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Full Length Research Paper

Antimicrobial Effect of Mangifera Indica Woodash on Bacterial Isolates From Human Skin

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There is a concerted and continued search for interesting and novel biologically active compounds found in nature that could be used against pathogenic microorganisms. Ethanol extracts of Mangifera indica woodash were investigated for in-vitro antibacterial activity against isolated skin bacteria, namely Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli and Streptococcus spp. The agar well diffusion method was used to assay for the antibacterial properties of the woodash extracts. Data obtained from the susceptibility tests showed that the woodash extracts of Mangifera indica at different concentrations inhibited the growth of all test organisms showing varying zones of inhibition. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the ethanol woodash extract were determined against these microorganisms using tube dilution. All test woodash extract of Mangifera indica exhibited a maximum MBC value of 7.0 mg/mL and MIC value of 13.0 mg/mL.

Keywords: Mangifera indica woodash, Inhibitory effect, plant nanomaterials, ethanol extracts.

INTRODUCTION

There is a concerted and continued search for interesting and novel biologically active compounds found in nature that could be used against pathogenic microorganisms. Current research has documented the therapeutic values of natural compounds in a bid to validate claims of their biological activity (Abraham et al., 1996). Because of its constant exposure to and contact with the environment, the skin is particularly apt to contain transient microorganisms. Nevertheless, there is a constant and well defined resident flora modified in different anatomic areas, secretions, habitual wearing of clothing or proximity to mucus membrane (Maisbach et al., 1981). The predominant microorganisms on the skin are aerobic and anaerobic diphteroids (e.g. Corynebacterium, Propionibacterium), non-hemolytic aerobic and anaerobic Staphylococci (Staphylococcus epidermidis, Staphylococcus aureus, Streptococcus species), Woodash is composed of many major and minor elements which are extracted from plants from the soil during growth. As a product of the combustion of organic matter, plant woodash has a lot of constituents with chemical, physical and biological applications (Abrahams et al., 1996). Data from research studies show the utility of natural products as sources of novel structures, but not necessarily the final drug entity (Newman et al., 2007).

The objective of this research project is to investigate the effect of the broad spectrum antimicrobial activity of woodash obtained from combusted woody parts of Mango (Mangifera indica) on cultures of skin bacterial colonies. Mangifera indica (mango) has been an important herb in indigenous medical systems for 400 years. Reports have shown the antibacterial activity of M. indica. In an in-vitro agar-diffusion technique, M. indica extract showed activity on seven bacterial species ; Bacillus pumilus, Bacillus cereus, Staphylococcus aureus, Staphylococcus citrens, Escherichia coli, Salmonella agona, Klebsiella pneumonia, one yeast (Sacchcromyces cerevisiae) and four fungi (Thermoasans autantiacus, Trichoderma raesei, Aspergillus flavus and Aspergillus fumigatus. The antimicrobial activities of methanolic extracts of M. indica has shown to exhibit antimicrobial activities at a concentration of 20mg/mL (). Ethano-medical studies indicates that 15 different bioactive compounds extracted from different parts of the i.e. seeds, fruits, resins, exudates, sap are possessing antimicrobial properties ().

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MATERIALS AND METHODS

Stem, bark and root parts of M.indica were obtained from the Forest Reserve of the Michael Okpara University of Agriculture, Umudike, Umuahia, Abia State. The plants were identified by a Plant taxonomist in the Department of Plant Science and Biotechnology, This was to authenticate the botanical identity of the plants by an authority as to validate findings of this research.

The woody parts of Mangifera indica were burnt completely aerobically after sun-drying to reduce its moisture content. The resulting charcoal was further subjected to combustion at elevated temperature in a laboratory electric furnace until the woodash was obtained. The woodash particles were shaken and sieved using a mechanical shaker with different mesh sizes. This was to remove detrital materials and to get the finest particle size of woodash. Sieved woodash particles were suspended in de-ionized sterile water at room temperature and centrifuged at 750 rpm for 3 minutes, to settle fine woodash particles using a laboratory centrifuge. This suspension was further centrifuged at 1000 rpm for 2 minutes to settle fine particle. Purification and fractionation of the woodash samples were done in triplicates after which all fractionate and collected woodash particles were dried at 600C, sterilized by autoclaving at 1210C for 1 hour, cooled and then stored in airtight s sample bottles until required for use (Washington, 1981).

The method of extraction used is the ethanol extraction method. ().

The powdered woodash of M.indica was weighed and 50g of each dispensed in triplicates into clean beakers and 200mL of 70% Ethanol added and allowed to stand for 72 hours. This solution was then filtered on Whatman No. 1 filter paper to get the ethanolic extract.

Exactly 10g of both woodash of M.indica was also placed in triplicates into clean beakers containing 100mL of distilled water, which was allowed to stand for 72 hours at room temperature and filtered using Whatman No.1 Filter paper, to obtain on aqueous extract (Konoman et al., 1992).

The filtered solvents were then placed on a laboratory hot plate and evaporated at 100C to complete dryness. The concentrate of each ethanolic and aqueous extracted woodash sample were kept in a sterile universal bottle, from which quantities were weighed to prepare various concentrations of both the aqueous and ethanol extracts which were used in the biological assay (Eloff, 1998b).Test solution made from both woodash samples of M.indica were prepared with care using well calibrated digital weighing balance. This was also done for the reference standard and control solutions (Ciprofloxacin) so as to ensure accurate potency estimation of the different wood ash samples (Humphrey et al., 1952). Data of colorimetric and spectrophtometric properties of the M.indica woodash concentrations of 1:9 dilutions in water at π=220 were collated.

The following bacterial species Staphylococcus aureus , Staphylococcus epidermidis, Streptococci species and Escherichia coli were isolated from different individuals within the Hostels of Michael Okpara University of Agriculture, Umudike, Umuahia, Abia State. Age, sex and occupation of volunteers were varied to obtain a representative sample. The skin isolates were collected using standard microbiological procedures and precautions. Bacterial samples were obtained by swabbing a given area of the lower arm and leg using a sterile swab stick. The swab stick was removed from its sterile tube and rubbed into the skin surface until; it was saturated with exudates and sweat from the skin surface. The swab stick was then inserted back into the culture tube and labelled appropriately before being transported back to the laboratory. Bacterial species in sampled skin isolates were involved, identified using cultural techniques, microscopy and biochemical methods.

The inoculums used for the agar diffusion assay consist of suspension containing 106 viable cells or CFU/mL from an overnight culture from a stock suspension that was preserved and used throughout the duration of the work (Innocent, 1989). Stock cultures of test microorganisms were maintained aseptically by culturing them in sterilized peptone water and maintained at 100C in refrigerator.

Assay test tubes and Bijou bottles used to establish the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the wood ash samples were sterilized by dry heat at 1500C for 1 hour. Sterile sampling bottles and tubes had sealed and screw caps.

Bacterial isolates were cultured on pre-dried nutrient, blood and MacConkey agar incubated for 16 hours. The agar well diffusion method was adopted as the most appropriate technique for the antimicrobial susceptibility test (AST) considering the range of concentrations of the different woodash samples used to determine the efficacy of the nanomaterials contained in the woodash (Vincent, 1989).

The 16 hours bacterial cultures were washed with diluted sterile physiological saline solution (0.85%) (w/v). To obtain a standard plate count of inoculum, reference was made to the McFarland's standard.

Four wells were made on the inoculated plates using sterile 6mm diameter cork borer at well spaced points on the plate. Space between adjacent wells is at least 30mm and also the nearest Petri-dish edge (Hewitt, 1977). Applications of different concentrations of woodash solution to the agar well were done within 2 minutes to minimize atmospheric contamination.

Three drops of 25mg/mL, 50mg/mL, 100mg/mL and 200mg/mL concentrations of each woodash were aseptically placed into each agar well separately using a sterile hypodermic syringe Inoculated cultures were allowed to stand for 1 hour at room temperature for samples to diffuse across the surface. The plates were then incubated at 370C for 24 hours and 48 hours, respectively.

Equal amounts of ciprofloxacin and sterile water were used as positive and negative controls in each series of independent experiments. Assays were done in triplicates.

The diameter of each zone was measured to the nearest 0.1mm. This was done by viewing the plate illuminated from below against a dark background.

Zones of inhibition were measured from the circumference of the wells to the circumference of the inhibition zones. This is recorded as the difference in the diameter between the wells and the growth free zone around the wells.

The diameters of the zones of inhibition were read after 6 to 8 hours of incubation and the results confirmed by taking readings again after overnight incubation. (Baron et al., 1994).

The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) were determined using the tube dilution method described by Stratton et al, 1990.

STATISTICAL ANALYSIS

Data derived from the antimicrobial susceptibility assays, minimum inhibition concentration (MIC) was analyzed using a one-way analysis of variance (ANOVA) to determine the

difference in the mean diameters of the zone of inhibition and significance statistically (Daniels, 1990).

RESULTS

Based on these susceptibility experiments performed in triplicates with woodash extract from M.indica resulted in a maximum zone of inhibition of 12.01mm in Staphylococcus aureus, 12.69mm in Staphylococcus epidermidis, 19.76mm in Streptococcus spp., and 13.02mm in Escherichia coli (table 1) Positive and negative controls used in the antimicrobial susceptibility assay.

TABLE 1: Mangifera indica woodash extract

Concentration (mg/mL)	S. aureus Mean	S.D	S.epidermidis Mean	S.D	Streptococcus spp Mean	S.D	Escherichia coli Mean	S.D
200	12.01^{c}	1.723	12.69^{b}	1.502	19.76^{c}	1.437	13.02^{bd}	2.01
100	8.95^{b}	0.218	8.36^{a}	3.041	18.56^{bc}	0.835	10.72^{bc}	1.172
50	8.97^{b}	0.800	9.42^{ab}	1.507	15.91^{b}	1.501	9.91^{b}	1.484
25	6.74^{a}	0.468	6.01^{a}	0.990	9.78^{a}	0.641	6.72^{a}	1.825
Control	13.51^{c}	0.510	27.34^{c}	2.086	17.71^{bc}	3.055	15.62^{d}	1.022

Values in the table above are the mean standard deviation of three replicate samples. Values with the same superscripts are not significantly different using Duncan Multiple Range Test (DMRT) at 0.05 significance level.

TABLE 2: Minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) of Mangifera indica woodash extract on test organism

Microorganisms	Concentrations of woodash extract (mg/mL)							
	6.25	12.5	25	50	100	200	ciprofloxacin	Distilled water negative control
Staphylococcus aureus	0.0 ± 0.0	0.75 ± 0.11	6.75 ± 0.05	8.97 ± 0.80	8.95 ± 22	12.01 ± 1.723	13.51 ±10.51	0.00 ± 0.0
Staphylococcus epidermidis	0.0 ± 0.0	0.74=9 ± 0.09	6.01 ± 0.99	9.42 ±1.51	8.36 ± 3.04	12.69 ± 1.50	27.34 ± 2.09	0.0 ± 0.0
Streptococcus spp	0.0 ±0.0	1.24 ± 0.9	9.78 ± 0.64	15.91 ± 1.50	18.56 ± 0.84	19.76 ± 1.44	17.71 ± 3.06	0.0 ± 0.0
Escherichia coli	0.0 ± 0.0	0.81 ± 0.13	6.72 ± 1.83	9.91 ± 1.48	10.72 ± 1.17	13.02 ± 2.01	15.62 ± 1.02	0.0 ± 0.0

TABLE 3: Physical Properties of Ethanolic Woodash Extract of Mangifera indica at 1:9 Dilutions in Water

Concentration (mg/mL)	Absorbance π = 220	Concentration π = 220	Transmittance π = 220	pH	Factor π= 220
200	1.246	1240	005.6	7.45	1000
100	1.214	1220	006.0	7.59	1000
50	0.902	0800	014.5	7.75	1000
25	0.228	0.380	048.8	7.80	1000

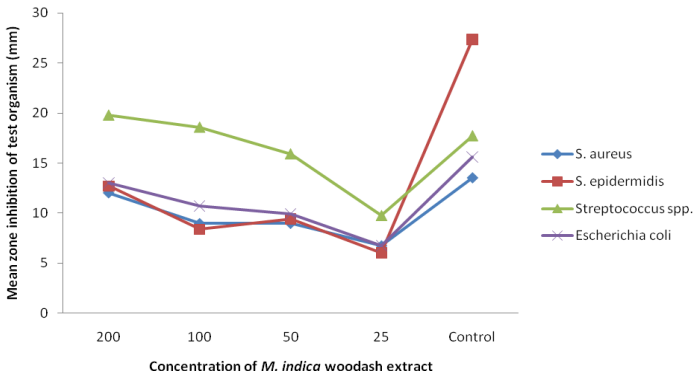

Fig. 1: Graph of mean zone of inhibition of test organisms against concentration of M. indica woodash extract

DISCUSSION

The uniqueness of woodash of M.indica is evident considering its pharmacology and shown to significantly decrease the growth of isolated skin bacteria. However, it's a combination of constituents of the woodash and not a single component of woodash that makes it posses' antibacterial properties may be responsible for the observed inhibitory effects of the woodash extracts. Woodash can affect bacterial metabolism indirectly by altering the physiochemical properties of a specific environment or directly through surface interaction. It was demonstrated that the nano-particle size of M. Indica posses inhibitory activities on all test bacterial species at a given concentration.

Therefore, it is hypothesized that the physical-chemical properties of the ethanolic woodash extract of M.indica at specified concentrations inhibits bacterial growth, by generating an unfavorable environment. Thermal activation and hydration further aided the extraction of critical components that confers antimicrobial properties of woodash extracts of M. indica Reactive oxygen ions and radicals are byproducts of aerobic bacterial metabolism with demonstrated toxic effects on bacteria (ImLay et al 1998). Numerous transition elements which are inherent in woodash in combination with elevated levels of reduced numerous macro elements and microelements in woodash with excessive free radical production in the presence of oxygen could cause oxidative stress and damage to bacterial cells, resulting in death (Aruoma, 2003).

CONCLUSION

This study confirms that the wood ash extract of M. indica have antimicrobial properties, however the result should be further strengthened by fractioning and assessment of constituents and particle size interference with bioactivity it was noticed that woodash extract of M. indica showed different inhibitions on all test bacteria with varying minimum inhibition concentration (MIC) and the minimum bactericidal concentration (MBC) which includes:

S. aureus with MIC =12.5 mg/mL and MBC= 6.25 mg/mL
S. epidermidis with MIC=13.0 mg/mL and MBC=7.0 mg/mL
Streptococcus spp with MIC = 12.5 mg/mL; MBC=6.5 mg/mL and

E.coli with MIC=12.5 and MBC=7.0 mg/mL
These inhibitory activities may be considered sufficient for further studies aimed at isolating and identifying active principles and evaluating possible antibacterial activity (Lampinen, 2005).

REFERENCES

Abrahams, P.W, Parsons J, A. Geophagy in the Tropics: A Literature Review. Geographical Journal (1996)162:67-72

Adjanohoun E., Aliyi M.R.A., Ake-Assi, L. Draman L.K., et.al (1991). Contributions to Ethanobotanical Floristic Studies in Western Nigeria. OAU/STRC, Lagos, Nigeria.

Al-bakri, A.C., Afif, F.U, (2007). Evaluation of Antimicrobial Activity of Selected by Rapid XTL Colorimetry and Bacterial Enumeration. Journal of Microbiological Methods.68:19-25.

Aruoma, O.I, (2003). Methodological Consideration for Characterizing Plants. Mut. Res. 523-524:9-20.

Baron, E.J, LR Peterson, S.Y, Finegold, (1994). Diagnostic Microbiology, Ed 9, Mosby, St.Louis.

Boyer-Joubert, C. Lorthiois, E; Moreau, F., in Annual Reports in Medicinal Chemistry. Doherty, A.M. Ed; Academic Press, Amsterdam, 2003; Vol. 38, pp 347-374.

Brantner, A., Pfeiffer, K.P. Brantner, H. (1994). Applicability of Diffusion Methods Required by the Pharmacopoeias for Testing Antimicrobial Activity of Natural compounds. Pharmazie 49:521-516.

Campbell A.G. (1990). Recycling and Disposing of woodash. Tappi Journal 9:141-145.

Carretero, M.I,. (2002). Clay Minerals and Their Beneficial Effects upon Human Health. A Review of Applied Clay Science 21:155-163.

Cos P, Vlietinick, A.J., Van de Berghe, D., Maes, L (2006). Antinfective Potential of Natural Products: How to Develop a Stronger In Vitro Proof of Concept. Journal of Ethanopharmocology.106: 290-302.

Cragg, G. M, Newman, D.J, Snader, K.M. Journal of Natural Products (1997)

Daniel, W.W., Biostatistics (1987). A Foundation for Analysis in the Health Sciences, 5th Ed. New York John Wiley and Sons Inc. 528-558.

DesForage K.M, Meyers N.L, (1992). Physical Properties of Woodash, p.8. In: Agronomy Abstracts American society of Agronomists, Madison, Wisconsin US.

Eloff, J.N. (1998a). A Sensitive and Quick Microplate Method to Determine the Minimal Inhibitory Concentration of Plant Extracts for Bacteria. Planta medical 64:711-713.

Eloff, J.N. (1998b). Which Extractant Should Be used for the Screening and Isolation of Antimicrobial Components from Plants? Journal of Ethanopharmocology 60:1-8.

Etiagni, L., Campbell, A.G (1991). Physical and Chemical Characteristics Of Woodash. Resources technology 37:173-178.

Etkin, N.L. (2001). Perspective in Ethanopharmocology: Forging A Closer Link Between Bioscience And Traditional Empirical Knowledge. Journal of Ethanopharmocology .76: 177-182.

EUCAST Definitive Document (2000). Terminology Relating To Methods for the Determination of Susceptibility of Bacteria to Antimicrobial Agents. Clinical Microbiology and Infections.6 (9):503-508.

EUCAST Discussion Document (2003). Determination of Minimum Inhibition Concentrations (MICs) Of Antibacterial Agents by Broth Dilution. Clinical Microbiology and Infections.9 (8): 1-3.

Gibbons, S (2004). Anti-staphylococcal Plant Natural Products. Natural Products Report 21:263-277.

Gibbons, S. (2003). An Overview of Plant Extracts as Potential Therapeutics. Expert Opinions Therapeutic Patterns.13 (4): 489-497.

Gottlieb,O.R,(1990). Phytochemical Differentiation and Functions Phytochemistry. 24: 1715-1724.

Grace O.M, Light M.E, Lindsey K.L, Van Staden J, Jager A.K (2002). Anti-bacterial Activity and Isolation of Active Compounds from Fruit of the Traditional African Medicinal Tree, Kigelia africana. South African Journal of Botany 68:220-222.

Hewitt H.W, Vincent, S (2003). Theory and Application of Microbiological Assay. Academic Press (Inc) London, UK

Hewitt W,(1977) Microbiological Assay: An introduction to Quantitative principles and Evaluation New York. Academic Press.

Humphrey, J.H, Lighthorn, J.W.(1952). A General Theory for Plate Assay of Antibiotics with Some Principal Applications. Journal of General Microbiology.7:129.

Hunter J.M. (1973). Geophagy in Africa and in the United States. Geographical Review 63: 170-195.

ImLay J.A. Chin S, M, Linn S,. DNA Damage and Oxygen Radical Toxicity. Science, 1988; 240: 1302-9.

Iwu, M.M, Chiori, C.O. (1984). Antimicrobial Activity of Eupatorium Odoratum extracts. Fitolerapia 6:354-356.

Jager, A.K (2003). Evaluation of Antibacterial Activity of Traditionally Prepared South African Remedies for Infections. South African Journal of Botany 69:595-598

Kavanagh F.W. (1989). Theory And Practice of Microbiological Assaying For antibiotics. J. Assoc. of Anal. Chem. 72:6.

Kohanski MA, Dwyer DJ, Hayete B. A Common Mechanism of Cellular Death Induced By Bactericidal Antibiotics. Cell, 2007; 130: 797-810.

Konoman EW, SD Allen, V.R. Dowell Jr., HM Sommers (Ed) 1992. Color Atlas and Textbook of Diagnostic Microbiology. J.B. Lippincott C. Philadelphia.

Lambert, R.J.W., Pearson, J (2000). Susceptibility Testing Accurate and Reportable Minimum Inhibitory Concentration (MIC) and Non-Inhibitory Concentration (MIC) values. Journal of Applied Microbiology 88.784-790.

Lampinen, J (2005). Continuous Antimicrobial Susceptibility Testing in Drug discovery. Drug plus International.

Larkins N, Wynn S. (2004). Pharmacognosy: Phytomedicines and their Mechanisms. Vet. Clin. North America Small Animal Practice 34(1): 291-327.

Maisbach H.C, and R. Aly (Eds): Skin Microbiology, Relevance To Clinical Infection, Soringer-Verlay, New York.

Marfield, R.P, Blunt J.W, Cardelina II, J.H, MaMahon J.B (1991). Journal of Medical Chemistry, 34. 3402-3405.

Marston A, Hostettman K, (1999). Biological and Chemical Evaluation of Plants Extracts and Subsequent Isolation. Strategy In: Bohlin L, Bruha, J.G (Eds). Bioassay Methods in Natural Products Research and Drug Development, Kluwer Academic Publishers, USA

Mitscher LA, Leu R, Bathala MS, Wu W, Beal JL (1972). Antimicrobial Agents from Higher Plants: Introduction, Rational and Methodology. Lioydia. 35:157-166.

Moellering, R.C, Mandell, G.L. Bennett, J.E. Dolin, R. (2000).Anti-infective Therapy. Volume 1, part 1, Section E, Churchill, Livingstone, England.

Murray PR., EJ Baron, MA Pfaller, FC Tenover, RH Yolken (eds). Manual of Clinical Microbiology, ed. 6, American Society of Microbiology, Washington. D.C.

Ndir, O, Pousset, J.L. (1981). African Medicinal Plants VII: In vitro Testing Of Euphorbia hirta Against Entamoeba histolytica. Plantes Medicinales Phtotherapie. 15:113-125.

Newman D.J, Cragg G.M, O Keefe, B.R. In Modern Biopharmaceuticals: Design, Development and Optimization. Knablein J. Ed, Wiley VCH Weinheim 2005, Vol. 2, pp 451-496.

Newman, D.J, Cragg, C.M (2007). Natural Products as Sources of New Drugs over 25 years. Journal Of Natural Products 70:461-477.

Newman, D.J, Cragg, G.M, Snader, K.M. Journal of Natural Products 2003, 66: 1022-1037.

Newman, D.J. Cragg, G.M. Snader, K.M. Natural Products Report, 2000, 17:215-254.

OAU/STRC (1986). African Pharmacopoeia, Vol. 2, OAU/STRC Lagos, Nigeria.

Omodamiro O. D., Unekwe P. C., Nweke I. N, Jimoh M. A. (2014)
Evaluation of diuretic activity of ethanol extract and its fractions of Agave sisalana
Peak Journal of Pharmacy and Pharmaceutical Sciences, Peak Journals

Phillipson J.O, Nell MJO. New Leads to Treatment of Protozoa Infections Based on Natural Product Molecules. Acta Pharm. Nord 1987:1:131-144.

Potential Antioxidant Actions of Bioactive Components in Food Abrahams, P.W., Parsons J.A. Geophagy in the Tropics: A Literature Review, Geographical Journal (1996) 162:63-72.

Rios, J.I, Recio, M.C (2005). Medicinal Plants and Antimicrobial Activity. Journal of Ethanopharmocology. 100:80-84.

Rojas G, Levaro J, Tontoriello J, Navarro V (2001). Antimicrobial Evaluation of Certain Plants Used in Mexican Traditional Medicine for the Treatment of Respiratory Diseases. Journal of Ethanopharmocology 74, 97-107.

Shah, PM. The Need for New Therapeutic Agents: What is the Pipeline? Clinical Microbiological Infections 2005; 11:36-42.

Sweetman S.C, Martindale. The Complete Drug Reference, 33 ed; The Pharmaceutical Press London, 2002.

Tenover, F.C, Swenson, J.M, O Hara C.M, Stocker, S.A (1995). Ability of Commercial and References Antimicrobial Susceptibility Testing Methods to Detect Vancomycin Resistance in Enterococci. Journal of Clinical Microbiology 33 (6): 15-24-1527.

Valsaraj R, Pushpangadan P, Smitt, UW. Antimicrobial Screening of Selected Medicinal Plants from India. Journal Of Ethanopharmocology 1997; 58:75-83.

Vincent, S (1989). Theory And Application Of Microbiological Assay, edited by W. Hewitt and S. Vincent, San Diego, Academic Press.

Wallace, J.R (2004). Antimicrobial Properties of Plant Secondary Metabolites, Proceeding of the Plant Secondary Metabolites. Proceeding Of The Nutritional Society Of America 63:621-629.

Washington, J.A (1981). Laboratory Procedures In Clinical Microbiology. Springer-Verlag, New York NY.

Wemuth-Wegner L, Kalinowski, H.O. and Jark, H. Plants Medica 61:196-197 (1995).

Yu, V.L, Marigan T.C. Jr, Barriere, S.L (1999). Antimicrobial Therapy and Vaccines. Williams & Wilkins, England.