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

Vincent Imieje¹,²,⁶, Ahmed A. Zaki²,³, Pius S. Fasinu⁴, Zulfqar Ali⁵, Ikhas A. Khan⁶, Babu Tekwani ²,⁵, Shabana I. Khan ², Egiebor O. Nosa⁶, Abiodun Falodun¹

¹Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Benin, Benin City, 300001, Nigeria.
²National Center for Natural Products Research, Institute of Pharmaceutical Sciences, University of Mississippi, 38677, USA.
³Pharmacognosy Department, Faculty of Pharmacy, Mansoura University, Mansoura 35516, Egypt.
⁴Department of Pharmaceutical Sciences, College of Pharmacy and Health Sciences, Campbell University, Buies Creek, NC 27506, USA.
⁵Department of Bio-Molecular Sciences, School of Pharmacy, University of Mississippi, 38677, USA.
⁶Office of Global engagement, University of Mississippi, 38677, USA.

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 antiparasitic activity with IC₅₀ values < 5.28 µg/mL at concentration ranges of 47.600 - 5.288 µg/mL. The hexane fractions exhibited the least activity with IC₅₀ 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 IC₅₀ < 0.8 and 15.2 µg/mL, respectively. Chromatographic analysis of the butanol and EtOAc fractions led to the isolation of the isooquinoline alkaloids; jatrohrrhizine (1), palmatine (2), columbamine (3) together with β-sitosterol (4). The isolated compounds where significantly active in inhibiting *P. falciparum* parasites with IC₅₀ values ranging between 1.7464 and > 1.487 µg/mL against D6 and W2 strains. Compounds 1 and 2 showed significant inhibition of *T. brucei* with IC₅₀ and IC₉₀ 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.

Keywords: Leishmanicidal, promastigotes, antiplasmodial, cytotoxicity.

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.

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 replace 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

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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 E. chlorantha MeOH (6: 4) 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].

Enanta 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, ricketsia, 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. chlorantha resulted in the isolation of berberine and protoberberine alkaloids possessing antimalarial [14,15], antibacterial [16], trypanocidal [17], and HIV [20], hepatoprotective [18], cytoprotective and ulcer healing [21] properties. Similarly, Fasola et al. reported significant activity of water extract of the stem bark of E. chlorantha against the African yellow fever virus [22].

Other phytochemical investigations on this plant also revealed the presence of isoquinoline, aporphine and phenanthrene alkaloids and sesquiterpenes [24].

Enanta chlorantha is locally known as Awogbo, Òsò pọpọ or Dokia-ibgo (Yoruba), Osooluma (Yoruba), Kukerin (Boki) and Ethenu-hogho (Bini). It is indigenous to the coast of Nigeria and the Bayelsa where it is very common in the forest regions of Nigeria [10]. This study aims to evaluate the anti-malarial, antileishmanial, and antitypocosomal 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-OD or CDCl_3. Chemical shifts are expressed in ppm relative to the solvent peaks serving as an internal standard.

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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 antiprotozoal 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 Indochna 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 [34], 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. IC50 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 °C in a 5% CO2 incubator for 48 h. After 48 h, 5μl of AlamarBlue was added to each well and the plates were incubated further for 24 h. 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 these concentration ranges were further tested at lower concentrations. IC50 and IC90 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 °C in an atmosphere of 95% humidity, 5% CO2. Concentration ranges tested were between 0.19 – 48 μg/mL for crude extracts. IC50 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 (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 antimalarial compounds 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) that is not only affordable but also provides effectiveness, safety and easy administration. The need for antimalarial compounds 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.

**Figure 1:** Isolated compounds 1–4

with IC50 < 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 trypanomastigote form. Fractions and compounds were also tested against THP1 cells for determination of general toxicity using standard experimental procedure. 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 IC50 values of < 0.370 μg/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 IC50 value of 14.57 μg/mL D6 and 11.23 μg/mL for W2. The EF and BF showed significant inhibition of parasites at IC50 values of < 5.28 μg/mL against the D6 and W2 clones while the AF had IC50 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 IC50 values of 1.8975, 2.1563, 2.2033 and >11.487 μM for Jatrorrhizine, palmatine, columbamine and β-sitosterol, 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 trypanosomical activities against L. donovani amastigotes and T. brucei blood stage trypanomastigotes with IC50 <0.8 and IC90 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 trypanomastigotes with IC50 of 1.7 μg/mL. Standard drugs Amphotericin B and Pentamidine were used with IC50 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 IC50 value >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 IC50 values >0.8 μg/mL and IC90 value 1.39 μg/mL for AMAST/THP1, respectively. Similarly, fractions EF gave IC50 and IC90 of 16.69 μg/mL, and >20 μg/mL, respectively for AMAST/THP1. The BF of the stem bark of E. chlorantha gave IC50 and IC90 of 19.4 μg/mL and >20 μg/mL, respectively for LDonovani 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 phytochemistry is needed to achieve optimal antiplasmodial activity. The IC50 values ranging from >20
Table 1: Antiplasmodial activity of extracts and fractions 47.6-5.28 µg/mL and their SI values

<table>
<thead>
<tr>
<th>Extract/Fractions</th>
<th>P. falciparum D6</th>
<th>P. falciparum W2</th>
<th>VERO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC₅₀ (µg/mL)</td>
<td>SI</td>
<td>IC₅₀ (µg/mL)</td>
</tr>
<tr>
<td>TE</td>
<td>0.37 &gt;0.13</td>
<td>0.32 &gt;0.14</td>
<td>4.760</td>
</tr>
<tr>
<td>HF</td>
<td>14.57 &gt;3.3</td>
<td>11.23 &gt;4.2</td>
<td>4.760</td>
</tr>
<tr>
<td>EF</td>
<td>&lt;5.28 &gt;9.0</td>
<td>&lt;5.28 &gt;9.0</td>
<td>4.760</td>
</tr>
<tr>
<td>BF</td>
<td>&lt;5.28 &gt;9.0</td>
<td>8.40 &gt;5.7</td>
<td>4.760</td>
</tr>
<tr>
<td>AF</td>
<td>&lt;5.28 &gt;9.0</td>
<td>&gt;0.13</td>
<td>4.760</td>
</tr>
</tbody>
</table>

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

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

<table>
<thead>
<tr>
<th>Compounds</th>
<th>P. falciparum D6</th>
<th>P. falciparum W2</th>
<th>VERO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC₅₀*</td>
<td>SI</td>
<td>IC₅₀</td>
</tr>
<tr>
<td>1</td>
<td>2.2033 &gt;6.4</td>
<td>3.7397 &gt;4.2</td>
<td>14.0774</td>
</tr>
<tr>
<td>2</td>
<td>1.8975 &gt;7.1</td>
<td>1.7464 &gt;7.7</td>
<td>13.5170</td>
</tr>
<tr>
<td>3</td>
<td>2.1563 &gt;6.5</td>
<td>2.6726 &gt;5.3</td>
<td>14.0774</td>
</tr>
<tr>
<td>4</td>
<td>&gt;11.487 1.0</td>
<td>&gt;11.487 1.0</td>
<td>&gt;11.4870</td>
</tr>
<tr>
<td>Artemisinin</td>
<td>&lt;0.1062 &gt;9.0</td>
<td>&lt;0.1062 &gt;9.0</td>
<td>&gt;16.8597</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>&lt;0.0937 &gt;9.0</td>
<td>0.4698 &gt;1.4</td>
<td>14.8810</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Samples</th>
<th>L. donovani Promastigotes</th>
<th>L. donovani axenic amastigotes</th>
<th>L. donovani Amastigotes + TPH1</th>
<th>T. brucei</th>
<th>THP1 cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC₅₀</td>
<td>IC₉₀</td>
<td>IC₅₀</td>
<td>IC₉₀</td>
<td>IC₅₀</td>
</tr>
<tr>
<td>TE*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>&lt;0.8</td>
</tr>
<tr>
<td>HF*</td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>&gt;20</td>
</tr>
<tr>
<td>EF*</td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>&gt;20</td>
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<tr>
<td>BF*</td>
<td>19.4</td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>&gt;20</td>
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<tr>
<td>AF*</td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>&gt;20</td>
</tr>
<tr>
<td>1*</td>
<td>&gt;29.57</td>
<td>&gt;29.57</td>
<td>&gt;29.57</td>
<td>&gt;29.57</td>
<td>&gt;29.57</td>
</tr>
<tr>
<td>3*</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Amphotericin B*</td>
<td>0.2315</td>
<td>0.2705</td>
<td>1.233</td>
<td>-</td>
<td>0.1937</td>
</tr>
<tr>
<td>Pentamidine*</td>
<td>4.4004</td>
<td>7.9519</td>
<td>29.366</td>
<td>-</td>
<td>9.303</td>
</tr>
<tr>
<td>DFMO*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* 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 IC50 and IC90 values of 1.7 µg/mL and 3.07 µg/mL, respectively. Compounds 1 and 2 (test concentrations 10.0-0.48 µg/mL) were also significant in reducing T. brucei growth against T. brucei (≥6.4, lower than that of the positive controls). The study validated the use of extracts, fractions, and isolated compounds in all instances 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 the investigational product in inhibiting parasites growth. It is important to establish that an investigational product has antiprotozoal and antimicrobial activity at concentrations that can be achieved in vivo without inducing toxic effects to cells. Cytotoxicity 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 cytotoxic concentration and is identified by IC50. 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). It is desirable to have a 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 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 > 6.4, lower than that of the positive controls.

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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