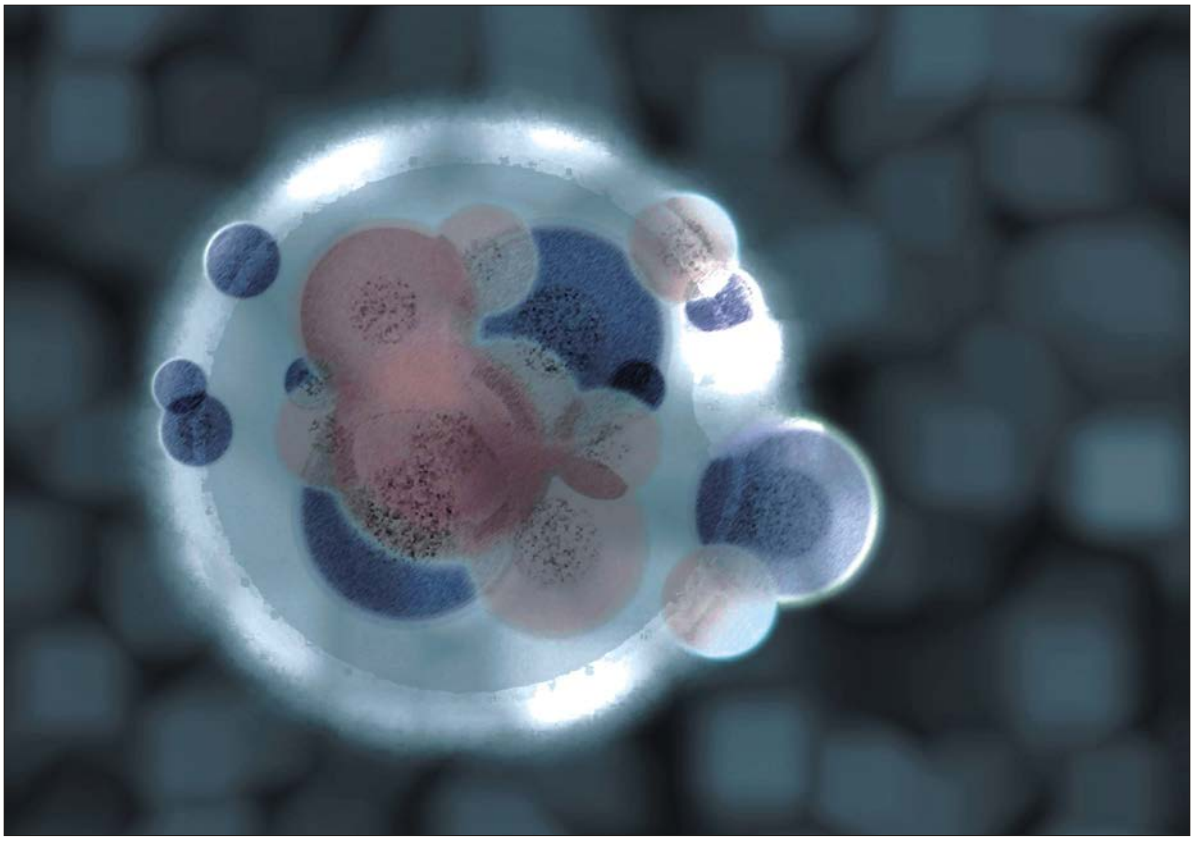

Journal of Biological Sciences

ISSN 1727-3048

science alert

ANSInet
an open access publisher
http://ansinet.com

Journal of Biological Sciences 8 (2): 356-361, 2008
ISSN 1727-3048

Antibacterial Activities of Crude Stem Bark Extracts of *Distemonanthus benthamianus* Baill

[1]O.A. Aiyegoro, [2]D.A. Akinpelu, [3]A.J. Afolayan and [1]A.I. Okoh
[1]Department of Biochemistry and Microbiology,
Applied and Environmental Microbiology Research Group (AEMREG),
University of Fort Hare, Alice 5700, South Africa
[2]Department of Microbiology, Obafemi Awolowo University, Ile-Ife, Nigeria
[3]Department of Botany, University of Fort Hare, Alice 5700, South Africa

Abstract: *In vitro* antibacterial activities of four fractions of stem bark of *Distemonanthus benthamianus* Baill. against some bacterial isolates implicated in oro-dental infections were investigated using standard microbiological methods. The aqueous and chloroform fractions exhibited significant inhibitory action against all twelve bacterial isolates tested at a concentration of 10 mg mL^{-1}. The zones of inhibition due to the aqueous fraction ranged between 10 and 15 mm while that of chloroform fraction ranged between 8 and 13 mm. The Minimum Inhibitory Concentration (MIC) exhibited by aqueous fraction against the bacterial isolates ranged between 0.625 and 2.5 mg mL^{-1} while that of chloroform fraction ranged between 0.313 and 5.0 mg mL^{-1}. Phytochemical analysis of *Distemonanthus benthamianus* extract revealed the presence of tannins, steroids, saponins and alkaloids. Between 18 and 76% of *Streptococcus mutans* were killed within 120 min contact time in aqueous extract concentration of between 0.3125 and 2.50 mg mL^{-1}, while between 15 and 60% of *Bacteroides gingivalis* were killed within the same period and concentration by the aqueous fraction of the crude extract. The same concentrations of extracts resulted in protein leakages in the test organisms and we proposed disruption of cell membrane as a mechanism of action of the plant extract.

Key words: *Distemonanthus benthamianus*, antibacterial activity, minimum inhibitory concentration, protein leakages, extracts

INTRODUCTION

Distemonanthus benthamianus belongs to the family Leguminosae (Nguelefack *et al*., 2005) which comprises the largest family of flowering plants, numbering some 400 genera and 10,000 species. The plant is sometimes named African or yellow satinwood and thus confused with *Aformosia laxiflora* which it resembles and share the same vernacular name. Other common names of *D. benthamianus* are: Movingui (Gabon), Barre (Ivory Coast), Bonsamdua (Ghana), Eyen (Cameroon), Ayaran (Nigeria). The plant is rich in flavonoids compounds (Nguelefack *et al*., 2005), such as Oxyayamin A, Oxyayanin B, Ayanin and Distemonanthin. These components have been implicated in antitumor activity (Arisawa *et al*., 2006), antioxidative activity (Ndukwe *et al*., 2005), anti-adrenergic activity (Guerrero *et al*., 2002) and in contact dermatitis, respectively.

D. benthamianus is a tree used in traditional African medicine to treat bacterial, fungal and viral infection (Nguelefack *et al*., 2005). The root is locally known as Orin ayan by the Yorubas of Western Nigeria and it is used as chewing stick for oro-dental hygiene. Recent interest in chewing sticks and their extracts has focused on their effects on organisms that are involved in oral infections. Africans that use chewing stick have fewer carious lesions than those that use toothbrush and their use has been encouraged by the World Health Organization (Ndukwe *et al*., 2005). Of all the works done on the antimicrobial potentials of *D. benthamianus* (Ndukwe *et al*., 2005), none has reported on the biological activity of the stem bark of the plant on oral bacterial pathogens. In this study, we assess the *in vitro* antibacterial activities of crude extracts of the stem bark of *D. benthamianus* against some bacterial strains that are implicated in oro-dental infections.

Corresponding Author: A.I. Okoh, Department of Biochemistry and Microbiology,
Applied and Environmental Microbiology Research Group (AEMREG), University of Fort Hare,
Private Bag X1314, Alice 5700, South Africa
Tel: +27406022365, +2782224970 Fax: 0866286824

MATERIALS AND METHODS

Plants material and preparation of extract: Fresh stem bark of *D. benthamianus* was collected from Ile-Ife in Osun state, Nigeria, in the month of September 2005 and authenticated at the herbarium of the Department of Botany, Obafemi Awolowo University, Ile-Ife, Nigeria. The bark was later air-dried, pulverized in a mill (Chisty Lab Mill, Chisty and Norris Ltd., Process Engineers, Chelmsford, England) and stored in an air-tight container for further use. Exactly 500 g of the pulverized bark of the plant was cold extracted using 60% methanol for 4 days with occasional shaking (Harborne, 1998). The mixture was then filtered (using Whatman No. 1 filter paper); the filtrate was concentrated to dryness *in vacuo* using a rotary evaporator. This gave a yield of 20 g of the crude extract.

Preparation of microorganism for the experiment: The following test microorganisms obtained from Obafemi Awolowo University, Ile-Ife, Department of Microbiology culture collections were used: *Bacillus subtilis* (NCIB 3610), *Staphylococcus aureus* (NCIB 8588), *Staphylococcus aureus* (ATCC 5538), *Staphylococcus aureus* (LIO), *Streptococcus mutans* (ATCC 6569), *Streptococcus mitis* (NCIB 196), *Bacteroides gingivalis* (ATCC 33277), *Bacteroides melaninogenicus* (ATCC 33184), *Pseudomonas aeruginosa* (ATCC 10145), *Proteus vulgaris* (NCIB 67), *Porphyromonas gingivalis* (NCIB 1377) and *Klebsiella pneumoniae* (ATCC 6380). The aerobic test organisms were sub-cultured in nutrient broth and nutrient agar (Oxoid, Ltd.), while the anaerobes were maintained on brain heart infusion agar (BBL Microbiology Systems, Cockeysville, Md.).

Phytochemical analysis of the plant extract: A small portion of the dry extract was used for preliminary phytochemical screening tests for tannins, saponins, steroids and alkaloids in accordance with the Trease and Evans (1989) and Harborne (1998) with little modifications.

Fractionation of the crude extracts with organic solvents of different polarity: Fractionation of the crude extract was carried out as described by Harborne (1998) with little modification. Exactly 20 g of the crude extract of *D. benthamianus* was dissolved in 100 mL sterile distilled water in a separation funnel and later fractionated using different organic solvents in order of their polarity. First, about 500 mL of n-Hexane was used to extract part of the resolved extract. The extraction was done thrice until the n-Hexane layer became colourless. The n-Hexane layer was later separated from the aqueous layer. The aqueous layer left was re-concentrated to eliminate the residual n-Hexane. Five hundred milliliter of chloroform was then added to the aqueous layer for further extraction. The extraction followed the same procedure with n-Hexane. The chloroform layer separated from the aqueous layer. Lastly, 500 mL of butanol was used for the extraction of the residual material and all extracts were concentrated to dryness and kept in the freezer until ready for use.

***In vitro* assay:** The antibacterial sensitivity assay of the crude fractions was determined using agar-well diffusion method (Irobi *et al*., 1996). The aerobic and anaerobic bacterial isolates used in this test were first grown for 18 h in nutrient broth and 48 h in brain heart infusion broth (Oxoid Ltd.) respectively and standardized to 0.5 McFarland standards (10^6 cfu mL^{-1}). Two hundred microliter of the standardized cell suspensions were spread onto the surface of Diagnostic Sensitivity Agar (Oxoid Ltd.). Wells were then bored into the agar using a sterile 6 mm diameter cork borer. Approximately 100 µL of the crude fractions at 10 mg mL^{-1} were introduced into the wells, allowed to stand at room temperature for about 2 h and then incubated at 37°C. The plates were observed for zones of inhibition after 24 h for the aerobes and 48 h for the anaerobes. The effects were compared with those of streptomycin and ampicillin standard antibiotics at a concentration of 1 and 10 µg mL^{-1}, respectively. The Minimum Inhibitory Concentration (MIC) of the plant extracts was determined as described by Betoni *et al*. (2006) with modifications. The reconstituted plant extracts was diluted to give between 5.0 and 0.025 mg mL^{-1} final concentration in nutrient broth or Brain Heart Infusion broth for anaerobes. Using a standard pipette, about 1 mL of 18 h old (48 h for anaerobes) bacterial broth (10^6 cfu mL^{-1}) culture was introduced into appropriately labeled test tubes. A set of tubes containing only growth medium and each of the test bacteria was set up separately to serve as controls. All tubes were incubated at 37°C for 24 h (48 h for anaerobes). The minimum inhibitory concentration was taken as lowest concentration that prevented growth of the bacterial strains. The Minimum Bactericidal Concentration (MBC) of the plant extracts was determined by a modification of the method of Spencer and Spencer (2004). Samples were taken from the test tubes with no visible turbidity (growth) in the MIC assay and subcultured onto freshly prepared Mueller Hinton II agar plates (or Brain Heart Infusion agar for anaerobes). After incubation for 48 h (5 days for anaerobes) at 37°C, the minimum bactericidal concentration was taken as the lowest concentration of the extract that did not allow any bacterial growth on the surface of the agar plates.

Rate of kill and protein leakage assays: The killing rate and protein leakage from the bacterial cell due to exposure to the aqueous extract were carried out using the methods of (Okeke *et al*., 2001; Ultee *et al*., 1999), respectively. Rate of kill was determined by assay of bacterial cell-death time. Approximately 5×10^5 cfu of test bacterial strain was introduced into 10 mL of Mueller Hinton Broth (MHB) and Brain Heart Infusion broth (for anaerobes) containing 1/2×MIC, MIC, 2×MIC or 4×MIC of aqueous extract and incubated on a horizontal shaker at 37°C. Exactly 0.5 mL volume of each suspension was withdrawn at the appropriate intervals and transferred to 4.5 mL of Mueller Hinton broth and (or Brain Heart Infusion broth for anaerobes) recovery medium containing 3% Tween-80 to neutralize the effects of the antibacterial compounds' carry-overs from the test suspensions. The suspension was then diluted serially and exactly 0.2 mL of each dilution was plated in triplicate on Mueller Hinton Agar (MHA) and (or Brain Heart Infusion agar for anaerobes). After 24 h (48 h for anaerobes) incubation at 37°C, emergent bacterial colonies were counted and compared with the count of the culture control. Protein leakage was determined by treatment of various concentrations of the extracts (relative to MIC) with bacterial cells washed three times in physiological saline by centrifugation at 10,000 rpm for 10 min followed by re-suspension in physiological saline. At intervals, each suspension was centrifuged at 7000 rpm and the supernatant obtained was assayed for protein using Bradford reagent. The concentration of protein was estimated from the established standard curve obtained using Bovine Serum Albumin (BSA). For these assays, *Streptococcus mutans Bacteroides gingivalis* were used to represent Gram positive and Gram negative strains, respectively.

RESULTS AND DISCUSSION

All four fractions of the crude extracts of the plant tested showed varying degrees of antibacterial activities against the test bacteria species (Table 1). The antibacterial activities of the aqueous and chloroform fractions compared favourably with that of two standard antibiotics (streptomycin and ampicilin) and have appeared to be broad in nature as its activities were independent on Gram reaction. The n-hexane and butanol fractions showed low antibacterial activities with zones of inhibition ranging between 0 and 10 mm. The MIC of the aqueous fraction of the plant ranged between 0.625 and 2.5 mg mL^{-1} while that of chloroform fraction ranged between 0.313 and 5.0 mg mL^{-1} (Table 2).

Phytochemical analysis of the plant revealed the presence of tannins, steroids, alkaloids and saponins (Table 3). These compounds are known to possess antimicrobial activities. Tannins have been found to form irreversible complexes with proline-rich proteins (Shimada, 2006) resulting in the inhibition of the cell protein synthesis. Furthermore, tannins are known to react with proteins to provide the typical tanning effect

Table 1: The antibacterial activities of four extracts of D. *benthamianus*

	Zone of inhibition (mm)					
Test organisms	AQ	BL	CL	HX	ST	AMP
	(10 mg mL^{-1})					(10 µg mL^{-1})
Bacillus subtilis (NCIB 3610)	10±1.00	6±2.00	11±1.00	7±1.55	20±2.00	0±0.00
Staphylococcus aureus (NCIB 8588)	13±0.55	0±0.00	11±2.00	0±0.00	20±0.55	0±0.00
Staphylococcus aureus (ATCC 5538)	11±1.55	0±0.00	8±1.05	0±0.00	20±0.00	0±0.00
Staphylococcus aureus (LIO)	14±2.00	0±0.00	12±2.00	0±0.00	22±0.55	0±0.00
Streptococcus mutans (ATCC 6569)	15±1.05	0±0.00	13±0.00	0±0.00	23±1.00	16±0. 05
Streptococcus mitis (NCIB 196)	12±0.00	9±1.55	11±1.05	0±0.00	18±0.00	12±2.00
Bacteroides gingivalis (ATCC 33277)	15±1.00	0± 0.00	13±1.00	0±0.00	0±0.00	0±0.00
Bacteroides melaninogenicus (ATCC 3756)	10±0.55	0±0.00	10±0.50	0±0.00	30±0.55	22±0.55
Pseudomonas aeruginosa (ATCC 10145)	11±0.55	0±0.00	8±2.00	0±0.00	21± 2.00	0±0.00
Proteus vulgaris (NCIB 67)	11±0.00	0±0.00	10±0.00	0±0.00	19±1.00	20±1.55
Porphyromonas giugivalis (NCIB 1377)	11±0.00	0±0.00	10±2.00	0±0.00	15±2.50	16±1.00
Klebsiella pneumoniae (ATCC 6380)	10±2.00	10±0.55	11±0.55	0±0.00	0±0.00	0±0.00

Values are mean±standard error of the mean; AQ: Aqueous fraction; BL: Butanol fraction; CL: Chloroform fraction; HX: n-hexane; ST: Streptomycin; AMP: Ampicilin; LIO: Locally Isolated Organism

Table 2: Minimum inhibitory concentrations (MICs) of aqueous and chloroform extracts *Distemonanthus benthamianus*

	MIC (mg mL^{-1})		
Test organisms	AQ	CL	ST
Bacillus subtillis (NCIB 3610)	1.250	1.250	0.0313
Staphylococcus aureus (NCIB 8588)	1.250	1.250	0.0625
Staphylococcus aureus (ATCC 6538)	2.500	2.500	0.2500
Staphylococcus aureus (LIO)	1.250	2.500	0.2500
Streptococcus mutans (ATCC 6569)	0.625	0.313	0.0625
Streptococcus mitis (NCIB 196)	1.250	2.500	0.2500
Bacteroides gingivalis (ATCC 33277)	0.625	1.250	-
Proteus vulgaris (NCIB 67)	2.500	5.000	0.2500
Klebsiella pneumoniae (ATCC 6380)	2.500	5.000	-

AQ: Aqueous fraction; CL: Chloroform fraction; LIO: Locally Isolated Organism; ST: Streptomycin; MIC: Minimum Inhibitory Concentration.

Table 3: Preliminary Phytochemical screening of extracts of *Distemonanthus benthamianus*

Test	*D. benthamianus*
Saponin	++
Tannin	++
Steroids	+
Alkaloids	++

+: Present; ++: Very positive (reaction positive within sec)

which is important for the treatment of inflamed or ulcerated tissues (Parekh and Chanda, 2007). Herbs that have tannins as their main component are astringent in nature and are used for treating intestinal disorder such as diarrhea and dysentery (Dharmananda, 2003). Tannins are reported to possess broad antimicrobial properties by means of different mechanisms that include enzyme inhibition, oxidative phosphorylation reduction and iron deprivation, among others (Parekh and Chanda, 2007). These observations thus support the use of *D. benthamianus* in herbal cure remedies. Alkaloids have amazing effect on humans and thus had led to the development of powerful pain killer medications (Kam and Liew, 2002). Quinlan *et al.* (2000) worked on steroidal extract from some medicinal plant which exhibited antibacterial activities on some tested bacterial isolates. Neumann *et al.* (2004) also confirmed the antiviral property of steroids. Thus, the presence of these compounds in *D. benthamianus* corroborates the antibacterial activities observed.

Different concentrations of the aqueous fraction exhibited significant bactericidal effects on *Bacteroides gingivalis* and *Streptococcus mutans*. At a concentration of 0.3125 mg mL^{-1} of the aqueous fraction of *D. benthamianus* about 18% of the *B. gingivalis* cells were killed within 30 min of the interaction with the fraction, while the percentage killed increased to 31% within 90 min of the cells interaction with the extract (Fig. 1). When the concentration of the fraction was increased to 2.50 mg mL^{-1}, the percentage of the cells death was 60% within 90 min. A similar trend of reaction occurred when the *Strept. mutans* was subjected to the interaction with the aqueous fraction (Fig. 2) with approximated 40 to 76% of cells killed in 120 min in the different concentrations of the extracts.

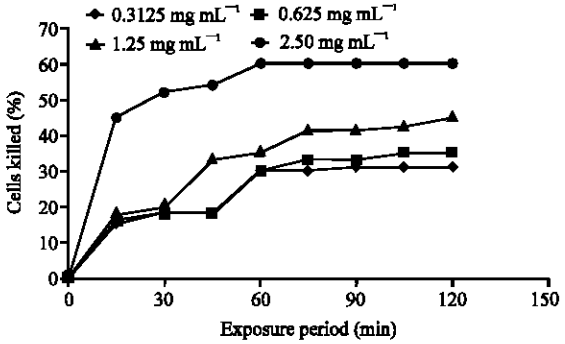

Fig. 1: Rate of kill profile of *Bacteroides gingivalis* (ATCC 33277) by aqueous extract of *Distemonanthus benthamianus*

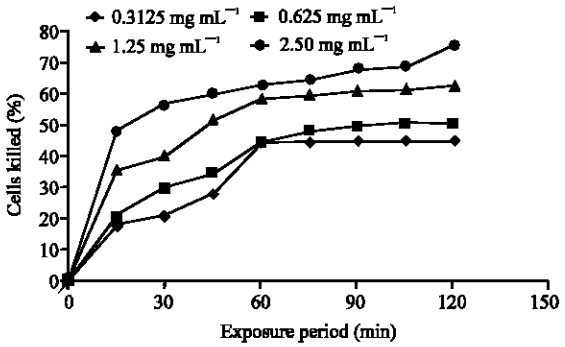

Fig. 2: Rate of kill profile of *Streptococcus mutans* (ATCC 6569) by aqueous extract of *Distemonanthus benthamianus*

Both aqueous and chloroform fractions of the plant exhibited broad spectrum antibacterial activities, although the aqueous fraction was more active thus supporting its usefulness in herbal treatment of various infections and its use as chewing stick for cleaning teeth in some parts of the world. The aqueous fraction has caused the leakage of considerable amount of proteins from *Bacteroides gingivalis* and *Streptococcus mutans* cells. Between 5 to 17 μg mL^{-1} of protein leaked out of *Bacteroides gingivalis* cells within 120 min contact time in extracts concentrations of between 0.3125 and 2.50 mg mL^{-1}, while the amount of protein that leaked out of *Streptococcus mutans* cells under similar conditions ranged between 3 and 13 μg mL^{-1} (Fig. 3, 4). With longer durations of exposure, cell viability decreased with a more significant increase in protein leakage and this suggests that membrane components are the primary targets of these two active fractions of the plant which ultimately resulted in bacterial death as previously suggested (Cao *et al.*, 2002). We therefore propose that a probable mechanism of action of aqueous and chloroform

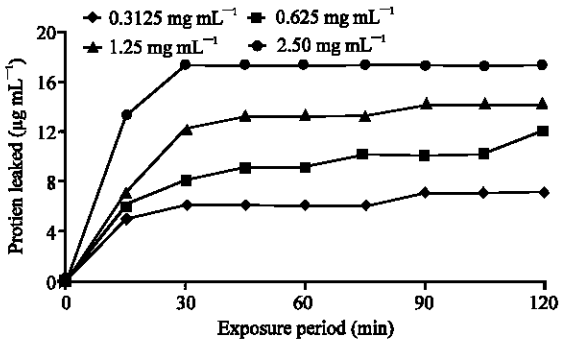

Fig. 3: Protein leakage in *Bacteroides gingivalis* (ATCC 33277) in the presence of different concentrations of aqueous extract of *Distemonanthus benthamianus*

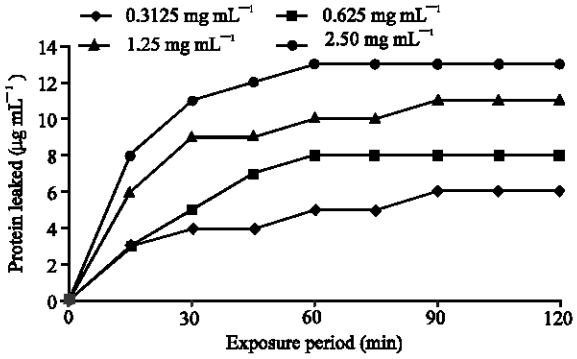

Fig. 4: Protein leakage in *Streptococcus mutans* (ATCC 6569) in the presence of different concentrations of aqueous extract of *Distemonanthus benthamianus*

fractions of *D. benthamianus* are by way of cell membrane disruption to bacterial cells. This is without prejudice to other possibilities that were not exploited by this study.

ACKNOWLEDGMENTS

We are grateful to the National Research Foundation (NRF) of the Republic of South Africa for financial support (Grant Ref. TTK2006061400023) and to the Herbarium unit of the Department of Botany, Obafemi Awolowo University Ile-Ife, Nigeria, for the identification of the plant.

REFERENCES

Arisawa, M., M. Shimizu, Y. Satomi, A. Nishino, H. Nishino and A.I. Washima, 2006. Inhibition of tumour-promoter-enhanced ^{32}Pi-incorporation into cellular phospholipids by flavonols from genus *Chysosplenium*. Phytother. Res., 9 (3): 222-224.

Betoni, J.E.C., R.P. Mantovani, L.N., Barbosa, L.C. Di Stasi and A. Fernandes Junior, 2006. Synergism between plant extract and antimicrobial drugs used on *Staphylococcus aureus* diseases. Mem. Inst. Oswaldo Cruz., 101 (4): 387-390.

Cao, M., T. Wang, R. Ye and J.D. Helmann, 2002. Antibiotics that inhibit cell wall biosynthesis induce expression of the *Bacillus subtilis* σW and σM regulons. Mol. Microbiol., 45 (5): 1267-1276.

Dharmananda, S., 2003. Gallnuts and the uses of Tannins in Chinese Medicine. In: Proceedings of institute for Traditional Medicine, Portland, Oregon.

Guerrero, M.F., P. Puebla, M.L. Martin, R. Carron, L. San Roman, M.T. Reguero and L. Arteaga, 2002. Inhibitory effect of N (G)-nitro-L-arginine methyl ester on the anti-adrenergic response elicited by ayanin in the pithed rat. Planta Med., 68 (4): 322-325.

Harborne, J.B., 1998. Phytochemical Methods. A Guide to Modern Techniques of Plant Analysis. Chapman and Hall, London.

Irobi, O.N., M. Young and W.A. Anderson, 1996. Antimicrobial activity of Annato (*Bixa orella*) extract. Int. J. Pharmacog., 34: 87-90.

Kam, P.C.A. and S. Liew, 2002. Traditional Chinese herbal medicine and anaesthesia. Anaesthesia, 57 (11): 1083-1089.

Ndukwe, K.C., I.N. Okeke, A. Lamikanra, S.K. Adesina and O. Aboderin, 2005. Antibacterial activity of aqueous extracts of selected chewing sticks. J. Contemp. Dent. Pract. 3 (6): 86-94.

Neumann, U.P., T. Berg, M. Baha, G. Puhl, O. Guckelberger, J.M. Langreh and P. Neuhaus, 2004. Long-term outcome of liver transplants for chronic hepatitis C: A 10-year follow-up. Transplantation, 77 (2): 226-231.

Nguelefack, E.M.P., K.P. Ngu, A. Atchade, T. Dimo, N. Tsabang and J.T. Mbafor, 2005. Phytochemical composition and *in vitro* effects of the ethyl acetate bark extract of *Distemonanthus benthamianus* Baillon (Caesalpiniaceae) on *Staphylococcus aureus* and *Streptococcus agalactiae*. Cameroon J. Exp. Biol., 1 (1): 50-53.

Okeke, M.I., C.U. Iroegbu, E.N. Eze, A.S. Okoli and C.O. Esimone, 2001. Evaluation of the root of *Landolphia owerrience* for antibacterial activity. J. Ethn., 78: 119-127.

Parekh, J. and S. Chanda, 2007. *In vitro* antibacterial activity of the crude methanol extract of *Woodfordia fruticosa* Kurz. flower (Lythaceae). Braz. J. Microbiol., 38: 2.

Quinlan, M.B., R.J. Quinlan and J.M. Nolan, 2000. Ethnophysiology and herbal treatments of intestinal worms in Dominica, West Indies. J. Ethnopharmacol., 80: 75-83.

Shimada, T., 2006. Salivary proteins as a defense against dietary tannins. J. Chem. Ecol., 32 (6): 1149-1163.

Spencer, A.L.R. and J.F.T. Spencer, 2004. Public Health Microbiology: Methods and Protocols. Human Press Inc. New Jersey.

Trease, G.E. and W.C. Evans, 1989. Textbook of Pharmacognosy. 12th Edn. Balliere, Tindal, London.

Ultee, A., E.P. Kets and E.J. Smid, 1999. Mechanisms of action of carvacrol on the food-borne pathogen *Bacillus cereus*. Applied Environ. Microbiol., 65 (10): 4606-4610.