Nigerian Journal of Science Vol 44 (2010): 57-62

Cytotoxicity and Antimicrobial Activity of Methanol Extract and Fractions of *Entandrophragma angolense* (Welw.) C. Dc. (Meliaceae) Leaves

*P.A. IDOWU[1], B.A. ADENIYI[1] AND J.O. MOODY[2]
[1] Department of Pharmaceutical Microbiology, Faculty of Pharmacy, University of Ibadan
[2] Department of Pharmacognosy, Faculty of Pharmacy, University of Ibadan.
*Corresponding Author
E-mail: igbo·:ega@yahoo.com

Abstract

The antibacterial activity and brine-shrimp lethality (BSL) assay of methanol extracts of *Entandrophragma angolense,* used to treat gastro-intestinal tract (GIT) infections in South-Western Nigeria was investigated. The extract and chromatographic fractions were tested at 20 and 10 mg/ml respectively against *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhi* (clinical isolate), *Salmonella typhimurium* ATCC 14028, *Escherichia coli* (ATCC 25922) and *Bacillus subtilis* (ATCC 6633) using agar-well diffusion method. Minimum Inhibitory Concentrations (MIC) were determined by agar dilution method. Further, the extract was fractionated on silica gel (70-230 mesh) using column chromatography. The diameter of zones of inhibition were between 15.0 – 30.5mm which was comparable with gentamicin (10μg/ml) used as control. The MIC was 80.0μg/ml on *Staph. aureus* and 350μg/ml on *Salmonella typhi*. The more polar fractions contained the antimicrobial compounds as the less polar fractions showed no antimicrobial activity as tested. Phytochemical screening revealed the presence of tannins, flavonoids, reducing sugars and steroidal compounds. Brine shrimp lethality assay gave a value of 62.5μg/ml. Therefore *E. angolense* leaves contains antimicrobial agents and cytotoxic principles to justify its folkloric uses and phytotherapeutic potentials in treating infections.

Introduction

Increasing drug resistance of pathogenic microbes against conventional antibiotics has necessitated a search for new and effective antimicrobial agents from plants natural products (Cowan, 1999; Newmann *et al.*, 2003; R'ios and Recio, 2005). Screening ethnomedicinal plants for bioactive constituents is the classical way of discovering new drugs (Kinghorn *et al.*, 2011). *Entandrophragma angolense* (Welw.) C. DC. is indigenous to West-African coast and employed medicinally to treat infections of gastrointestinal nature (especially GIT ulcer), pains and malaria (Burkill, . 1997). The plant's leaf decoction is orally consumed for treatments and it is therefore important to establish its pharmacological and toxicological values. Phytochemical analysis, antiulcer, and antimalarial studies of some of the plant's parts have been reported (Akinsanya *et al.*, 1960; Njar *et al.*, 1995; Orisadipe *et al.*, 2001 and

Keywords: *Entandrophragma angolense*, phytochemistry, fractions, antibacterial, cytotoxicity

UNIVERSITY OF IBADAN LIBRARY

2005; Bickii *et al.*, 2007;). However little or no reports are available on the antimicrobial and cytotoxicity studies. As part of our investigation into the scientific basis of using plants for treating infectious diseases locally with a view to discovering new antimicrobial agents (Cox and Balick, 1994; Hostettman *et al.*, 2000), the methanolic extract of *E. angolense* leaves was examined for antimicrobial and cytotoxicity activity. Further, since many chemical constituents of a plant may be responsible for an observed antimicrobial activity of an extract (Idowu, *et al.*, 2006) the chromatographic fractions were also tested for antimicrobial activity.

Materials and Methods

Plant Materials

Entandrophragma angolense was collected from Olokemeji forest reserve in Nigeria, and was identified in Forestry Research Institute of Nigeria (FRIN) with a deposited voucher specimen number FHI – 10432. The air-dried leaves (500g) were pulverized and extracted with methanol using soxhlet apparatus, to give a yield of 15.06% (75.3g).

Test Organisms

Bacterial species (*Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhi* (clinical isolate), *Salmonella typhimurium* ATCC 14028, *Escherichia coli* ATCC 25922 and *Bacillus subtilis* ATCC 6633) were obtained from the Department of Pharmaceutical Microbiology, University of Ibadan. All bacteria were cultured on nutrient agar (No. 2) and nutrient broth (pH 7.4) (Oxoid) and were maintained on agar slope at 4°C before testing. Brine shrimp (*Artemia salina* Sander®) eggs were purchased from Great Salt Lake Company, USA.

Phytochemical Screening

Phytochemical screening was done using the methods of Harbone (1991) and Edeoga *et al.*, (2005). The leaves were tested for the presence of the following secondary metabolites: alkaloids, tannins, glycosides, flavonoids, saponins, steroids and reducing sugars.

Chromatographic Fractionation

Silica gel 70 – 230 mesh (Merck), precoated aluminum sheet silica gel $60F_{254}$ (Merck), and a sintered glass filter column (porosity 3, diameter 1 x 3) were used for the fractionation of the extract. A mixture of hexane, ethyl-acetate and methanol in varying proportion were used as eluent. The loaded extract (6.0g) gave 50 fractions (each 50ml) which were monitored by TLC and viewed under UV lamp (254nm) and iodine tank. Fractions with similar R_f were pooled together to give 8 fractions which were tested for antimicrobial activity using *Staph. aureus, Bacillus subtilis, E. coli* and *Ps. aeruginosa* as test organisms.

Determination of Antimicrobial Activity

The antibacterial activity of the extracts and fractions was determined using the agar-well diffusion technique of Perez *et al.* (1990) with slight modifications. Nutrient agar or *Salmonella-Shigella* (SS) agar plates were seeded with 100 µL of an overnight culture of each bacterial isolate (equivalent to 10^7-10^8 cfu/ml according to the 0.5 MacFarland standards). The seeded plates were allowed to set and a standard cork borer of 8.0 mm diameter was used to cut uniform wells on the surface of the agar. The wells were then filled with 100 µl of each extract/fraction at a concentration of 10 or 20 mg/ml in 40% methanol. Control wells containing antibiotic gentamicin (Sigma) at 10µg/ml and 40% methanol were used as positive and negative control respectively. A pre-incubation period of 60min at 4°C was allowed for diffusion of extracts and test solutions before incubation at 37°C for 24 h after which the diameter of zones of inhibition were measured. Each test was carried out in triplicate.

Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration was determined using the agar dilution method of Andrews (2001), with slight modifications. The extract at 20 mg/mL was serially diluted in test tubes to give a final concentration in the range of 10.0, 5.0, 2.5, 1.25, 0.625, 0.313, 0.157, 0.079 and 0.04 mg/mL respectively. One milliliter of each dilution of the extract was mixed with 9mL of Mueller Hinton agar, poured into 10cm diameter Petri dishes and allowed to set. After allowing the agar to dry for about 30min, each plate was inoculated with 1: 100 dilution of overnight broth cultures of each test organisms (containing 1.0 x 10^8cfu/mL according to 0.5 MacFarland standards) and incubated for 24 h at 37°C. Nutrient agar plates with extract but without an organism and one containing only the organism without extract served as positive and negative controls respectively. Each test was carried out in duplicate. The plates were then examined for the presence of growth after the incubation period. The least concentration that gave no visible colonies of the test organism was taken as the minimum inhibitory concentration (MIC) of the extract.

Determination of Cytotoxicity (Brine-Shrimp Lethality Assay)

The cytotoxicity of *E. angolense* methanolic extracts was done according to the methods of Meyer *et al.* (1982) and Rahman *et al.* (2008). Brine shrimp eggs (*Artemia salina*, Sanders[R] ,U.S.A.) were hatched in natural sea water collected from Badagry Beach in Lagos in a plastic chamber. The eggs were sprinkled into the larger compartment which was darkened, while the smaller compartment was illuminated. After 48 hours incubation at room temperature (25-29°C), nauplii (larvae) were collected by pipette from the lighted side whereas their shells were left in another side. The crude extracts were dissolved in dimethylsulphoxide (DMSO) at a maximum concentration of not exceeding 0.05% and then diluted with sea water (4.5ml of sea water + 0.5ml of dissolved extract) to final concentrations of 1000, 100 and 10 ppm respectively. Ten nauplii were used to test each dose of extracts and each dose was tested in triplicate. . Tubes containing 10 nauplii in sea water with quinidine sulphate (Sigma) at 200µg/ml and those without extract were set up as positive and negative controls respectively. After 24hr, the number of nauplii that survived was counted under a magnifying glass, and these were used to determine the numbers killed. The numbers of dead nauplii were recorded and Finney probit analysis program was used to determine the lethal concentration (LC_{50}) to half of the test organisms.

Results and Discussions

Phytochemical screening of the leaves revealed the presence of tannins, steroids, terpenes, flavonoids and reducing sugar while alkaloid, saponins and antraquinone glycoside were conspicuously absent (Table 1). These secondary metabolites representing the major classes of the bioactive components of *E. angolense* leaves especially tannins, phenolics, and flavonoids have been previously reported to have exhibited potent antimicrobial activities on bacteria and fungi (Scalbert, 1991 and Rauha *et al.*, 2000).

Table 1: Results of Phytochemical Screening of *Entandrophragma angolense* Leaf

Secondary metabolites	Tests performed	Colour reaction	Result
Alkaloids	Draggendorff,	No reddish-brown	-
	Meyer	No ream Precipitates	-
Tannins	Ferric- Cl test	Blue-black colour	+ + +
Anthraquinone glycoside	Molisch test	No pink Colouration	-
Flavonoids	Ammonia Tests	Yellow colouration	+ +
Steroids	Liebermann- Burchard	Green Layer	+ + +
Reducing Sugar	Fehling's Test	Brick-red	+ +
Saponins	Frothing-Test	No-frothing	-
	Emulsifying-Test	No Emulsion	-
Terpenoids	Salkowski Reaction	Reddish-brown	+ + +

Key:
- \+ = present in low concentration;
- ++ = present in moderate concentration
- +++ = present in high concentration;
- \- = absent or not detected

UNIVERSITY OF IBADAN LIBRARY

Antibacterial screening of the methanolic extract and fractions of *E. angolense* leaf have demonstrated a potent broad spectrum antimicrobial activity on common pathogenic bacteria like *Staph. aureus, E. coli, Pseudomonas aeruginosa* and specifically on typhoidal and non-typhoidal *Salmonella* strains (Table 2). The diameters of zone of inhibition were between 16.0 - 30.0 mm on the tested organisms at 20mg/ml. More importantly was the antimicrobial activity displayed by the methanolic extracts of *E. angolense* against MDR *Salmonella* strains. This confirms the presence of potent antimicrobial agents in the plant.

From the column chromatography fractions with the same TLC profiles pooled together into 8 fractions (labeled ID1-ID8 as shown in Table 2) were tested for antimicrobial activity on *Staph. aureus*, *Bacillus subtilis*, *E. coli* and *Pseudomonas aeruginosa*. From the results, ID1-ID5 did not show significant antimicrobial activity on any of the tested organisms at 10mg/mL, ID6 showed little antimicrobial activity on *Bacillus subtilis* and *Pseudomonas aeruginosa;* while ID7 and ID8 representing the most polar fractions showed good antimicrobial activity comparable to gentamicin at 10µg/ml. This showed that the major antimicrobial constituents of the plant are of high polarity which may be represented by the tannins and flavonoids revealed in the phytochemical screening (Table 1).

Table 2: Antimicrobial Activity of MeOH Extract and Fractions of *E. angolense* Leaves

Diameter (mm) of Zones of Inhibition of Extract/Fractions on Test Bacteria						
Extract/ Fractions	*Staph. aureus*	*Bacillus subtilis*	*Ps. aeruginosa*	*E. Coli*	*Salmonella typhi*	*Salmonella typhimurium*
MeOH (crude) 20mg/ml	25.5	30.5	30.0	30.0	25.5	25.0
MIC (µg/ml)	80.0	85.0	120.0	120.0	830.0	320.0
ID-1(1-10)	-	10.5	-	-	-	-
ID-2(11-16)	10.5	10.5	11.0	11.0	-	-
ID-3(17-18)	-	-	-	-	-	-
ID-4(20-23)	-	-	-	-	-	-
ID-5(24-27)	-	-	-	-	-	-
ID-6(28-32)	-	11.0	14.0	-	-	-
ID-7(37-43)	28.0	28.5	30.0	26.0	26.0	24.0
ID-8(44-50)	28.0	26.0	28.5	26.0	26.5	25.0
Gentamicin 10µg/ml	25.0	30.0	25.5	30.0	30.0	28.0

Key: - = no activity

UNIVERSITY OF IBADAN LIBRARY

Brine shrimp lethality (BSL) test has been used for screening plant extracts for cytotoxity activities (Rahman *et al.* (2008), Krishnaraju *et al.* (2005) and Pisutthanan *et al.* (2004); and results showed correlation with cytotoxicity and antitumor properties determined by cell lines as reported by McLaughlin *et al.* (1993) and Carballo *et al.* (2002). The MeOH extract of *E. angolense* leaf gave LC_{50} value of 62.5μg/ml (Table 3) which is in agreement with brine shrimp test values reported by Rahman *et al.* (2008) and Pisutthana *et al.* (2004) for some other Meliaceae plants of India similarly tested for cytotoxic activity (values LC_{50} of the methanolic fractions of the stem barks of *Azadirachta indica* was 181.53μg/ml while that of *Chukrasia tabularis* was 15.2μg/ml).

Extracts with LC_{50} less than 100 μg/ml are considered toxic and can contain cytotoxic compounds (Pisutthanan et al. (2004); Krishnaraju et al. (2005), while values ≥ 1000 μ g/ml are considered as non-toxic. Therefore at LC_{50} of 62.5μg/ml, methanolic extract *E. angolense* is considered toxic. However, the plant is widely consumed among local people for intestinal troubles and malaria (Burkhill, 1997; Bickii *et al.*, 2007) without recorded untoward side effects; probably due to the presence of other adjuvants in the pot-herbs. On the other hand, the toxicity of the plant extract showed that the plant may contains cytotoxic constituents that can be tested as anti-tumor agent in cancer chemotherapy.

Table 3: Results of Brine Shrimps Lethality Assay

Concentrations	1000 ppm		100 ppm		10 ppm	
Extract	0 min	24 h	0 min	24 h	0 min	24h
E1	10	0	10	8	10	10
E2	10	0	10	0	10	10
E3	10	0	10	0	10	10
No. killed	30		22		0	

According to Finney Computation, Lethal Concentration Lc_{50} = 62.463 = 62.5 μg/ml.

Conclusion

The study has shown that *Entandrophragma angolense* leaves contain cytotoxic and antimicrobial chemical constituents. The antimicrobial activity displayed justified the folkloric and traditional uses of the plant part in treating infections and gastro-intestinal ailments as reported by Burkill, 1997. The extract showed a high antimicrobial potency and broad spectrum of activity comparable with the control standard antibiotic, gentamicin. Polar fractions were more active than less-polar fractions. It is recommended that the antimicrobial constituents be isolated and characterized for possible therapeutic uses.

Acknowledgement

The authors acknowledge the contribution of Mr. Felix Uyang (late) of FRIN towards the collection and authentication of the plant; Prof. D. A. Okorie of Organic Chemistry Unit, Department of Chemistry, University of Ibadan for a lab space for phytochemistry; and staffs of Pharmaceutical Microbiology Department for technical assistance.

References

Akinsanya, A., Bevan, C.W.L., Hirst, J., Halsall, T.G. and Taylor, D.A.H.(1960): West African timbers. III. Petroleum extracts from the genus Entandrophragma. *J. Chem. Soc;* 3827–3829.

Andrews, J. M. (2001) Determination of Minimum Inhibitory Concentrations. *J. Antimicrob. Chemother.* 48, Suppl. SI. 5-10.

Bickii, J., Tchouya, G.R.F., Tchouankeu, J.C. and Tsamou E. *(2007):* The Antiplasmodial Agents of the Stem Bark of *Entandrophragma angolense* (Meliaceae). *Afr. J. Trad. CAM* 4(2): 135–139.

Burkill, H. M. (1997). *The Useful Plants of West Tropical Africa.* Vol. 4: Families M-R. Royal Botanic Gardens, Kew.

Carballo, J. L., Hernández-Inda, Z. L., Pérez, P. and García-Grávalos, M. D. (2002): A comparison between two brine shrimp assays to detect *in vitro* cytotoxicity in marine natural products. *BMC Biotechnology* 2: 17

Cowan, M. M. (1999): Plant products as antimicrobial agents. *Clin.Microbiol. Rev.* 12(4): 564-582.

Cox, P. A. and Balick, M. J. (1994): The

UNIVERSITY OF IBADAN LIBRARY

ethnobotanical approach to drug discovery. *Scientific American* 270 (6): 60-82.

Edeoga, H. O, Okwu, D. E. and Mbaebie, B. O. (2005): Phytochemical constituents of some Nigerian medical plants. *African Journal of Biotechnology* 4 (7): 685-688.

Harborne, J. B. (1991): *Phytochemical Methods* 2nd Edition. Oxford University Press London. pp 51-384.

Hostettmann, K., Marston, A., Ndjoko, K. and Wolfender, J. (2000): The potential of African plants as a source of drugs. *Current Organic Chemistry* 4: 973-1010.

Idowu, P. A., Moody, J. O. and Odelola, H. A. (2006): Antimicrobial activity of the crude extracts and isolated fractions of *Garcinia kola* Heckel stem bark. *Nigerian Journal of Science* 40: 81-85

Kinghorn, A. D., Chai, H., Sung, C. K. and Keller, W. J. (2011): The classical drug discovery approach to defining bioactive constituents of botanicals (Review). *Fitoterapia* 82: 71–79

Krishnaraju, A. V., Rao, T. V. N., Sundararaju, D., Vanisree, M., Tsay, H and Subbaraju, G. V. (2005): Assessment of Bioactivity of India Medicinal Plants using Brine Shrimp (*Artemia salina*) lethality assay. *International Journal of Applied Science and Engineering* 32: 125-134.

McLaughlin, J. L., Chang, C. J. and Smith, D. L. (1993): Simple bench-top bioassays (brine shrimp and potato discs) for the discovery of plant antitumor compounds. In: *Human medicinal agents from plants*. Kinghorn, A. D and Balandrin, M. F. (Eds.). *ACS symposium* 534, American Chemical Society, Washington, DC.: 112-137

Meyer, B. N., Ferrigni, N. R., Putnam, J. E., Jacobson, L. B., Nichols, D. E. and McLaughlin, J. L. (1982): Brine shrimp a convenient general bioassay for active plant constituents. *Planta Medica* 45: 31-34.

Newmann, D. J., Cragg, G. M., and Snader, K. M., (2003). Natural products as sources of new drugs over the period 1981–2002. *Journal of Natural Products* 66: 1022–1037.

Njar, V. C. O., Adesanwo, J. K., Raji, Y. (1995): Methyl angolensate: the antiulcer agent of the stem bark of *Entandrophragma angolense*. *Planta Medica* 61: 91–92.

Orisadipe, A. T, Amos, S., Adesomoju, A. A., Binda, L., Emeje, M., Okogun, J. I., Wambebe, C. and Gamaniel, K. (2001): Spasmolytic Activity of Methyl Angolensate: a Triterpenoid Isolated from *Entandrophragma angolense*. *Biol. Pharm. Bull.* 24 (4): 364-367.

Orisadipe, A. T., Adesomoju, A. A., Ambrosio, M., Guirrerio, A. and Okogun, J. I. (2005): Tirucallane triterpenes from the leaf extract of *Entandrophragma angolense*. *Phytochemistry* 66: 2324–2328

Perez, C., Pauli, M. and Bazerque, P. (1990): An antibiotic assay by the agar-well diffusion method. *Acta Biol. Med. Exp.* 15: 113–115.

Pisutthanan, S., Plianbangchang, P., Pisutthanan, N., Ruanruay, S. and Muanrit, O. (2004): Brine Shrimp Lethality Activity of Thai Medicinal Plants in the Family Meliaceae. *Naresuan University Journal* 12(2): 13-18.

R'ios, J. L. and Recio, M. C. (2005): Medicinal plants and antimicrobial activity. Perspective Paper. *J. Ethnopharmacol.* 100: 80-84.

Rahman, M. S., Begum, B., Chadhury, R., Rahman, M. K. and Rashidi, M. A. (2008): Preliminary Cytotoxicity screening of some medicinal plants of Bangladesh. *Dhakar Univ. J. Pharm. Sci.* 7 (1): 47-52.

Rauha, J., Remesa, S., Heinonenb, M., Hopiab, A., Kahkonenb, M., Kujalac, T., Pihlajac, K., Vuorelaa, H. and Vuorelaa, P. (2000): Antimicrobial effects of Finnish plant extracts containing flavonoids and other phenolic compounds. *Intn. J. Food Microbiology* 56: 3–12.

Scalbert, A. (1991): Antimicrobial properties of tannins. *Phytochemistry* 30: 3875–3883.

UNIVERSITY OF IBADAN LIBRARY