Research Journal of Medical Sciences 2 (4): 178-181, 2008
ISSN: 1815-9346

In vitro Antileishmaniasis, Phyto and Cytotoxicity of *Pycnanthus angolensis* Methanolic Extracts

[1]P.A. Onocha, [2]E.O. Ajaiyeoba and [3]M.S. Ali
[1]Department of Chemistry, [2]Department of Pharmacognosy,
University of Ibadan, Ibadan, Nigeria
[3]H.E.J. Research Institute of Chemistry, International Center for Chemical Sciences,
University of Karachi, Karachi, 75270, Pakistan

Abstract: Antileishmaniasis, phytotoxicity and cytotoxicity of the methanolic extracts of the root, stem and leaves of *Pycnanthus angolensis* was evaluated *in vitro*, as part of the screening of ethno - medically useful plants from the Nigerian flora for biological activity and constituents. Brine shrimp lethality has been extensively used as a tool to screen active natural products. 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 antileishmanial activity was assessed using promastigote culture of Pakistani leishmanial strain (*L. major*) in 96 well micro titer plate bioassay, phytotoxicity using the *Lemna* bioassay and cytotoxicity using brine shrimp lethality assay. The methanolic extract of the root and stem exhibited dose dependent phytotoxicity while the leaf methanolic extract only displayed significantly phytotoxicity at the highest dose investigated. The stem methanolic extract was found to be leishmanicidal with an IC_{50} of 70.59 μg $mL^{-1}$and exhibited no cytotoxicity. The root methanolic extract exhibited cytotoxicity with a positive lethality of LD_{50} 727.70 μg mL^{-1} and was not leishmanicidal. The leaf methanolic extract was neither leishmanicidal nor cytotoxic. These results could be considered a valuable support of the ethnomedical uses of the plant.

Key words: *Pycnanthus angolensis*, Myristicaceae, methanolic extracts, antileishmanial, phytotoxicty, cytotoxicity

INTRODUCTION

Pycnanthus angolensis (Wewl.) Warb. (Myristicaceae), commonly known as wild African nutmeg is a lowland tree native to West and East Africa. It is reputed for its analgesic, stomachic, aperative, carminative, anthelmintic, anti-inflammatory, haemostatic, antimalarial and antimicrobial actions in African ethno medicine (Burkill, 2000; Ancolio *et al*., 2002). Other reported ethno - medical indications include its use in the treatment of female sterility, gonorrheal infertility, rheumatism, hemorrhoids, sore throat, rhino pharyngeal and broncho pneumonia, chronic skin diseases, leprosy, to promote healing of wounds and as a poison antidote (Burkill, 2000; Keay *et al*., 1964). It is also used to treat chronic fungal infections, a clinical problem commonly seen in patients with uncontrolled hyperglycemia (Gill, 1991). Its potential utility in the treatment of type 2 diabetes has been demonstrated (Luo *et al*., 1999).

Allantoin (Prista *et al*., 1960), Kombic acid (Lok *et al*., 1983), flavonoids (Omobuwajo *et al*., 1992), dihydroguaiaretic acid (Njoku *et al*., 1997) and novel anti hyperglycemic terpenoid- type quinones (Luo *et al*., 1999; Fort *et al*., 2000) have been isolated from this plant.

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 (Bogdan, 1996). The disease presents as fever, weight loss and hepato-splenomagly with biochemical abnormalities of hyper-γ-globulinemia and pancytopenia (Pearson and Sousa, 1996). It has received increasing attention in developed countries because of the growing number of cases seen in AIDS patients (Bogdan, 1996; Saleheen, 2004) and the occurrence of viescerotropic *L. tropica* disease among Persian Gulf War participants (Saleheen, 2004). Prevalent antimonial

Corresponding Author: P.A. Onocha, Department of Chemistry, University of Ibadan, Ibadan, Nigeria

drugs have remained standard treatment for visceral leishmaniasis since the 1940s. These 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 relapse (Saleheen, 2004). Amphoteracin 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. Thus there is still need for development of new drugs.

To the best of our knowledge, there is no previous ethno-medical report on the leishmanicidal activity of *Pycnanthus angolensis*. Our decision to explore this possibility was based on the observation of its use in the treatment of chronic skin diseases, sores/wounds, as a haemostatic, anti-inflammatory and as a healing dressing. This may be the basis for the traditional use of the plant which retrospectively could at least in some cases have been caused by leishmaniasis.

In continuation of our studies of the biological activity and constituents of ethno -medically useful plants from the Nigerian flora (Onocha *et al*., 2003, 2005; Ajaiyeoba *et al*., 2005) as source for development of new drugs, we now present the results of the *in vitro* antileishmaniasis, phytotoxicity and cytotoxicity of *P. angolensis*.

MATERIALS AND METHODS

Plant material: Different parts of *P. angolensis* (root, stem and leaves) were collected in May 2004 from Barth road, University of Ibadan. A voucher specimen (FHI 1064) was identified by Mr. Felix Usang ofForest Research Institute of Nigeria (FRIN) where it is deposited. The air dried roots (1 kg), stem (1.2 kg) and leaves (900 g) of *P. angolensis* were extracted with methanol for 48 h, respectively and the resulting plant extracts were stored in the refrigerator prior to use.

Parasite culture: The promastigote culture of Pakistani leishmanial strain (*L. major*) were maintained in blood agar based modified NNN diphasic medium supplemented with RMPI-1640 (Sigma R-7388), with 20 mM HEPES and L-glutamine without $NaHCO_3$ at 25°C (Ash and 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 min, 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^{-1}. Subsequently 100 μL of culture was added in all wells except first column which received 180. The last 2 rolls 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. angolensis* methanolic extracts were performed in 96 well micro titer plates in triplicates. Total 20 μL of solubilized extracts were added into the first wells and mixed well by micropipette. A total of 100 μL of sample was removed and added into the next well, mixed well and repeated till the 8th well was reached. Remaining 100 μL was discarded. By doing this, the first well received a final concentration of 100 μg mL^{-1} while the last had 0.78 μg mL^{-1} of crude extracts to be tested. The plates were incubated in the dark at 22°C for 72 h on an orbital shaker. After 5 days exposure, drug activity (IC_{50}) was assessed microscopically using improved Neubauer-counting chamber programme (Ash and Orithel, 1987).

Phytotoxicity: The *Lemna* bioassay was carried out using the modified protocol of Mclaughlin (Maclaughlin, 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 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^{-1} medium flasks to which 20 mL of E-medium and 10 plants of *L. 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°C for 7 days and examined daily during incubation. The number of fronds per flask was counted on day 7 to determine the growth inhibition or proliferation of fronds in the flasks. The percentage growth regulation was therefore analysed with reference to the negative control (Atta-ur-Rahman, 1991).

Cytotoxicity: The eggs of brine shrimp *Artemia salina* were readily available as fish food in pet shops. The eggs hatched within 48 h 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^{-1} in vials containing 5ml of brine and ten shrimps (*Artemia salina*) in each of three replicates using the modified method of Mc laughlin (MacLaughlin,1991; Hopp *et al*., 1996). Survivors were counted after 24 h. The data were processed using a Finney computer programme (MacLaughlin, 1991) and LD_{50} values were obtained. Solvent and the reference cytotoxic drug (etoposide) served as negative and positive controls, respectively.

RESULTS AND DISCUSSION

Leishmanicidal activity: Concentrations of *P. angolensis* extracts ranging from 0.78-100 μg mL^{-1} in triplicates were tested for their antileishmanial activity. The stem methanolic extract as shown in Table 1, was found to be leishmanicidal at an IC_{50} value of 70.59 μg mL^{-1}. IC_{50} ≤100 μg mL^{-1} for extracts was considered significant (Saleheen, 2004).

Phytotoxicity: The 10, 100 and 1000 μg mL^{-1} 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 proliferatory activity on *Lemna* fronds. The stem and root methanolic extracts of *P. angolensis* exhibited significant dose dependent phytotoxicity, with low activity at 10 μg mL^{-1}, moderate activity at 100 μg $mL^{-1}$and significant activity at 1000 μg mL^{-1}. The leaf methanolic extract on other hand displayed marked activity at 1000 μg mL^{-1} as indicated in Table 1.

Cytotoxicity: *In vitro* cytotoxicity assay of *P. angolensis* using brine shrimp indicated a positive lethality of the root methanolic extract with LD_{50} value of 727.70 μg mL^{-1}. Results are as shown in Table 1.

P. angolensis species are widely used in various classical and herbal formulations worldwide. Its potential utility in the treatment of type 2 diabetes has been demonstrated (Luo *et al*., 1999).

To the best of our knowledge, there is no previous ethno - medical report on the leishmanicidal activity of *P. angolensis* extracts. Our decision was based on the observation of their use in the treatment of chronic skin ulcers, sores, swellings/itching as well as their anti-inflammatory property.

The present findings demonstrate that the stem methanolic extract of *P. angolensis* possesses a good leishmanicidal activity. The use of *P. angolensis* extracts in the treatment of skin ulcers, sores, swelling and itching (Burkill, 1997; Keay *et al*., 1964) might be explained in the light of these results. The root methanol extract of *P. angolensis* although not leishmanicidal was found to be bioactive with a positive lethality LD_{50} of 727.70 μg mL^{-1}. In addition, the significant phytotoxicity exhibited by all the extracts in inhibiting the growth of *Lemna minor* is indicative of the presence of biologically active natural products in all the extracts. Their use can particularly become more important when standardized therapies induce resistance to various infections. Further studies will be necessary to investigate the effect of these active plant extracts when combined with antileishmanial agents commonly used.

Table 1: Antileishmaniasis, phytotoxicity and cytotoxicity assays of methanolic extracts of *Pycnanthus angolensis*

Methanolic Plant extracts/ Standards	Inhibition (%) IC_{50} (μg mL^{-1})[a,b] (Leishmaniasis)	Conc. (μg mL^{-1})	Growth (%) inhibition[c] (*Lemna minor*)	Inhibition[a] (%) (Brine shrimp lethality)	LD_{50} (μg mL^{-1}) (Brine shrimp Lethality)
Stem	70.59±0. 4	1000	100	-	
		100	41.60±0.9	-	-
		10	33.33±0.3	-	
Root	>100	1000	100	60±0.6	
		100	43.33±0.7	6.67±0.8	
		10	-10.0	0	727.70±0.8
Leaves	>100	1000	75.0±0.1	-	
		100	0	-	-
		10	-8.35		
Amphotericin B	0.12±0.01				
Paraquate		0.0176±0.01	100		
Etoposide				100	7.4625±0.01

[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^{-1}), [c]Negative inhibition means growth promoter / proliferation

ACKNOWLEDGEMENT

The authors are grateful to Mr. Felix Usang of the Forest Research Institute (FRIN), Ibadan, Nigeria, for authenticating the plant material. PA Onocha acknowledges with thanks the Senate Research Grant of the University of Ibadan, Nigeria (SRG/FSC/2000/2A) and H.E.J. Research Institute of Chemistry, International Centre for Chemical Sciences, University of Karachi, Karachi, Pakistan for collaborative research.

REFERENCES

Ajaiyeoba, E.O., O.M. Bolaji and B.O. Akinboye, 2005. *In vitro* antiplasmodial and cytotoxicity activities of plants used as antimalarial agents in the southwest Nigerian ethnomedicine. J. Nat. Remedies, 5: 1.

Ancolio, C., M.V. Azas and E. Ollivier, 2002. Antimalarial activity of extracts and alkaloids isolated from six plants used in traditional medicine in Mali and Sao. Tome. Phytother. Res., 16: 646-649.

Ash, L.R. and T.C. Orithel, 1987. Parasite: A guide to laboratory procedure ASCP press; Chicago, pp: 128-130.

Atta-ur-Rahman, 1991. Studies in Natural Products Chemistry. Elsevier Science Publishers; Netherlands, Vol. 9.

Burkill, H.M., 2000. The useful plants of west tropical Africa Royal botanic Garden; Kew, 4: 235-238.

Bogdan, C., A. Gessner, W. Solbach and M. Rolinghoff, 1996. Invasion, control and persistence of Leishmania parasites. Curr. Opin. Immunol., 8: 517-525.

Fort, D.M., R.P. Ubillas and C.D. Mendez *et al*., 2000. Novel antihyperglycemic terpenoid-quinones from *Pycnanthus* angolensis. J. Org. Chem., 65: 6534-6539.

Gill, G.V., 1991. Non-Insulin dependent *Diabetes mellitus*. In: Textbook of Diabetes, Pickup, J. and G. William, (Eds). Blackwell Scientific Publications; London, pp: 24-34.

Hopp, D.C., L. Zeng, Z.M. Gu and J.L. McLaughlin, 1996. Squamatacin: An annonaceous acetogenin with cytotoxic selectivity for the human prostrate tumor cell lines (PC-3). J. Nat. Prod., 59: 97-99.

Keay, R.W., C.F.A. Onochie and D.P. Stanfield, 1964. Nigerian trees, Federal Department of forest Publication; Ibadan, Nigeria.

Lok, C.M., A. Groenewegen, J.B.A. Stroink, J.P. Ward, 1983. Kombic acid, a hydroquinone polyisoprenoic carboxylic acid from *Pycnanthus kombo* seed fat. Phytochem., 22: 1973-1976.

Luo, J., J. Cheung, E.M. Yevich and J.P. Clark *et al*., 1999. Novel terpenoid-type quinines isolated from *Pycnanthus angolensis* of potential utility in the treatment of type 2 diabetes. Pharm. Exp. Therap., 288: 529-534.

McLaughlin, J.L., 1991. In: Methods in Plant Biochemistry. In: Hostettman, K. (Ed.). Academic Press; London, 5: 1-35.

Njoku, C.J., D.C. Hopp and F. Alli *et al*., 1997. Dihydroguaiaretic Acid: a bioactive component of the stem bark of *Pycnanthus angolensis*. Planta Medica, 63: 580-581.

Omobuwajo, O.R., S.A. Adesanya and G.O. Babalola, 1992. Isoflavonoids from *Pycnanthus angolensis* and *Baphia nitida*. Phytochem., 31: 1013-1014.

Onocha, P.A., A.O. Opegbemi and A.O. Kadri *et al*., 2003. Antimicrobial evaluation of Nigerian Euphorbiaceae Plants 1: *Phyllanthus amarus* and *Phyllanthus mullerianus* leaf Extracts. J. Nat. Prod. Med., 7: 9.

Onocha, P.A., E.O. Audu, O.O. Dosumu and O. Ekundayo, 2005. Phytochemical and Antimicrobial Properties of Extracts of *Combretum racemosun* Acta Horticul., 675: 1.

Onocha, P.A., D.A. Okorie and H.C. Krebs *et al*., 2005. *In vitro* Cytotoxic Pentacyclic Triterpenoids of *Newbouldia laevis*. Congress on Medicinal and Aromatic Plants for Human Welfare, Thailand.

Pearson, R.D. and A.Q. Sousa, 1996. *In vitro* activity of atavaquone against *Leishmania chagasi* promastigotes. Clin. Infec. Dis., 22: 1.

Prista, L.N., A.C. Alves and M.C. Fatima, 1960. Allantoin in some plants used in the treatment of Wounds. *Garcia Orta*, 8: 315-26.

Saleheen, D., S.A. Ali and S.M. Yasinzai, 2004. Antileishmanial activity of aqueous onion extract *in vitro*. Fitoterapia, 75: 9-13.