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ANTIMICROBIAL ACTIVITY OF METHANOL EXTRACTS AND FRACTIONS OF THE LEAF AND STEM BARK OF VITEX DONIANA SWEET (LAMIACEAE)

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ABSTRACT: *The objective of this study was to investigate the antimicrobial activity of leaves and stem bark of Vitex doniana Sweet (Lamiaceae) in vitro on clinical isolates of Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus and Candida albicans. Fresh dried leaves and stem bark of Vitex doniana were extracted by cold maceration which yielded a mucilaginous methanol extract. Fractionation of the crude extract was done with hexane, ethyl acetate, butanol and water in that order. Phytochemical analysis and lethality tests (LD_{50}) were carried out using standard procedures. Antimicrobial activity of the extracts and fractions at 50, 100, 200 and 400 mg/ml were evaluated using the agar well diffusion method. Phytochemical analysis revealed the presence of alkaloids, saponins, tannins, flavonoids, carbohydrates, steroids, cardiac glycosides. Lethality was not observed in the mice even at 5000 mg/kg. Results showed significant ($P < 0.05$) antimicrobial activity as well as a broad spectrum activity. This study therefore supports claims by traditional health practitioners.*

KEYWORDS: Vitex Doniana, Antimicrobial, Bacteriostatic, Phytochemical, Acute Toxicity

INTRODUCTION

The acceptance of traditional medicine as an alternative form of health care, and the development of microbial resistance to the available antibiotics has led researchers to investigate the antimicrobial activity of medicinal plants (Lis-Balchin and Deans, 1996; Maoz and Neeman, 1998; Hammer *et al*., 1999). Antimicrobial susceptibility tests are used to determine which specific antibiotics a particular bacteria or fungus is sensitive to. Antimicrobial susceptibility tests can guide the physician in drug choice and dosage for difficult-to-treat infections (Levinson, 2010). Common methods used in the evaluation of the antibacterial and antifungal activities of plant extracts and essential oils, include the agar diffusion method (paper disc and well), the dilution method (agar and liquid broth) (Yagoub, 2008; Okigbo *et al*., 2009; El-Mahmood, 2009; Aiyegoro *et al*., 2009), and the turbidimetric and impedimetric monitoring of microbial growth (Rìos and Recio, 2005). These methods are simple to carry out under laboratory conditions.

The output from antimicrobial susceptibility testing is either in the form of a zone size (in µg/ml) or MIC, which is the lowest concentration of drug that inhibits the growth of the organism. For certain infections, it may be important to know the concentration of drug that actually kills the organism rather than just inhibiting its growth; this concentration is called the minimal bactericidal concentration (MBC). Bactericidal antibiotics usually have an MBC equal or very similar to the MIC, whereas bacteriostatic antibiotics usually have an MBC significantly higher than the MIC (NCCLS, 1998; Levinson, 2010). It is worthy of note that antimicrobial activity results of the same plant part tested most of the time varied from

researcher to researcher. This is possible because concentration of plant constituents of the same plant organ can vary from one geographical location to another depending on the age of the plant, differences in topographical factors, the nutrient concentrations of the soil, extraction method, as well as method used for antimicrobial study (James, 2012).

Vitex doniana is a tree that is well adapted to the tropical climate but occur in temperate zones (Padamalatha *et al*., 2009; David, 2008). It belongs to the family Lamiaceae and is commonly called black plum, African oak, prune fingerleaf, Vitex (Glew *et al.*, 1997; Aigbokhan, 2014). Various parts of the plant are used in diverse ways both for food and therapeutic purposes. Some of such ailments are commonly caused by regular pathogenic microorganisms and include *Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus* and *Candida albicans*. Against the backdrop of growing drug resistance and increasing search for newer drugs with reduced (or no) side effects and enhanced pharmacological activity, it is pertinent to establish a scientific backing for claimed potency of herbals in disease management.

METHODOLOGY

Plant Collection, Identification and Preparation

The fresh leaves and bark of *Vitex doniana* were collected in the month of June 2015 from Eha-Alumona, Nsukka Local Government Area, Enugu State, Nigeria.

They were identified and authenticated by Mr A.O. Ozioko of the International Centre for Ethnomedicine and Drug Development, InterCEDD. It was also authenticated at the department of Plant Science and Technology, University of Jos, by the taxonomist, Mr. Agyeno Otuwose. A voucher specimen was deposited with a voucher specimen sample Number UJH15000239.

The plant specimens were washed under running tap water to remove soil and extraneous materials, dried in shade or sun (at a low intensity) for 120 hr (Mukherjee, 2002). They were powdered and stored in airtight containers until required for use.

Source of Microorganisms

Pure cultures of clinical isolates of *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Candida albicans were* obtained from the microbiology unit of the Central Diagnostic Laboratory, National Veterinary Research Institute (NVRI), Vom, Jos, Plateau State. These were sub-cultured and re-identified to ensure the purity of the isolates. The inoculum size of each test strain was standardized according to the *National Committee for Clinical Laboratory Standards* (NCCLS, 1998).

Extraction

A 500 g of the powdered leaf and 1000 g of the powdered stem bark were macerated with 80 % methanol: 2.5 and 5 litres respectively. The residue was rinsed and filtered repeatedly with fresh solvents to attain some level of exhaustive extraction; as judged by loss of colour of the filtrate (Sofowora, 2008). The collective filtrate was evaporated to dryness using a rotary vacuum evaporator at a controlled temperature of 40 – 45^{o} C. The extracts were transferred into sterile sample containers and preserved in a refridgerator at 4^{o} C.

Fractionation

For both leaf and stem bark, 100 g each of the crude extract was initially dissolved in a methanol: water (80:20) v/v mixture and sequentially extracted with solvents of increasing polarity starting with n-hexane, followed by chloroform, ethyl acetate, butanol and ended with water as prescribed by Harborne (1998). For each fractionation step, extraction was performed with the solvent until judged by loss of colour of the filtrate. The separated organic layers were concentrated in a rotary evaporator, except for water that was evaporated using a freeze dryer. The dried fractions were weighed and kept in sterile sample containers and preserved in a refridgerator.

Phytochemical Screening

Preliminary qualitative chemical tests were carried out using standard methods (Brain and Turner, 1975; Harborne, 1998; Evans, 2002). Crude extracts and fractions were screened for the presence of alkaloids, saponins, flavonoids, tannins, cardiac glycosides, anthraquinones and carbohydrates.

Acute Toxicity Studies

The Lorke's method (Lorke 1983) and Duffus modules (Duffus, 1993) were used. Administration of extracts was oral using a feeding needle (Hassan *et al*., 2007).

Antimicrobial Assay

The extracts and fractions were evaluated for antimicrobial activity using the agar well diffusion method (Okeke *et al*., 2001). Nutrient agar plates and nutrient broth (Oxoid) were used. 0.5 McFarland turbidity standard (CLSI, 2006) was used to prepare the organisms. 8mm diameter cork borer was used; 2mg/ml Gentamycin and Fluconazol were reference standards for bacteria and *C. albicans*. Extracts and fractions were reconstituted in normal saline to obtain concentrations (mg/ml) of 50, 100, 200, and 400. Incubation was at 37^{o} C for 24 hours. The Inhibition Zone Diameter (IZD) was measured to the nearest mm. The MIC was determined using the turbidity method according to the *National Committee for Clinical Laboratory Standards* NCCLS, (1998). The MBC or MFC was also determined according to standard (NCCLS, 1998).

Statistical Analysis

All values were expressed as mean ± S.E.M. (Standard Error of Mean). Statistical significance was determined using the Kruskal-Wallis test for independent samples and the Wilcoxon paired sample test for related samples. Values with $P < 0.05$ were considered significant.

RESULTS

Table 1: Yield of Extraction and Fractionation

Plant part	Extraction solvent	Yield (g)	Percentage Yield (%)
Leaf	Methanol (80%)	157.15	31.43
	n-hexane	7	7
	Ethyl acetate	13.5	13.5
	Butanol	16	16
	Water	17	17
Stem bark	Methanol (80%)	83.1	8.31
	n-hexane	4.5	4.5
	Ethyl acetate	10.5	10.5
	Butanol	9	9
	Water	31	31

Table 2: Result of Phytochemical Screening

	Leaf					**Stem bark**				
Constituents	CE	HF	EAF	BF	WF	CE	HF	EAF	BF	WF
Alkaloids	+	–	–	–	–	++	–	++	+	+
Saponins	+	–	+	+++	+	+	–	+	++	+
Tannins	+++	–	++	+++	++	++	–	+	+++	+++
Flavonoids	+++	+	+++	+++	++	++	–	+++	+	+
Carbohydrates	++	+	–	++	+	++	+	++	++	++
Steroids	++	+++	–	–	–	++	+++	+	–	–

Key:

- : Absent

+ : Slightly Present

++ : Present

+++ : More present

Table 3: Antimicrobial Effect of Methanol Crude Extract and Fractions of *Vitex doniana* Leaf determined from the Mean of Inhibition Zone Diameter using Agar Well Diffusion Method

ORGANISMS	CONCENTRATIONS (mg/ml)														
	50			100			200			400			Standard (2mg/ml)		
	MCE	BF	EAF	MCE	BF	EAF	MCE	BF	EAF	MCE	BF	EAF	FLUCONAZOL/ GENTAMYCIN		
Candida albicans	0	0	0	0	0	9	0	9.5	11	0	12	13	26	26.5	26
Bacillus subtilis	0	13	20	0	16	22.5	0	18.5	25	10	21	27	30.5	30	30.5
Pseudomonas aeruginosa	0	10.5	15.5	0	14	18.5	0	17	21	0	19	23	32	30.5	31
Escherichia coli	0	0	18	0	11	21	0	13	24	0	15.5	26	30	30	30.5
Staphylococcus aureus	0	13	20	0	16	22	9.5	18	24	14	20.5	25	29	28.5	28

Key:

MCE: Methanol Crude Extract

BF: Butanol Fraction

EAF: Ethyl Acetate Fraction

Diameter of cork borer used: 8mm

Table 4: Antimicrobial Effect of Methanol Crude Extract and Fractions of *Vitex doniana* Stem Bark determined from the Mean of Inhibition Zone Diameter using Agar Well Diffusion Method

ORGANISMS	CONCENTRATIONS (mg/ml)														
	50			100			200			400			Standard (2mg/ml)		
	MCE	BF	EAF	MCE	BF	EAF	MCE	BF	EAF	MCE	BF	EAF	FLUCONAZOL/ GENTAMYCIN		
Candida albicans	0	11	12.5	15.5	15	15	18	18	18	21	21.5	22	25.5	26.5	26
Bacillus subtilis	0	16.5	18.5	0	19	21	14	21	23.5	16.5	23.5	26	31	30	30.5
Pseudomonas aeruginosa	0	15	11	0	17.5	14	12.5	20	17	15	22	20	32.5	30.5	31
Escherichia coli	0	10	17	10.5	13.5	20	15	17	22	20	20	24	30	30	30
Staphylococcus aureus	0	16	18	14	18	20.5	16.5	20.5	23	19	23	25	28.5	28.5	28

Key:

MCE: Methanol Crude Extract

BF: Butanol Fraction

EAF: Ethyl Acetate Fraction

Diameter of cork borer used: 8mm

DISCUSSION

The result of phytochemical analysis agrees with previous analyses ((Arokiyaraj *et al.*, 2009; Agbede and Ibitoye, 2007; Ejikeme and Henrietta, 2010; Adejumo *et al.*, 2013). These indicate a dominance of polar secondary metabolites and negligible steroidal components. An LD_{50} > 5000 mg/kg implies that the plant is safe for consumption. This is particularly interesting as per the search for newer drugs with little or no side effects. The varying degrees of antimicrobial activity may be due to the different solvents adopted for extraction and fractionation (Freiburghausa *et al*., 1996). The ability of the extracts to inhibit the growth of the pathogens might be as a result of the presence of bioactive substances such as alkaloids, saponins, tannins, flavonoids, cardiac glycosides, steroids, etc. (Elujoba, 1996; NCCLS, 1998), which acted in synergy (Elujoba, 1996; NCCLS, 1998; Kilani, 2006). The leaf crude extract was more or less inactive but the fractions exhibited antimicrobial activity. This may be due to antagonism among the phytochemicals in the crude extract, which were separated by fractionation. This result agrees with earlier findings (Ejikeme and Henrietta, 2010; Kilani,

2006) using a different approach. The Plant parts inhibited both gram positive and gram negative bacteria, and pathogenic yeast. *Vitex doniana* Sweet could therefore be said to be a broad spectrum antibiotic plant (Todar, 2005). The Minimum Inhibitory Concentrations and Minimum Bactericidal or Fungicidal Concentrations shows that at least 50 mg/ml of the plant extract and /or fractions is necessary for the observed antimicrobial activity. Statistical analysis showed that the test organisms were generally equally affected in terms of zone diameter of inhibition (IBM SPSS Statistics 21).

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