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Anti-leishmanial, anti-inflammatory and antioxidant potential of *Omphalocarpum ahia* A. Chev

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ABSTRACT

Omphalocarpum ahia A.Chev. is an evergreen medium-sized tree, about 30 m tall, and exudes a whitish fluid from the incised bark. It is used in folklore medicine for the treatment of pain, inflammation, bacterial and parasitic diseases. Reports of its pharmacological activities are not widespread. This study sought to evaluate its anti-leishmanial, anti-inflammatory and antioxidant potential. Its anti-leishmanial activity in comparison to Amphotericin B was evaluated in vitro against promastigote forms of Leishmania donovani using a hemocytometer counting chamber and a high field microscope with mobility of the parasite as a marker of cell viability. The extract was tested at concentrations between 15.6-500 µg/mL. The anti-inflammatory activity of the extract was evaluated using the carrageenan-induced foot edema in chicks whereas the 2,2-diphenyl-1-picrylhydrazyl radical scavenging assay was used for the antioxidant evaluation. O. ahia showed a lower anti-leishmanial activity (IC_{50}=124.0±0.67 µg/ml) than the standard drug amphotericin B (2.4± 0.67 µg/ml). It also showed moderate anti-inflammatory activity (ED_{50}=75.9±0.667 mg/kg BDW) compared to diclofenac (ED_{50}=3.74±0.333). The radical scavenging activity of O. ahia was just three times less potent than Vitamin E used as positive control. These activities may be due to the presence of tannins, glycosides, saponins, terpenoids and phytosterols found in the phytochemical analysis of the plant. Thus from the observed activity, barring any toxicity issues, O ahia may be harnessed and integrated into the treatment of leishmanias is caused by L. donovani as it may present a cheaper and safe alternative to the toxic synthetic analogues.

Keywords: Leishmanicidal, anti-inflammatory, antioxidant, *L. donovani*, DPPH radical, *O ahia*

INTRODUCTION

Leishmaniasis is a major public health problem especially in the developing countries. According to the World Health Organization (WHO)[1], about 88 countries are threatened by leishmaniasis and about 350 million people are at risk for the disease [2]. Kwakye-Nuako et al [3] reported the outbreak of human cutaneous Leishmaniasis in the Volta region of Ghana. DNA sequencing of three isolates revealed them to be Leishmania, identical to each other but different from all other forms of Leishmania species. Phylogenetic analysis indicated the parasites to be new members of the *Leishmania enriettii* complex, a new subgenus of Leishmania parasites. This development is

worrying at a time where the healthcare delivery system of Ghana is at breaking point and may have dire consequences on our ailing economy.

The antimonial drugs in current use are expensive, toxic and reports of resistance are widespread [4]. Other drugs like pentamidine and amphotericin B which are also used to treat the disease have been limited due to their high toxicity and cost [5]. Because of the adverse side effects of these treatment regimens, considerable attention has been given to the discovery and development of new, less toxic agents [6]. In an ongoing search for less toxic and cheaper Leishmanicidal agents, plant-derived products present a viable option. These plants and their products are readily available and accessible in the communities and majority of the populace rely on herbal medicines.

Omphalocarpum ahia occurs mostly in West Africa but majority of them are found in Sierra Leone and Ghana. In Ivory Coast, it is called 'Abe aguia' or 'akyeahia'. In Ghana the Ashanti's called it 'osonodokono' while the Nzemas called it 'asoro'.It is an evergreen medium-sized tree, about 30 m tall, and exudes a whitish fluid from the incised bark[7]. It is used in folklore medicine for the treatment of pain, inflammation, bacterial and parasitic diseases. A closely related species, *Omphalocarpumelatum*Miers, has been reported to have weak antibacterial activity [8]. The dichloromethane-methanol (1:1) extract of the fruit pericarp of *Omphalocar pumprocerum* exhibited antiplasmodial, antileishmania and anti trypanosomal activity [9]. Thus the genus *Omphlaocarpum* may have a great potential for the treatment of parasitic diseases, inflammation among a host of others. *Omphalocarpum ahia* has not been investigated to validate scientifically it uses in folklore medicine. Phytochemical analysis of the plant has by far not being forthcoming. Therefore in this research, the anti-leishmanial, anti-inflammatory and antioxidant potential of *O. ahia* was evaluated to give scientific credence to its alleged folklore uses.

MATERIALS AND METHODS

Plant material collection and identification

The stem bark of *O. ahia* (Sapotaceae) was harvested from Adukrom, a village in Nzema East Metropolis of the Western region of Ghana, in October 2014 and was identified by curators of the University of Cape Coast herbarium (Ghana). A voucher specimen (BHM/Omph/018A/2014) has also been identified and deposited at the herbarium of the Department of Herbal Medicine, Faculty of Pharmacy, Kwame Nkrumah University of Science and Technology, Ghana.

Extraction

The stem bark of *O. ahia* was air dried for 7 days followed by oven drying at 40^{o}C 48 hours. The dried material was milled into powder and packed into brown paper bags and kept until needed for extraction. The powdered air-dried stem bark (600 g) was cold macerated with 70% ethanol for 72 hours. The resulting extract was then filtered and concentrated under reduced pressure (40^{o}C) to give the crude extract (yield of 4.2% ($^{w}/_{w}$).

***In vitro* anti-leishmanial activity**

Cultivation of parasite

Promastigote strain of *Leishmania donovani* (LV90) was obtained from the Division of Biomedical and Life Sciences, Faculty of Health and Medicine, Lancaster University, U.K. The promastigotes were cultivated at 25°C in medium 199 (Gibco, Invitrogen) supplemented with 5% penicillin/streptomycin, 10% heat-inactivated fetal bovine serum (FBS), 10 mM adenine (pH 7.5), and 5 Mm L-glutamine Using a pipette, 500 microlitre of parasite (containing 14.4x106 parasites) were picked and added to 15 mL of M199 media in a culturing flask. The mixture was incubated at 25°C for five days. The number of parasites was counted daily using a hemocytometer (Z359629 SIGMA) according to the method described by Adade et al, [10].

Anti-leishmanial assay

The anti-leishmanial activity of the selected plants in comparison to Amphotericin B was evaluated *in vitro* against the promastigote forms of *Leishmania donovani* using a hemocytometer counting chamber and a high field microscope with mobility of the parasite as a marker of cell viability. A stock solution of the total crude methanol extract of the plant (1000 µg/mL) was prepared in DMSO. The different concentrations of the extract were prepared by two fold dilution of the stock as follows: 1 ml of the stock solution was dispensed into the first well of the 24 well micro titre plates. It was topped up with 1 mL of M199 media mixing thoroughly. 1 ml of this solution was again dispensed into the second cell and procedure repeated in subsequent cells. Each cell was then topped with 20 µl of *Leishmania donovani* culture containing 95.5×106 cells/mL of the promastigotes. Finally, each well was topped up with a suitable

amount of the medium (M199) to make a final volume of 2 mL, thereby achieving concentrations of 15.6, 31.2, 125, 250 and 500 µg/mL. The total concentration of DMSO was thus reduced to 0.5%V/V, a concentration which has negligible effect on parasite growth rate and morphology [11]. Total solution contained M199, DMSO, plant extract and 20µl promastigotes of Leishmania parasite. The same procedure was repeated for the positive control amphotericin B. The negative control consisted of the medium M199, DMSO and promastigotes of *L. donovani* mixed evenly in a cell without the extract. The parasites were incubated up to 72 hours at 25°C. All the *in-vitro* experiments were run in triplicate and the results expressed as a % inhibition in parasite numbers.

Anti-inflammatory assay

Experimental animals

Cockerels (*Gallus gallus*; Akate farms, Kumasi, Ghana) were obtained 1-day post-hatch and were housed in a stainless steel cages (34 × 57 × 40cm^3) at a population density of 10 chicks per cage. They were fed with chicks-feed (Chick mash, GAFCO, Tema, Ghana) and clean water was also available. The chicks were routinely maintained for seven days at a room temperature of 30^{o}C. They were tested at 7 days of age.

Anti-inflammatory assay

The experimental model used in assaying the anti-inflammatory activity of the extract was the carrageenan-induced foot edema in chicks [12]. The chicks were randomly selected and grouped into 5 per cage. Their initial foot volumes were determined by using a digital vernier caliper[13]. The anti-inflammatory activities of the extracts were compared to diclofenac and dexamethasone, used as standard drugs.

The chicks were weighed and their doses calculated per their body weight. 10µL of 2% carrageenan, freshly prepared in normal saline, was injected sub-plantar into the left foot of the chicks. One hour after the carrageenan challenge, the foot volume was measured again. The 70% ethanol extracts of *Omphalocarpum ahia* (EOA) given orally at 30, 100, and 300 mg/kg body weight. The standard drugs, dexamethasone (0.3, 1, and 3mg/kg body weight) and diclofenac (10, 30 and 100mg/kg body weight) were also given orally. The control groups received only the vehicle (normal saline). The foot volumes were measured at hourly intervals for 6 hours. The edema component of the inflammation was quantified by measuring the difference in foot volume before carrageenan injection and at the various time intervals. The percentage inhibition of edema was calculated based on the following equation:

$$\text{Percentage (\%) inhibition of edema} = \left[{}^{(AUC^\circ - AUC)}/_{AUC^\circ}\right] \times 100\%$$

Where: AUC^o is the Area under the curve for the control (non-treated group)

AUC is the Area under the curve for the test samples (treated group).

In vitro antioxidant assay

2,2-diphenyl-1-picrylhydrazyl radical scavenging assay

In this assay, the method described by Govindarajan et al [14] with few modifications by Amponsah et al., [15]was followed. The extracts (1 ml quantity of each) at concentrations of 500, 250, 125 and 62.5 µg/ml was added to 3 ml methanol solution of DPPH in a test tube and incubated at 25°C for 30 minutes. The ability of the extract to decolourise the purple colour of DPPH was determined at 517 nm in a spectrophotometer (Cecil CE 7200 ms pectrophotometer, Cecil instrument limited, Milton Technical Centre, England). The control was prepared by adding 1 ml of methanol and 3 ml of DPPH, incubating at 25°C for 30 minutes. Vitamin E (100, 50, 25, 12.5 and 6.25 µg/ml) was used as positive control. Results were expressed as percentages of blank. The EC_{50}which is the concentration required to scavenge 50 % of the DPPH molecule was calculated. Each test was carried out using three replicates

$$\text{\% DPPH Scavenging activity} = \left[{}^{(A^\circ - A)}/_{A^\circ}\right] \times 100\%$$

Where: A^o is the absorbance of the blank (negative control)

A is the absorbance of the test sample (positive control).

Phytochemical analysis
Phytochemical analysis on the plant for the presence of secondary metabolites such as tannins, alkaloids, glycosides, terpenoids and phytosterols was done according to the method described by Trease and Evans, [16].

STATISTICAL ANALYSIS
Doses and concentrations responsible for 50 % of the maximal effect (EC_{50} and IC_{50}) for the extract was determined using an iterative computer least squares method, with the following nonlinear regression (three-parameter logistic) equation.

$$Y= \frac{a+(b-a)}{\left(1+10^{(LogEC50-X)}\right)}$$

Where, *X* is the logarithm of dose and *Y* is the response. *Y* starts at *a* (the bottom) and goes to *b* (the top) with a sigmoid shape. The fitted midpoints (ED_{50}/IC_{50} values) of the curves were compared statistically using *F* test [16-17]. Graph Pad Prism for Windows version 5.0 (Graph Pad Software, San Diego, CA, USA) was used for all statistical analyses. $P < 0.05$ was considered statistically significant [18].

RESULTS AND DISCUSSION

Anti-leishmanial activity
The study looked at the anti-leishmanial activity of ethanol extract of *O. ahia* using promastigotes of *L. donovani* at concentrations 500µg/ml, 250µg/ml, 125µg/ml, 62.5µg/ml, 31.25µg/ml and15.63µg/mL.It showed a concentration dependent activity. The 500µg/ml extract produced 59.4%, 89.4%, 99.5% and 100% leis hmanicidal activity at 6h, 12h, 24h, 48 and 72h respectively (Figure 1). The lower concentrations were practically inactive. The overall anti-leishmanial activity, as measured by the IC_{50}(124.0±0.67µg/ml) was lower than that of Amphotericin B (2.4± 0.67µg/ml) used as positive control (Figure 2). This is the first report of the leishmanicidal activity of *Omphalocarpum ahia*. Drugs with potential leishmanicidal activity have been found to inhibit the activity of trypanothione synthetase (TryS) which synthesizes trypanothionebis(glutathionyl) spermidine; ($T[SH]_2$), a metabolite that keeps the cellular redox homeostasis of the parasite [19]. A similar mechanism is thus anticipated for *O. ahia*.

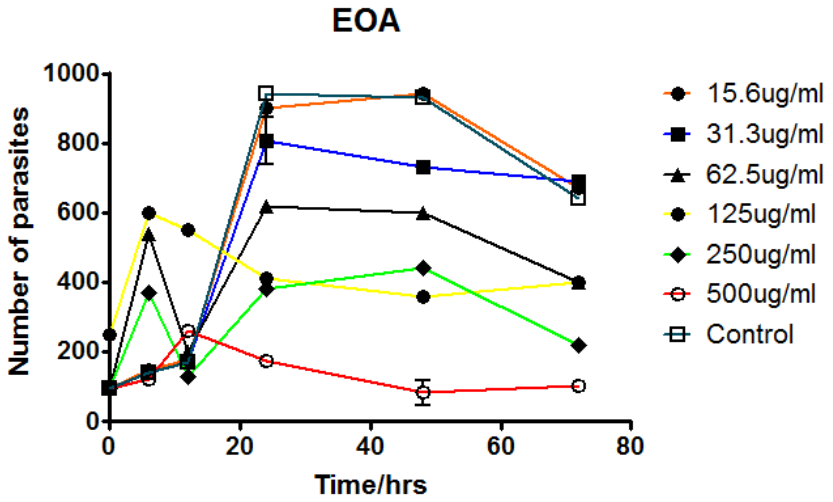

Figure 1: The time-course effect of *Omphalocarpum ahia* alcoholic extract on *L. donovani* parasites

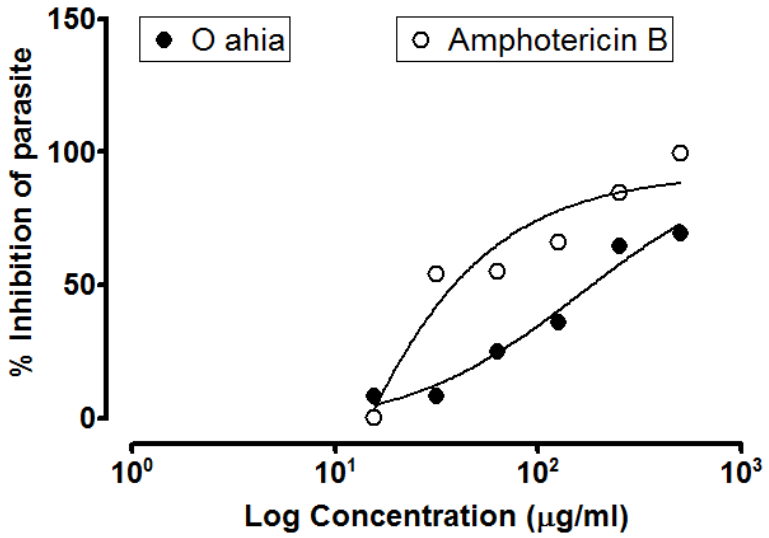

Figure 2: Concentration response curve for *O. ahia* and Amphotericin B

Leishmania donovani is the main cause of the deadly visceral leishmaniasis, the most severe type of the disease that can cause death in almost 100% of the cases accounting for nearly 50000 deaths per year; a death toll surpassed only by malaria [1]. The high cost and nephrotoxicity of Amphotericin B has limited it use as the main agent for the treatment of this type of leishmaniasis. Thus from the observed activity, barring any toxicity issues, *O ahia* may be harnessed and integrated into the treatment ofleishmaniasis caused by *L. donovani* as it may present a cheaper and safe alternative to the toxic synthetic analogues.

Anti-inflammatory and antioxidant activities

In this research, the anti-inflammatory potential of the methanol extracts of (EOA), using the chick carrageenan induced inflammation model was evaluated. The anti-inflammatory activity of the extract was compared to diclofenac and dexamethasone, used as standard drugs. Induction of oedema begun one hour after sub-planter injection of carrageenan (2%) with the inflammation reaching its peak within 2-3 hours (Figure 3). Oral administration of the extracts (30-300 mg/kg body weight) resulted in the reduction of the oedema induced by carrageenan in the chick paw from the second hour. All the extracts and standard drugs showed a significant ($p<0.05$) dose-dependent reduction in oedema (Figure 3). The anti-eodematogenic activity was quantified using the ED_{50}. This is the dose required to reduce the inflammation by 50%. *O ahia* showed moderate activity (ED_{50}=75.9±0.667 mg/kg BDW) compared to diclofenac (ED_{50}=3.74±0.333 BDW)(Table 1).The extract also showed a concentration dependent DPPH radical scavenging activity (Figure 4). The decrease in the absorbance of DPPH was due to phytoconstituents in the plant extracts acting as antioxidants by hydrogen donation. The radical scavenging activity of *O ahia* was three times less potent than Vitamin E used as positive control.

Immunological response to Leishmania parasite infection includes inflammation with cytokines and leukotriene B4 (LTB4) playing active roles in the process. Many of the molecules that promote inflammation also activate phagocytes leading to the production of the reactive oxygen species nitric oxide (NO), which acts directly to kill the parasite [20]. However, an exacerbated production of these molecules may also lead to tissue damage. These molecules seem to have a beneficial role at early infection but worsen the disease outcome in established infections [21]. Studies on Leishmaniasis showed that higher frequency of pro-inflammatory cytokine production leads to larger lesions. Anti-inflammatory agents provide a balance between pro-inflammatory and anti-inflammatory cytokines and that determines the outcome of the infection [22]. Thus the antioxidant and anti-inflammatory activity of *O ahia* may be beneficial in the treatment of leishmaniasis *in vivo*. These activities may be due to the presence of tannins, glycosides, saponins, terpenoids and phytosterols found in the phytochemical analysis of the plant

Table 1 Effect of *O ahia* and standard drug on carrageenan-induced oedema

Extracs/Drug	ED50±SEM (mg/kg body weight)
Diclofenac	3.74±0.333
Dexamethasone	1.98±0.133
Omphalocarpum ahia	75.9±0.667

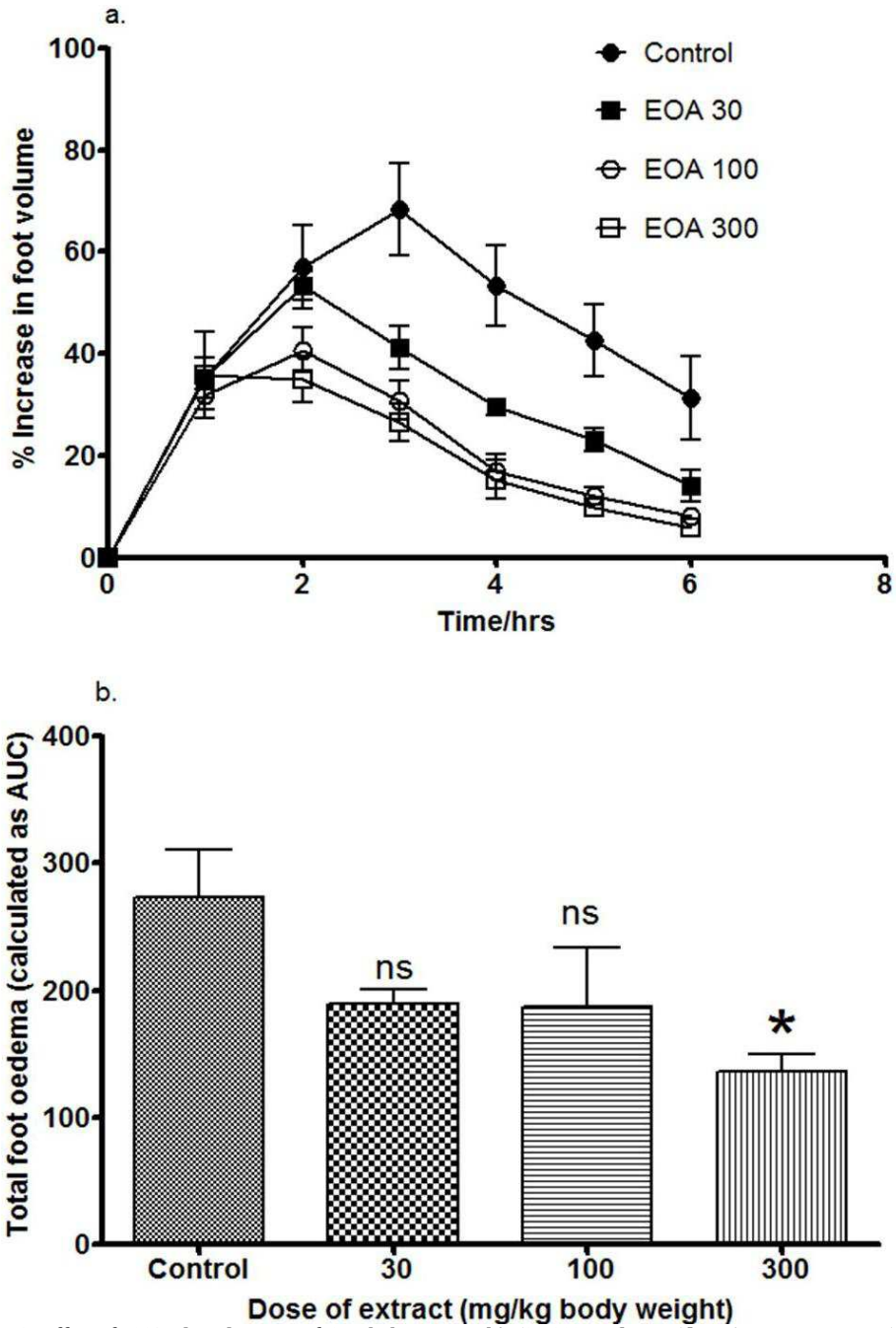

Figure3: Effect of 70% ethanol extract of *Omphalocarpum ahia* (30-300 mg/kg; p.o)] on time course curve (a) and the total oedema response, calculated as AUC's, for 6 h, in carrageenan induced paw oedema in chicks (b). Values are means ± S.E.M (n=5), *** p < 0.001, ** p < 0.01. *P < 0.05 compared to vehicle-treated group (One-way ANOVA followed by Newman-Keul's post hoc test)

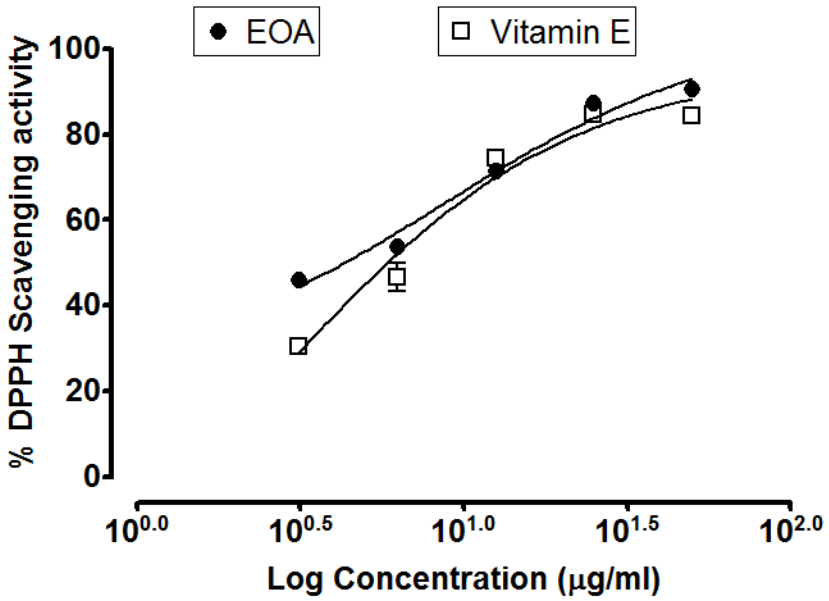

Figure 4: Free radical scavenging activity of *O. ahia* and Vitamin E

Table 2 Secondary metabolites of *O ahia*

Plant constituents	O. ahia
Tannins	Positive
Flavonoid	Negative
Terpenoids	Positive
Alkaloids	Negative
Glycosides	Positive
Saponins	Positive
Phytosterols	Positive

CONCLUSION

The present study has shown that ethanol extract of *O. ahia* exhibits considerable anti-leishmanial, anti-inflammatory and antioxidant activities and thus justifies it use in folklore medicine. If the toxicity profile is established, *O ahia* may be useful in the treatment of leishmaniasis. Isolation of its bioactive compounds is currently on going in our laboratory.

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