.7 (*d*, C-11), 124.6 (*d*, C-12), 131.4 (*s*, C-12a), 119.01 (*d*, C-13), 136.9 (*s*, C-13a), 118.9 (*s*, C-13b), 55.9 (*q*, 3-OCH_3), 61.9 (*q*, 9-OCH_3), 57.4 (*q*, 10-OCH_3). HRESI-MS: *m/z* 352.1571 (Cal. 352.1543). The HRESI-MS of compound **3** gave a molecular ion at *m/z* = 338.1399 corresponding to the molecular formula $C_{20}H_{20}NO_4$. Comparison with literature data identified compound **3** to be columbamine [20, 25, 26].

β-sitosterol (**4**): ^{13}C NMR (100 MHz, $CDCl_3$) δ 140.76, 121.72, 71.81, 56.78, 56.06, 50.14, 45.84, 42.33, 39.78, 37.26, 36.52, 36.16, 35.89, 33.95, 32.43, 31.91, 31.67, 29.16, 28.26, 26.08, 24.31, 23.08, 21.22, 19.83, 19.41, 19.04, 18.79, 11.99, 11.87. The MS spectrum of *β*-sitosterol confirmed its molecular mass at *m/z* = 414.7, which would be related to the molecular formula $C_{29}H_{50}O$. The compound further confirmed by comparing its data with those reported in literature [28-31].

Activity screening

Antiplasmodial, trypanosomicidal and leishmanicidal effects of crude methanol stem bark extract, fractions and isolated compounds of *E. chlorantha* were investigated *in vitro* against *Plasmodium falciparum* (chloroquine sensitive D6 and chloroquine resistant W2 strains) and *ex vivo* against *Leishmania donovani* (promastigotes, axenic amastigotes and

intracellular amastigotes in THP1 cells) and *Trypanosoma brucei* using standard procedures as described. The selectivity indices (a measure of samples cytotoxicity on mammalian cells) of the extracts were determined using VERO cell lines (monkey fibroblast).

Antiprotozoal assay

The *in vitro* antiplasmodial activity was determined using an assay protocol based on a colorimetric method that determines the parasite lactate dehydrogenase (pLDH) activity [32, 33]. The assay was performed in a 96-well microtiter plate and included two *P. falciparum* strains [Sierra Leone D6 (chloroquine sensitive) and Indochina W2 (chloroquine-resistant)]. DMSO was used as vehicle while artemisinin and chloroquine were included in each assay as positive drug controls.

The parasite-rescue and transformation assay with differentiated transformed human acute monocytic leukemia (THP1) cells infected *in vitro* with *Leishmania donovani* as described [8], was adopted. In this assay, *Leishmania* amastigotes growth was evaluated by an alamar Blue fluorometric assay in 96-well microplates. Extracts and compounds were prepared in DMSO, while Amphotericin B and Pentamidine were used as standard anti-leishmanial drugs. IC_{50} values were computed from the dose response curve by XLfit version 5.2.2.

Plant extracts and isolated compounds were screened again *T. brucei brucei* using a method previously described [34]. Briefly, a 2 day old culture of *T. brucei* in the exponential phase was diluted with IMDM medium to 5 X 10^3 cells/ml and dispensed in 384 well culture plates with 98µl in each well plus 2 µl of test samples and incubated at 37 ºC in a 5% CO_2 incubator for 48h. After 48 hr, 5µl of AlamarBlue was added to each well and the plates were incubated further for 24 hrs. Standard fluorescence was measured on a Fluostar Galaxy fluorometer (BMG LabTechnologies) at 544 nm ex, 590 nm em. Extracts were screened at concentrations ranging from 20 – 0.8 µg/mL. Pure compounds were screened at concentrations ranging from 10 – 0.4 µg/mL. Pentamidine and α- difluoromethylornithine (DFMO) were used as standards. Active extracts or compounds crossing this concentration range were further tested at lower concentrations. IC_{50} and IC_{90} values were computed from dose response growth inhibition curve by XLfit version 5.2.2.

In vitro Cytotoxicity Test

Cytotoxicity assay was performed in 96-well microtiter plates using neutral red uptake method as described by [35, 36]. The cytotoxicity of the plant extracts was assessed against VERO cell line (monkey kidney fibroblast) cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and 0.2% NaHCO3 at 37 ºC in an atmosphere of 95% humidity, 5% CO_2. Concentration ranges tested were between 0.19 – 48 µg/mL for crude extracts. IC_{50} was calculated from dose-response curve as earlier described. The selectivity indices (SI) were determined by measuring the cytotoxicity of samples on mammalian cells (VERO; monkey kidney fibroblast). The selectivity index (the ratio between the cytotoxicity (VERO cells) and antiparasitic activities were calculated.

Results and Discussion

The last decade has witnessed an explosion in the discovery of potent chemotypes against protozoal diseases, especially *plasmodium* species. Notwithstanding, challenges still abound. There is rapid development of resistance of the *plasmodium* parasites to currently used antimalarials even to the artemisinins [37]. Others include the issues of rapid onset of action, safety, especially in children and pregnant women and compliance i.e. cure malaria in a single dose. Hence, there is an increasing and urgent need for alternative drugs for malaria and other parasitic diseases (leishmaniasis and trypanosomiasis) treatment that is not only affordable but also provides effectiveness, safety and easy administration. The need for antimalarials that can target all three checkpoints in the pathophysiology of the disease: the blood, liver and transmission stages in order alleviate the symptoms, prevent re-lapses, and to protect other humans, respectively cannot be over emphasized.

Traditional herbal practitioners in Nigeria have achieved success with the use of *E. chlorantha* as remedy against malaria and other infectious diseases and several studies have shown the potential of extracts and isolated compounds of *E. chlorantha* in inhibiting *plasmodium* species and other parasitic diseases (leishmaniasis and trypanosomiasis) both *in vitro* and *in vivo* [11, 12, 16, 19, 38-45]. WHO guidelines and previous studies [46, 47], classified the activity of antiplasmodial screening of extracts and isolated compounds into four classes based on IC_{50} values: highly active extracts

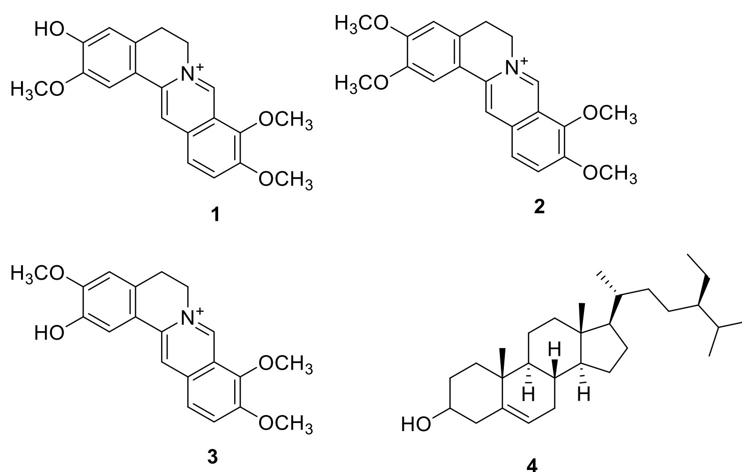

Figure 1: Isolated compounds 1-4

with IC_{50} < 5 µg/mL, promising activity at 5-15 µg/mL, moderate activity at 15-50 and µg/mL and inactivity at > 50 µg/mL.

In this study, we evaluated the *in vitro* activities of crude methanol extract (TE) of *E. chlorantha*, different fractions (HF, EF, BF and AF) and isolated compounds (Jatrorrhizine, palmatine, columbamine and β-sitosterol) against *Plasmodium falciparum* [D6 (chloroquine sensitive) and W2 (chloroquine resistant) strains], *L. donovani* (promastigotes, axenic amastigotes and intracellular amastigotes in THP1 cells) and *T. brucei brucei* trypomastigote form. Fractions and compounds were also tested against THP1 cells for determination of general toxicity using standard experimental procedures. Secondary antimalarial evaluation of the methanol extract (total extract, TF) revealed significant inhibition of parasites growth in chloroquine sensitive *P. falciparum* D6 and resistant W2 clones with IC_{50} values of < 0.370µg/mL and < 0.3200 µg/mL, respectively at tested concentration ranges of 47.6-5.288 µg/mL. Of all the fractions, the HF exhibited moderate activity with IC_{50} value of 14.57 µg/mL D6 and 11.23 µg/mL for W2. The EF and BF showed significant inhibition of parasites at IC_{50} values of < 5.28 µg/mL against the D6 and W2 clones while the AF had IC_{50} of 8.40 µg/mL against the D2 clone. Similarly, antiplasmodial screening of the isolated compounds (Table **2**) showed that these compounds where highly active in inhibiting *plasmodium* parasites growth with IC_{50} values of 1.8975, 2.1563, 2.2033 and >11.487 µM for Jatrorrhizine, palmatine, columbamine and β-sitoserol, respectively against D6 and 1.7464, 2.6726, 3.7397 and >11.487 µM against W2 clone. In the other studies, the extracts and isolated compounds exhibited selective and significant leishmanicidal and trypanosomicial activities against *L. donovani* amastigotes and *T. brucei* blood stage trypamastigotes with IC_{50} <0.8 and IC_{90} 1.39 µg/mL for fractions and 15.19 -29.57 µM for isolated compounds, respectively. EF exhibited significant inhibition of *T. brucei* blood stage trypamastigotes with IC_{50} of 1.7 µg/mL. Standard drugs Amphotericin B and Pentamidine were used with IC_{50} 0.1937 - 1.233 µM and 0.0058 - 29.366 µM, respectively, for all test organisms. The antileishmanial activity of the isolated compounds was compared with those of the crude extract and fractions (Table **3**). The isolated compounds **1** and **2** gave IC_{50} values >29.57 µM (>10 µg/mL) for *Leishmania donovani* (promastigotes, axenic amastigotes, and intracellular amastigotes in THP1) compared with the crude methanol extract that gave IC_{50} values <0.8 µg/mL and IC_{90} value 1.39 µg/mL for AMAST/THP1, respectively. Similarly, fractions EF gave IC_{50} and IC_{90} of 16.69 µg/mL and >20 µg/mL, respectively for AMAST/THP1. The BF of the stem bark of *E. chlorantha*, gave IC_{50} and IC_{90} of 19.4 µg/mL and >20 µg/mL, respectively for *L.donovani* promastigotes. The results of this screening revealed that the crude methanol extract exhibited higher activity than the fractions and isolated compounds. This activity may be due to the so called “entourage effect”, wherein it is suggestive that the whole plant phyto-constituents or partially purified extract exerts better effects than individual components. This effect was reported in a study of cannabinoid receptors where a mixture of the inactive, fatty acid esters of glycerol (palmitoyl glycerol and linoleyl glycerol) with 2-arachidonoyl-glycerol (2-AG), was more potent than 2-AG in the inhibition of adenylyl cyclase in COS-7 cells transfected for either CB1 or CB cannabinoid receptors [48]. The fractions and isolated compounds exhibited strong *in vitro* inhibitory activity against *T. brucei* blood stage trypamastigotes at IC_{50} and IC_{90} values ranging from >20

Table 1: Antiplasmodial activity of extracts and fractions 47.6-5.28 µg/mL and their SI values

Extract/Fractions	*P. falciparum* D6		*P. falciparum* W2		**VERO**
	IC_{50} µg/mL)	**SI**	IC_{50} (µg/mL)	**SI**	IC_{50}
TE	0.37	>0.13	0.32	>0.14	>4.760
HF	14.57	>3.3	11.23	>4.2	>4.760
EF	<5.28	>9.0	<5.28	>9.0	>4.760
BF	<5.28	>9.0	<5.28	>9.0	>4.760
AF	<5.28	>9.0	8.40	>5.7	>4.760

TE: total extract, HF: hexane, EF: ethyl acetate, BF: butanol, AF: aqueous fractions

Table 2: Antiplasmodial activity of isolated compounds and their SI * µM

Compounds	*P. falciparum* D6		*P. falciparum* W2		**VERO**
	IC_{50}*	**SI**	IC_{50}*	**SI**	IC_{50}
1	2.2033	>6.4	3.7397	>4.2	>14.0774
2	1.8975	>7.1	1.7464	>7.7	>13.5170
3	2.1563	>6.5	2.6726	>5.3	>14.0774
4	>11.487	1.0	>11.487	1.0	>11.4870
Artemisinin	<0.1062	>9.0	<0.1062	>9.0	>16.8597
Chloroquine	<0.0937	>9.0	0.4698	>1.4	>14.8810

Table 3: Antileishmanial and antitrypanosomal activity of extract, fractions and isolated compounds

Samples	***L. donovani* Promastigotes**		***L. donovani* axenic amastigotes**		***L. donovani* Amastigotes + TPH1**		***T. brucei***		**THP1 cytotoxicity**	
	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}
TE*	-	-	-	-	<0.8	1.39	15.23	17.17	-	-
HF*	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20
EF*	>20	>20	>20	>20	16.67	>20	1.7	3.07	>20	>20
BF*	19.4	>20	>20	>20	>20	>20	4.50	11.41	>20	>20
AF*	>20	>20	>20	>20	>20	>20	-	>20	>20	>20
1#	>29.57	>29.57	>29.57	>29.57	>29.57	>29.57	18.31	27.71	>29.57	>29.57
2#	>28.39	>28.39	>28.39	>28.39	>28.39	>28.39	15.19	23.09	>28.39	>28.39
3#	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
4#	>24.13		>24.13	>24.13	>24.13	>24.13	>24.13	>24.13	>24.13	>24.13
Amphotericin B#	0.2315	0.2705	1.233	-	0.1937	0.3365	-	-	-	-
Pentamidine#	4.4004	7.9519	29.366	-	9.303	15.263	0.0058	0.0088	NT	NT
DFMO#	-	-	-	-	-	-	15.658	40.214	NT	NT

* test conc. (20.0-0.8µg/mL), # µM, NT (not tested).

μg/mL to 1.7 μg/mL. Out of the fractions tested (test concentrations 20.0-0.8 μg/mL) (Table **3**), EF was considered to be the most active against *T. brucei* with IC_{50} and IC_{90} values of 1.7μg/mL and 3.07 μg/mL, respectively. Compounds **1** and **2** (test concentrations 10.0-0.48μg/mL) were also significant in inhibiting *T. brucei* blood stage trypamastigotes in this study with IC_{50} and IC_{90} values of 18.31 μM and 27.71 μM, respectively for **1** and 15.19 μM and 23.09 μM for **2** (Table **3**). Compound **4** showed the least activity with IC_{50} and IC_{90} values >24.13 μM.

In a study [49], oxygenation at C-2, C-3 (ring A) and C-9, C-10 (ring D) of isoquinoline skeleton together with the presence of quaternary nitrogen atom at position 7 were identified as the structural motifs required for strong antiplasmodial activity. Vennerstrom and Klayman [16] reported the *in vitro* activity of Palmatine against *Plasmodium falciparum* D6 and W2 clones with IC_{50} values of 797 μM and 463 μM, respectively and Jatrorrhizine IC_{50} of 480 μM [50]. Palmatine isolated from the methanol extract of the stem bark of *E. chlorantha* was shown to exhibit *in vitro* activity against *Leishmania infantum* (IC_{50}) 790 μM [51] and 10.08 mg/mL against the same parasite by the crude methanol extract [43]. Also, Palmatine isolated from the methanol extract of the stem bark of *E. chlorantha* exhibited antitrypanosomal activity *in vitro* against *T. brucei brucei* [19, 51] with IC_{50} values of 280 mM and the MeOH stem bark extract of the plant showed inhibitory activity against *T. brucei brucei* with IC_{50} of 10.5 mg/mL [41] and 8.36 mg/mL [43]. In another study [26], Palmatine isolated from another specie *A. kummeriae* exhibited significant antiplasmodial activity against *P. falciparum* K1 strain (IC_{50} 0.08 ± 0.001 μg/mL). In our study, all the extracts and compounds were assessed for their cytotoxicity in order to determine the selectivity index (SI). It is important to establish that an investigational product has anti-protozoal and or antimicrobial activity at concentrations that can be achieved *in vivo* without inducing toxic effects to cells. Cytoxicity tests use a series of increasing concentrations of the investigational product to determine what concentration results in the death of 50 percent of the host cells. This value is referred to as the median cellular cytotoxicity concentration and is identified by IC_{50}. The relative effectiveness of the investigational product in inhibiting parasites growth compared to inducing cell death is defined as the therapeutic or selectivity index (SI) (i.e., CC_{50} value/EC_{50} value) [52]. It is desirable to have a high therapeutic index giving maximum activity against plasmodium parasites and pathogenic micro-organisms with minimal cell toxicity. In this study the SI of extracts, fractions and isolated compounds in all instances are as shown in tables 1, 2 and 3.

The extracts and compounds were subjected to cytotoxicity evaluation with an aim to establish the safety of these extracts usage in ethnomedicine, as shown in tables 1 and 2 above. The EF, BF and AF exhibited SI > 9.0 comparable to the positive control drugs artemisinin and chloroquine, and may therefore be relatively safe. However, with SI >0.13, the TE may be said to be cytotoxic, and thus care must be exercised when decoctions of this plant is used in the treatment of parasitic diseases. Isolated compounds also exhibited varying selectivity indices ranges 1 to > 6.4, lower than that of the positive controls.

Conclusion

This study has demonstrated the antiprotozoal potential of crude extract, fractions and isolated compounds of *E. chlorantha* with very significant *in vitro* and *ex vivo* inhibition of parasites growth and good selectivity indices. The study validates the ethnomedicinal use of the stem bark of this plant in the treatment of malarial and other parasitic diseases. With an IC_{50} value of 0.37 μg/mL (TE) and 1.8975 - >11.487μM (compounds), the extract and compounds could be considered to be highly active [46, 47] and very good candidates for further antiprotozoal studies.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article are original and that any liability for claims relating to the content of this article will be borne by them.

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