IJABR Vol. 3(2):23 -31 (2011)

Original Article

ANTIBACTERIAL EFFICACY OF THE PIGMENTED AND DE-PIGMENTED LEAF EXTRACTS OF *Alstonia boonei* de WILD.

* Fadipe, L. A., Jimoh, O. T., Otache S. A. and Shuaib, D. T.

Department of Chemistry, Federal University of Technology, Minna, Niger State, Nigeria,

Submitted: Jul.1 9, 2011; Accepted: Nov. 18, 2011; Published: Dec. 30, 2011.

ABSTRACT

Antibacterial efficacy of pigmented and de-pigmented petroleum ether and methanolic leaf extracts of *Alstonia boonei* was investigated. Phytochemical screening of these extracts using standard methods revealed that the pigmented and de-pigmented petroleum ether extract showed the strong presence of steroidal nucleus only, while the pigmented and de-pigmented methanol extracts revealed the presence of alkaloids, saponins, tannins, flavonoids, steroidal nucleus and cardiac glycosides with the de-pigmented extract showing a stronger presence of these constituents. The *in-vitro* antibacterial efficacy of these extracts revealed that the de-pigmented methanol extract showed appreciable activity against all the test organisms at 100mg/ml; a broad spectrum inhibitory effect that was quite similar to that produced by the standard drug, Erythromycin at 0.5mg/ml. The antibacterial efficacy of the active extract was only slightly enhanced at increased temperatures. The minimum inhibitory (MIC), minimum bactericidal (MBC) concentrations and MBC:MIC ratio of the active extract ranged between 6.25-50mg/ml, 25-100mg/ml and 1.0–4.0 respectively. Fractionation of the active extract by vacuum liquid chromatography gave rise to fractions that exhibited less inhibitory activities against the test organisms at 20mg/ml than the crude de-pigmented methanolic extract at 100mg/ml. MIC, MBC and MBC/MIC ratios of the active fractions also ranged between 100-200mg/ml, 50-200mg/ml and 1.0–2.0 respectively. The above findings suggest that the de-pigmented methanol extract of *A. boonei* might be a valuable source of antibacterial agents for the treatment of diarrhoea, typhoid fever, urinary and gastrointestinal infections.

Keywords: *Alstonia boonei*; leaf extracts; methanol; pigmented; de-pigmented; fractions; antibacterial.

*Correspondence: labsfad@yahoo.com, Tel: +234-803-406-4217.

INTRODUCTION

Alstonia boonei de Wild (Family Apocynaceae) is a lactiferous perennial tree that grows up to 45m in height and 1.2m in diameter. It is locally known as 'Ahun' (Yoruba) and 'Egbuora' (Igbo). It occurs in the tropics and subtropical regions (Cowan, 1999). The plant is widely used as an antiarthritic (Kweifo-Okai and Carroll, 1992; Taiwo *et al.*, 1998), antipyretic, analgesic and anti-inflammatory (Olajide *et al.*, 2000; Osadebe, 2002), antibacterial and antiviral (Anani *et al.*, 2001; Tan *et al.*, 2006), antifertility (Gupta *et al.*, 2005; Raji *et al.*, 2005), anti-oxidant (Akinmoladun *et al.*, 2007) and as an antimalarial (Majekodunmi *et al.*, 2008). A review of the literature reveals no report on comparison of the various classes of secondary metabolites present in the pigmented and de-pigmented petroleum ether and methanolic extracts of the leaves of *Alstonia boonei* and their antibacterial efficacy against selected bacteria. The development of microbial resistance to antibiotics makes it pertinent to constantly

search for new, active and safe compounds effective against pathogenic bacteria.

MATERIALS AND METHODS

Collection and Identification of Plant Material

Fresh leaves of *A. boonei* were collected from Ondo State Forest Reserve, Ifon, Nigeria in the month of March, 2009. The plant was duly identified and deposited at the Herbarium, Department of Biological Sciences, Faculty of science, Ahmadu Bello University, Samaru, Zaria, Nigeria.

Extraction Procedures

Five hundred grams of air-dried leaves of *A. boonei* was defatted by macerating it with 1.5L of petroleum ether (60-80°C) for a period of 6 days until the extracting solvent had become colourless. The resulting solution was concentrated in vacuo using a rotavapour and dried extract was labelled 'Pab'. Dried marc was again macerated with 2L of methanol and subjected to same procedure as above. The dried extract was dried and labelled 'Mab'.

De-pigmentation of Crude Extracts

The method of Hostettmann *et al.* (1998) was adopted. Fifteen gram of petroleum ether leaf extract of *A. boonei* (Pab) was dissolved in 500ml of petroleum ether and thoroughly mixed with 75g of activated charcoal until a right consistency was achieved. This was tightly sealed and kept aside for 72h. The mixture was filtered and the residue washed severally with petroleum ether to ensure a chlorophyll-free extract. Filtrate was concentrated in-vacuo, dried and labelled 'Pab-C'. For the methanolic-based extract, 38g of methanol leaf extract of *A. boonei* (Mab) was mixed with 700ml of methanol and mixed thoroughly with 190g of activated charcoal. The same procedure as above was repeated and the extract labelled 'Mab-C'. All extracts were subjected to antibacterial screening in comparison with Erythromycin.

Phytochemical Screening of the Extracts

All extracts (Pab, Pab-C, Mab and Mab-C) were screened for the presence of various phytoconstituents using standard methods (Sofowora, 1993; Evans, 1996).

Fractionation of De-pigmented Methanolic Extract

Twenty grams of active crude de-pigmented methanol extract, Mab-C was fractionated using vacuum liquid chromatography (Pelletier *et al.*, 1986). Silica gel (60-120 mesh) was used as the stationary phase, while varying proportions of increasing polarity of petroleum ether-chloroform and chloroform-methanol was used as the mobile phase. Obtained fractions were subjected to antibacterial testing.

Antibacterial Screening of the Extracts/Fractions

Source of Bacteria: Five bacterial strains; *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella typhi* (clinical strain) in overnight cultures (at 37°C) in nutrient broth were used in this study. All organisms were obtained from Microbiology laboratory, Federal University of Technology, Minna.

Assay of Antibacterial Activity of the Extracts/Fractions

The agar-well diffusion method was employed (Perez *et al.*, 1990; Dall'Agnol *et al.*, 2003). Standardized inoculums containing 10^6 cfu/ml 0.5ml McFarland standards were evenly streaked onto the surface of sterile agar plates for each organism. Eight millimeter wells were bored into the solidified agar using sterile cork borer at equidistant. Extracts/fractions were separately reconstituted to give concentrations of 100mg/ml (extracts), 0.5mg/ml (Erythromycin) and 20mg/ml (fractions). 0.5ml of each extract/fraction/drug was introduced into the wells with the aid of a Pasteur pipette individually. Plates were incubated

aerobically at 37°C for 24hr and zones of inhibition around the wells were measured to the nearest millimetre using a meter rule. Experiments were carried out in triplicates and results analyzed for statistical significance. Comparisons between groups were performed using two-way analysis of variance (ANOVA) on a statistical software package -Statistcal Package for Social Sciences (SPSS 15.0 for Windows, 2006 version) with Ryan-Einot-Gabriel-Welsch F Post hoc tests for separation of means. Differences were considered significant, if $p \leq 0.05$. A plant extract/fraction is considered 'active', when it has an inhibition zone of ≥ 14mm (Mothana and Linderquist, 2005).

Effect of Temperature on the Antibacterial Activity of Mab-C

The method of Doughari and Sunday (2008) was adopted. 100mg/ml of Mab-C was re-constituted in methanol and 5ml each of this mixture was transferred into 2 different test tubes and treated by heating the content of each test tube in a water bath to 50°C and 100°C respectively and subjected to antibacterial testing.

Determination of Minimum Inhibitory Concentration (MIC)

MIC was determined using the broth dilution method (Sahm and Washington, 1990). To 0.5ml varying concentrations of the active extract and fractions, 2ml of nutrient broth, followed by a loopful (0.5 McFarland turbidity standard) of the test organisms was added. A tube containing nutrient broth only seeded with the test organisms served as control. Tubes were incubated at 37°C for 24hr.The MIC was regarded as the lowest concentration showing no detectable growth/turbidity.

Determination of Minimum Bactericidal Concentration (MBC).

A loopful of broth was collected from those tubes showing no turbidity/ visible growth from the MIC tubes above and sub cultured onto freshly prepared plates. Inoculated plates were incubated at 37°C for 24hr. The least concentration showing no visible growth after incubation was taken as the MBC.

MBC/MIC ratios of the Active Extract /Fractions

The MBC/MIC ratio of the active extract/fractions was calculated by adopting the method of Agnese *et al* (2001).

RESULTS AND DISCUSSION

Phytochemical screening revealed the presence of several constituents in the pigmented and de-pigmented methanolic extracts with the de-pigmented methanolic extract showing a stronger presence of these constituents, while the pigmented and de-pigmented petroleum ether extracts responded only to the presence of steroidal nucleus as shown in Table 1.

Table 1: Phytochemical constituents of *A. boonei* leaves

Constituents	Pab	Mab	Pab-C	Mab-C
Alkaloids	-	++	-	++
Saponins	-	+	-	+++
Tannins	-	++	-	+++
Flavonoids	-	++	-	+++
Steroidal nucleus	+++	+++	+++	+++
Cardiac glycosides	-	+++	-	++
Carbohydrates	-	+++	-	+++

Key: +++= Highly present; ++= moderately present; += fairly present; -= absent.

Table 2: Antibacterial activity of leaf extracts of *A. boonei (100mg*/ml) against some bacterial strains.

Test Compound	Diameter of zones of inhibition of test organisms (mm)*				
	B. Subtilis	*S. aureus*	*E. coli*	*P. aeruginosa*	*S. typhi*
Pab	-	-	-	-	-
Pab-C	-	-	-	-	-
Mab	5.33±2.00	8.71±1.53	8.55±1.00	7.33± 0.58	7.67± 0.58
Mab-C	18.0±0.71	21.2±2.00	25.0±0.58	15.3± 1.00	25.0± 0.58
Erythromycin (0.5mg/ml)	20.2±1.00	25.0±0.00	27.3±2.41	12.5±2.00	26.2±1.41

*= mean values of three replicates with standard error shown as ±; p ≤0.05 was significant.
- = No measurable zone of inhibition.

Table 3: Effect of temperature on the antibacterial activity of de-pigmented methanol leaf extract of *A. boonei* (Mab-C) against test organisms

Temp(°C)	Diameter of zones of inhibition of test organisms (mm)*				
	B. subtilis	*S. aureus*	*E. coli*	*P. aeruginosa*	*S. typhi*
50	19.1±0.71	21.6±0.42	26.7± 0.58	16.0±1.00	27.2±0.26
100	22.1±1.00	26.5±1.15	28.6±0.65	12.8±1.00	28.2±0.58

Table 4: MIC, MBC and MBC/MIC ratio of active de-pigmented methanol leaf extract of *A. boonei* (Mab-C) against test organisms

MIC (mg/ml), MBC (mg/ml) and MBC/MIC values				
B. subtilis	*S. aureus*	*E. coli*	*P. aeruginosa*	*S. typhi*
50;100;2.0	25; 100;4.0	25;100;4.0	50;100;2.0	50;100;2.0

Table 5: Antibacterial activity of VLC fractions of active de-pigmented methanol leaf extract of *A. boonei* (20mg/ml) against test organisms.

	Diameter of zones of inhibition of test organisms (mm)*				
Test compound	*B. Subtilis*	*S. aureus*	*E. coli*	*P. aeruginosa*	*S. typhi*
hMab-C1	-	-	13.5±2.05	-	12.5±2.00
Mab-C2	-	-	-	-	-
Mab-C3	-	-	-	-	-
Mab-C4	-	-	12.2±1.67	17.3± 0.68	12.7± 0.58
Mab-C5	-	-	17.1±0.58	15.8± 1.00	-

Table 6: MIC; MBC and MBC/MIC ratios of active VLC fractions of de-pigmented methanol leaf extract of *A. boonei* (Mab-C) against test organisms

	MIC (µg/ml); MBC (µg/ml) and MBC/MIC ratios				
Test compound	*B. subtilis*	*S. aureus*	*E. coli*	*P. aeruginosa*	*S. typhi*
Mab-C1	ND	ND	100;200;2.0	ND	100;200;2.0
Mab-C2	ND	ND	ND	ND	ND
Mab-C3	ND	ND	ND	ND	ND
Mab-C4	ND	ND	200;200;1.0	100;200;2.0	200;200;1.0
Mab-C5	ND	ND	100;50;2.0	100,50;2.0	ND

ND = Not Determined

Generally, methanol has been reported to be a better solvent for more consistent extraction of plant bioactives from medicinal plants compared with petroleum ether (Eloff, 1998). This is probably because methanol is a mid-polar solvent. Result of the antibacterial activity of the extracts showed that the de-pigmented crude methanol extract (Mab-C) exhibited significant inhibitory activity against the test organisms as shown in Table 2. The pigmented and de-pigmented crude petroleum ether extracts expressed practically no activity against the test organisms. This is probably because of the absence of most bioactive compounds in these extracts (Table 1) or could be as a result of may be the extracts contain 'inactive substances' which probably antagonize/reduce the antibacterial action of one another (Ebi and Ofoefule, 1997) or probably, sometimes, the amount of active components in crude extracts from medicinal plants may be small or too diluted (Dall'Agnol *et al.*, 2003; Ndip *et al.*, 2009). The observed appreciable broad spectrum activity of Mab-C against both *Gram-positive* and *Gram-negative* bacteria could probably be due to the removal of chlorophyll pigment which is sometimes assumed to act as an inhibitory or masking substance (Iriyama *et al.*, 1974; Khackik *et al.*, 1986) which can interfere with the antibacterial property of some extracts (Khan and Saeed, 1998). The presence/exposure of the bioactives in the Mab-C, even in relatively low concentrations could contribute to the observed antibacterial activity (Dall'Agnol *et al.*, 2003), although chlorophyll, a green-coloured, Mg-containing pigment present in plants, especially the leaves has been reported to possess lots of biological importance (Indrajith and Ravindran, 2009). The antibacterial activity of Mab-C did not show a significant change with increase in temperature (Table 3). This implies that its inhibitory activity towards the test strains was not dependent on temperature, an indication that the phytoconstituents in the active fraction are thermo-stable (Doughari and Obidah, 2008).

Antibacterial assay of the five fractions collected from vacuum liquid chromatography of the active de-pigmented extract, revealed that fractions Mab-C4 and Mab-C5 at 20mg/ml produced significant inhibitory effects against *Gram* negative *P. aeruginosa* (17.3mm±0.58) and *E. coli* (17.1mm±0.68) respectively as shown in Table 5. Generally, fractions Mab-C4 and Mab-C5 which seemed to be the most active fractions, displayed better inhibitory activities against the *Gram* negative bacteria, with no visible zone of inhibition(s) against the *Gram* positive organisms. This implies that the fractions did not posses broad spectrum activity against the organisms, or probably the activity of the fractions could be enhanced at higher concentrations. It was observed that the fractions (20mg/ml) were less active than the crude active de-pigmented extract (100mg/ml). This could be as a result of possible synergistic effect between the phytoconstituents of the crude (Doughari and Obidah, 2008) or probably because of lower concentration.

The efficacy of the de-pigmented extract/fractions as an antibacterial was further supported by their low MICs and MBCs. Low MIC and MBC values are an indication of the efficacy of a plant extract and may serve as veritable sources for compounds with therapeutic potency (Fabry *et al.*, 1998). The calculated MBC/MIC ratio for the active extract/fractions was used to ascertain if the observed antibacterial effects were bacteriocidal or bacteriostatic in nature. Mab-C and its active fractions had MBC/MIC ratios that ranged between 2.0-4.0 and 1.0–2.0 respectively. This is indicative of a bacteriostatic effect.

Extracts/fractions with MBC/MIC ratio greater than one would indicate a bacteriostatic effect of the extract/fraction, while lower than one is indicative of a bacteriocidal effect (Agnese *et al.*, 2001).

CONCLUSION

The secondary metabolites which were more observed in the de-pigmented methanol leaf extract of *Alstonia boonei* were probably responsible for the promising broad spectrum antibacterial properties of this plant, suggesting that the plant on removal of chlorophyll pigment could be an effective source of antibacterial substances. Further studies on the isolation and characterization of the biologically active constituents of this plant can help provide cheaper and safer antibacterials.

ACKNOWLEDGEMENT

The authors wish to express their unreserved gratitude to Mal. Ali of the Department of Microbiology, Federal University of Technology, Minna, Niger State who was of great help in the antimicrobial aspect of this work.

REFERENCES

Agnese, A. M., Perez, C., Cabrera, J. L (2001). *Adesmia aegiceras*: Antimicrobial and chemical study. *Phytomedicine* 8 (5): 389-394.

Akinmoladun, A. C., Ibukun, E. O., Afor, E., Akinrinola, B. L., Onibon, T. R., Akinboboye, A. O., Obutor, E. M., Farombi, E. O (2007). Chemical constituents and anti-oxidant activity of *A. boonei*. *African J. of Biotechnology* 6 (10):1197-1201.

Anani, K., Hudson, J. B., de Souza, C., Akpagana, K., Tower, G. H. N., Arnason, J. T., Gbeassor, M. (2001). Investigation of medicinal plants of Togo for antiviral and antimicrobial activities. *Pharmaceutical Biology* 38 (1):40-45.

Cowan, M. M (1999). Plant products as antimicrobial agent. *Clinical Microbial Review* 12: 564-582.

Dall'Agnol, R., Ferraz, A., Bernardi, A. P., Albring, D., Nor, C., Sarmento, L., Lamb, L., Hass, M., von Poser G., Schapoval, E. E (2003). Antimicrobial activity of some Hypericum species. *Phytomed* 10: 511-516.

Doughari, J. H., Obidah, J. S (2008). Antibacterial potentials of stem bark extracts of *Leptadenia lancifolia* against some pathogenic bacteria. *Pharmacology online* 3: 172-180

Doughari, J.H., Sunday, D. (2008). Antibacterial activity of *Phyllantus muellerianus*. *Pharm. Bio* 46 (6):405.

Fadipe *et al*, 2011

Ebi, G.C., Ofoefule, S.I. (1997). Investigations into the forkloric antimicrobial activities of *Landolphia owerrience*. *Phytother Res.* 11:149–151.

Eloff, J. N. (1998b). Which Extract should be used for the Screening and Isolation of antimicrobial components from plants? *J Ethnopharmacol* 60: 1-8

Evans, W .C (1996). *Trease and Evans' Pharmacognosy*. 14th ed. London: WB Saunders Company Ltd, pp. 227, 245, 251.

Fabry, W., Okemo, P., Ansorg, R (1998). Antibacterial activity of East Africa medicinal plants. *J. Ethnopharmacol* 60 (1): 79-84.

Guptaa, R. S., Bhatnagerc, A.K., Joshib, Y.C., Shamab, M.C., Khushalania, V., Kachhawaa, J.B.S. (2005). Induction of antifertility with lupeol acetate in male albino rats. *Pharmacology* 75 (2):57-62.

Hostettman, K., Marston, A., Hostettman, M (1998). *Preparative Chromatography*

techniques: Applications in natural product isolation. UK: Springer, pp. 9-11.

Indrajith, A., Ravindran, K.C. (2009). Antioxidant potential of Indian medicinal plant- *Phyllantus amarus* L under supplementary UV-B radiation. *Recent Research in Sci. and Tech.* **1** (1): 034-042.

87 Iriyama, K., Ogura, N., Takamiya, A. A (1974). Simple method for extraction and partial purification of chlorophyll from plant material using dioxane. *J. of Biochem* 76 (4): 901-904.

Khachik, F., Beecher, G.R., Whittaker, N. F (1986). Separation, identification and quantification of the major carotenoid and chlorophyll constituents in extracts of several green vegetables by liquid chromatography. *J. Agric. Food Chem.* 34 (4): 603-616.

Khan, F.Z., Saeed, M.A (1998). Phytochemical and antimicrobial studies of *Salvia splendens* sello. *Pakistan J. of Pharm Sci.* **11** (2): 13-21.

Kweifo-Okai, G., Carroll, A.R (1992). Antiarthritic effects of *A. boonei* triterpenoids. In: Gopalakrishnakone, P; Tan, C.K. (editors). *Recent advances in toxicology research.* Singapore: VTRG 3 pp 19.

Majekodunmi, S.O., Adegoke, O. A., Odeku, O. A (2008). Formulation of the extract of the stem bark of *A. boonei* as tablet dosage. Tropical J. of Pharmaceutical Research 7 (2): 987-994.

Mothana, R.A., Lindequist, U. (2005). Antimicrobial activity of some medicinal plants of Island Soqotra. *J. Ethnopharmacol* 96: 177-181.

Ndip, R.N., Ajeagbelac, A.N., Wirna, T., Luma, H.N., Wirmum, C., Efange, S. M. N (2000). *In-vitro* antimicrobial activity of *Ageratum conyzoides* (Linn) on clinical isolates of *Helicobacter pylori*. *African J. of Pharmacy and Pharmacology* 3 (11): 585-592.

Olajide, O.A., Awe, S.O., Makinde, J. M., Ekhelar, A.I., Olushola, A., Morebise, O., Okpako, D. T (2000). Studies on the anti-inflammatory, antipyretic and analgesic properties of *A. boonei* stem bark. *J. of Ethnopharmacol* 71 (1-2): 179-186.

Osadebe, P. O (2002). Anti-inflammatory properties of the root bark of *A. boonei*. *Nig. J. Nat. Prod. Med.* **6:** 39-41.

Pelletier, S. W., Chokshi, H.P., Desai, H.K. (1986). Separation of diterpenoid alkaloid mixtures using Vacuum Liquid Chromatography. *J. of Natural Products* 49 (5): 892-900.

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

Sahm, D. F., Washington, J. A (1990). Antibacterial susceptibility test dilution methods. In: Lennette E. H, editor. *Manuals of clinical microbiology,* 5th Edition. Washington D.C: American society for Microbiology, pp. 1105-1116.

Sofowora, E.A (1993). *Medicinal plants and traditional medicine in Africa.* Ife, Nigeria, University press, pp. 12-23.

Raji, Y., Salman, T., Akinsomisoye, O. S (2005). Reproductive functions in male rats treated with methanolic extract of *Alstonia boonei* stem bark. *African J. of Biomedical Research* 8 (2): 105-111.

Taiwo, O. B., Van Der Berg, A. J. J., Kroes, B. H., Beukelman, C. J., Horsten, S. F. A. J., Van Ufford, H. C. Q., Van Den Worm, E., Makinde, J. M., Labadie, R. P (1998). Activity of *A. boonei* de wild (Apocynaceae) stem bark extract on human complement and polymorpho

nuclear leukocytes. *Indian J of Pharmacology* 30 (3): 169-174.

Tan, P. V., Boda, M., Sonke, B., Etoa, F., Nyasse, B. (2006). Susceptibility of *Helicobacter* and *Campylobacter* to crude extracts prepared from plants used in Cameroonian folk medicine. *Pharmacology on line* 3: 877-891.