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Antileishmaniasis, phytotoxicity and cytotoxicity of Nigerian Euphorbiaceous Plants 2: *Phyllanthus amarus* and *Phyllanthus muellerianus* Extracts.

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ABSTRACT: Methanolic extracts of the leaves of *Phyllanthus amarus* and stem of *Phyllanthus muellerianus* were studied for antileishmaniasis activity, phytotoxicity and cytotoxicity. The antileishmanial activity was evaluated *in vitro* using promastigote culture of Pakistani leishmanial strain (*L. major*) in 96 well micro titer plate bioassay, while phytotoxicity was assessed using the Lemna bioassay and cytotoxicity using brine shrimp lethality assay. Methanolic extract of stem of *P. muellerianus* was found to exhibit cytotoxicity with a positive lethality of LD_{50} 4.867 µg/ml, low phytotoxicity at 100 µg /ml and significant phytotoxicity at 1000 µg/ml. It showed no anti-leishmanial activity. On the other hand, the methanolic extract of leaf of *P. amarus* was found to be leishmanicidal with an IC_{50} of 78.27 µg/ml, low phytotoxicity at 1000 µg /ml and no cytotoxic activity. Bioactive compounds are often toxic to *Artemia salina* (shrimp eggs) and it has been observed that natural antitumor compounds can inhibit the growth of *Lemna minor*. The higher cytotoxicity exhibited by *P. muellerianus* when compared with the standard drug, etoposide used (LD_{50} 7.4605 µg/ml) as well as the good anti-leishmanial activity displayed by *P. amarus* justifies their ethno-medicinal uses and is suggestive of the presence of physiologically active natural products in both plants.

Keywords *Phyllanthus amarus, Phyllanthus muellerianus*, antileishmanial, phytotoxicty, cytotoxicity, extracts.

Introduction

Phyllanthus, a genus of Euphorbiaceae has wide application and importance in African traditional ethno-medicine. It has long been used as medicinal agents in cultures around the world. The antimicrobial evaluation of *Phyllanthus amarus* (Schum & Thonn) and *Phyllanthus mullerianus* (O. Ktze) Exell extracts have been described in part one (Onocha *et al*., 2003). Traditionally, *Phyllanthus* species have been used to treat jaundice, gonorrhoea, frequent menstruation, dysentery, diabetes, skin ulcers, sores, swelling and itching (Burkill 2000; Headstrom 1978; Oliver-Bever 1986; Iwu 2000). Friedelane type triterpenoids (Adesida *et al.*, 1972; Hui *et al.*, 1976), alkaloids (Hoii *et al*., 1972; Blanpui *et al*., 1967), tannins (Foo 1993a; 1993b) and flavones (Chawhan *et al*., 1977) are well represented in this genus. Research efforts with *Phyllanthus* species have focused on its potential for fighting viruses, specifically the hepatitis B virus (Thyagarajan *et al*., 1988; Venkateswaran *et al*., 1987; Lee 1996; Yeh *et al*., 1993; Jarayam & Thyagarajan 1996; Jayaram *et al*., 1997). This may be the basis for traditional use of *Phyllanthus* species against disease symptoms such as jaundice and skin ulcers/sores which retrospectively could in at least some cases have been caused by hepatitis B virus and leishmaniasis respectively. *P. amarus* has also been reported to have

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the ability to inhibit two of the pro-inflammatory enzymes (Keimer 2000). *Leishmania* species are intracellular parasitic haemoflagellates that infect macrophages of the skin and viscera to produce disease in their vertebrate hosts. Three major clinical manifestations of leishmaniasis are recognised: visceral, cutaneous and muco-cutaneous leishmaniasis (Bodgan 1996). These diseases have received increased attention in developed countries because of the growing number of cases seen in AIDS patients (Bodgan 1996; Berman 1997). Prevalent drugs not only have several adverse effects but drug resistance and treatment failures are becoming increasingly common especially in immuno-compromised patients who often fail to respond, or are prone to relapse (Davidson 1999). Amphotericin B and its new lipid formulations are used as second line of treatment. However these are severely limited due to prolonged length of therapy and adverse reactions (Davidson 1999; Yardley & Croft 1997; Saleheen 2004). Thus there is still need for development of new drugs. The antileishmanial, phyto and cytotoxicity activities of the *Phyllanthus* species in this study have hitherto not been investigated. In continuation of our studies of the biological activity and constituents of ethnomedically useful plants from the Nigerian flora (Onocha *et al*, 2003; Onocha *et al*., 2005a; Onocha *et al*., 2005b) as source for development of new drugs, we now present the results of the antileishmanicidal, phytotoxic and cytotoxic activities of *P. amarus* and *P. muellerianus*.

Materials and Methods

Plant Material

The leaves of *P. amarus* and stem of *P. muellerianus* were collected from the University of Ibadan campus and authenticated by Mr. Felix Usang of the Forest Research Institute of Nigeria (FRIN), Ibadan where voucher specimens were deposited under file numbers FHI106462 and FHI106463, respectively.

Preparation and Extraction of Plant Material

The air dried leaves of *P. amarus* (1.5 kg) and stem of *P. muellerianus* (1.7 kg) were extracted in 7.5 L of methanol for 48 hours, respectively. On concentration *P. amarus* and *P. muellerianus* afforded 300 and 200 g methanolic extracts, respectively. The extracts were stored in the refrigerator prior to use.

Parasite culture

The promastigote culture of Pakistani leishmanial strain (*L. major*) obtained from Bioassay laboratory of H.E.J. Research Institute of Chemistry, International Center for Chemical Sciences, University of Karachi, Pakistan were maintained in blood agar based modified NNN diphasic medium supplemented with RMPI-1640 (Sigma R-7388), with 20mM HEPES and L-glutamine without $NaHCO_3$ at 25^{o}C (Ash & Orithel 1987).

Leishmanicidal activity

Leishmanial promastigotes were grown in bulk early in modified NNN diphasic medium using normal saline. Parasites at log phase were centrifuged at 2000 rpm for 10 mins, washed three times with saline at same speed and time. Parasites were diluted with fresh culture medium to a final density of 10^6 cell/ml. Subsequently 100 µl of culture was added in all wells except first column which received 180 µl. The last two rows were left for negative and positive controls. Negative control received medium with solvents while the positive control contained varying concentrations of the standard antileishmanial compound Amphotericin B.

Serial dilutions of *P. amarus* and *P. muellerianus* methanolic extracts were performed in 96 well micro titer plates in triplicates. 20 µl of solubilized extracts were added into the first wells and mixed well. 100 µl of sample was removed and added into the next well, mixed well and the 100 µl removal repeated till the 8th well was reached. 100 µl removed from last well was discarded. By doing this, the first well received a final concentration of 100 µg/ml while the last had 0.78 µg/ml of crude extracts to be tested. The plates were incubated in the dark at 22^{o}C for 72 hrs on an orbital shaker. After 5 days, drug activity (IC_{50}) was assessed microscopically using improved Neubauer counting chamber programme (Ash & Orithel 1987).

Phytotoxicity

The Lemna bioassay was carried out using the modified protocol of Mclaughlin (Mclaughlin 1991; Hopp *et al*., 1996). The *Lemna minor* (Duckweed) were cultivated under optimum conditions for 1 or 2 days, briefly washed in water and transferred into the E-medium nutrient (a mixture of various constituents adjusted to pH 5.5-7.0 to provide nutrients for growth of plant) prior to use. The flasks used for the bioassay were initially inoculated with 10, 100 and 1000 µl in each of three replicates of the stock solution of the extracts (30 mg of crude dissolved in 1.5 ml MeOH/EtOH). The solvents were left to evaporate overnight, thus yielding 10, 100 and 1000 µg/ml medium flasks to which 20 ml of E-medium and 10 plants of *Lemna minor* each containing a rosette of 2-3 fronds was introduced. Other flasks containing solvent and reference/standard drug paraquate served as negative and positive controls, respectively.

The flasks were placed in growth cabinets maintained at 28±1^{o}C for 7 days and examined daily during incubation. The number of fronds per flask was counted on day 7 to determine growth inhibition or proliferation of fronds in the flasks. The percentage growth regulation was analysed with reference to the negative control (Atta-ur-Rahman 1991).

Cytotoxicity

The eggs of brine shrimp *Artemia salina* were purchased from fish food pet shop. The eggs hatched within 48 hours of being placed in artificial sea water. The Brine shrimp lethality assay on the extracts were carried out using initial concentrations of 10, 100 and 1000 µg/ml in vials containing 5 ml of brine and ten shrimps (*Artemia salina*) in each of three replicates using the modified method of Mclaughlin (Mclaughlin 1991; Hopp *et al*., 1996). Survivors were counted after 24 hours. The data were processed using a Finney computer programme and LD_{50} values were obtained. Solvent and the reference cytotoxic drug (Etoposide) served as negative and positive controls respectively.

Results

Leishmanicidal activity

Concentrations of *P. amarus* and *P. muellerianus* extracts ranged from 0.78 to 100 µg/ml in triplicates were tested for their antileishmanial activity. As shown in Table 1, *P. amarus* was found to be leishmanicidal at an IC_{50} value of 78.27 µg/ml while *P. muellerianus* was not. $IC_{50} \leq 100$ µgml^{-1} for extracts was considered significant (Ash & Orithel 1987).

Phytotoxicity

Ten (10), 100 and 1000 µg/ml in each of three replicates of the stock solution of the extracts (30 mg of crude dissolved in 1.5 ml MeOH/EtOH) were tested for their inhibitory or proliferative activity on *Lemna minor* fronds. *P. muellerianus* exhibited significant dose dependent phytotoxicity while *P. amarus* on the other hand indicated a low activity at 1000 µg/ml as indicated in Table 1.

Cytotoxicity

In vitro cytotoxicity assay of *P. muellerianus* using brine shrimp indicated a positive lethality with an LD_{50} value of 4.867 µg/ml. This was higher than that of standard drug etoposide used (LD_{50} 7.4605 µg/ml) Table 1. *P. amarus* was not toxic.

Discussion

Phyllanthus species are widely used in various classical and herbal formulations worldwide. Apart from their diverse uses, they have recently been shown to possess anthelmintic, antidiabetic (Headstrom 1978), antiviral - specifically the hepatitis B virus (Thyagarajan *et al*., 1988; Venkateswaran *et al*., 1987; Lee 1996; Yeh *et al*., 1993; Jarayam & Thyagarajan 1996; Jayaram *et al*., 1997), anti-inflammatory (Keimer *et al.,* 2000) and antimicrobial (Onoch *et al*., 2003) properties. There has been a lot of focus on the antiviral properties of *Phyllanthus* species recently. To the best of our knowledge, there is no previous ethno-

medical report on the leishmanicidal activity of *Phyllanthus* species. Our decision was based on the observation of their use in the treatment of chronic skin ulcers, sores, swellings and itching. This is thus, the first report on the antileishmanial, phyto and cytotoxicity assays of *P. amarus* and *P. mullerianus*.

The results of present study indicated that the methanolic extract of the leaf of *P. amarus* is leishmanicidal. The present use of Phyllanthus species in the treatment of skin ulcers, sores, swelling and itching (Onoch *et al*., 2003; Burkill 2000; Headstrom 1978; Oliver-Bever 1986) might be explained in the light of these results and the metabolites present in the extract (Onocha *et al*., 2003). This finding is also in accordance with the earlier report of anti-inflammatory activity (Keimer *et al*., 2000). The methanol extract of the stem of *P. muellerianus* on the other hand, was not leishmanicidal but was found to be cytotoxic bioactive with a positive lethality (LD_{50} 4.867 µg/ml), which is higher than the standard drug etoposide used (LD_{50} 7.4605 µg/ml) as well as significantly inhibiting the growth of *Lemna minor*. This is suggestive of the presence of physiologically active compounds in these plants. Their use can particularly become more important when standardized therapies are achieved for induce resistance to various infections.

Table 1. Antileishmanial activity, phytotoxicity and cytotoxicity of methanolic extracts of *Phyllanthus amarus* and *Phyllanthus muellerianus*

Methanolic Plant extracts/ Standards	**% Inhibition IC_{50} (µg/ml)[a,b] (Leishmaniasis)**	**Conc. (µg/ml)[c]**	**% Growth Inhibition[a,c,d] (*Lemna minor*)**	**% Inhibition[a,c] (Brine Shrimp lethality)**	**LD_{50} (µg/ml)[a] (Brine Shrimp lethality)**
P. amarus	78.27 ± 0.6	1000	48.33 ± 0.7	-	-
(leaves)		100	0	-	-
		10	-8.35	-	
P. mullerianus	>100	1000	100	100	
(stem)		100	55.2 ± 0.2	96.67 ± 0.7	
		10	-3.4	63.33 ± 0.8	4.867 ± 0.7
Amphotericin B	0.12± 0.01				
Paraquate		0.0176	100		
Etoposide				100	7.4625

[a]Values are mean ± S.E.M (n = 3), $p < 0.05$ (Student's t-test); [b]Assay in 96 well micro titer plates (serial dilutions from 100 to 0. 78 µg/ml); [c]Assays in concentrations of 10, 100 and 1000 μgml^{-1}; [d]Negative inhibition means growth promoter / proliferation

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