In-Vitro Evaluation of Stem Bark Extracts of *Daniellia oliveri* (Hutch and Dalz) for Antimicrobial Activity

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Abstract

A comparative study of the antimicrobial properties of ethanol, methanol, acetone, cold and hot water extracts of Daniellia oliveri was carried out. The extracts were assayed by the minimum inhibitory concentration broth microdilution and minimum bactericidal concentration methods before they were submitted to phytochemical screening. Three Gram positive, three Gram negative bacteria and yeast were used in the assay. All the extracts showed varied activity levels against the test microorganisms. Ethanol, methanol and acetone extract had better antimicrobial activity than the aqueous extracts within the concentration range of 6.25 – 100 mg/ml assayed. The activity was more pronounced against the Gram positive and fungal organisms than the Gram negative bacteria. This study shows that ethanol is the best solvent for extracting pharmacologically active compounds from plant materials. Its extract had the lowest bactericidal concentration of 6.25-25 mg/ml on organisms challenged. Results suggest that the D. oliveri has potential for use as pharmaceutical as well as in ethnomedicinal treatment of infections and diseases.

Keywords: *Daniellia oliveri,* Inhibitory concentration, Bactericidal concentration, Antimicrobial activity

Introduction

Plant extracts have been utilized for a variety of purposes since prehistoric time (Jones, 1996). Of all their wide ranging applications, their use in natural therapies and alternative medicine has been most profound and revolutionary (Cordell and Colvard, 2005). Plant extracts are also useful as perfumeries, flavorings, and preservatives of processed and stored food crops as well as the basis for many quality pharmaceuticals (Hammer *et al*., 1999; Smith-Palmer *et al*., 1998). While the antimicrobial properties of several plant extracts in use today have well documented '*in vitro'* activity, many others have few published data (Hili *et al*., 1997). One of such in the latter category is *Daniellia oliveri* Hutch and Dalziel. It is a large savanna plant belonging to the family Caesalpiniaceae and capable of reaching a height of 110ft and a trunk diameter of 4ft (Gilbert, 2000). Traditionally, *D. oliveri* is used in the treatment of breast tumors, vestibule vagina fistula (VVF), swellings and abscesses (Jegede *et al*.,2006) as well as in a variety of genito- urinary tract diseases (GTD), skin ailments (SA) (Raffauf, 1992) and gastrointestinal disorders (GID) (Ahmadu *et al.,* 2007). Moreover, its use as antirheumatic, antiseptic, diuretic as well as expectorant, laxative, purgative, vermifuge, vulnerary and hypotensive agent have been reported (Fleury, 1997).

The medicinal uses of *D. oliveri* is attributable to the presence of oleoresin, a brownish-yellow fluid which is readily expressed when the bark is bruised as deep as the wood (Jegede *et al*., 2006). Oleoresin is also produced in varying amounts in the leaves and trunk of the plant. Modern scientific data have confirmed the medicinal importance of oleoresin; especially its antinociceptive, anti-inflammatory and antioxidant properties (Basille *et al*., 1988; Jegede *et al*., 2006). Unfortunately, there is paucity of information on the antimicrobial qualities of *D. oliveri* despite its application in the treatment of GTD, SA and GID.

In order to contribute to the investigations on the antimicrobial properties of medicinal plants, this work is concerned with the evaluation of the antimicrobial quality of *D. oliveri* against a diverse range of organisms comprising Gram positive and Gram negative bacteria and a yeast. The purpose was to create directly comparable and quantitative antimicrobial data.

Materials and Methods

Organisms and growth conditions: Microorganisms were obtained from the culture collections of the Department of Microbiology, University of Nigeria, Nsukka as well as the Bioresources Development and Conservation Programme (BDCP), Nsukka, Enugu State, Nigeria. Organisms used are as follows: *Staphylococcus aureus NCTC 6571. Bacillus subtilis ATCC 6633, Escherichia coli ATCC 11775, Klebsiella pneumoniae NCTC 11228, Pseudomonas aeruginosa ATCC 10145, Enterococcus faecalis NCTC 8213* and *Candida albicans ATCC 10231.*

Organisms were maintained on Blood Agar (BA) (Oxoid). Cells from 3-4 isolated colonies of each organism were inoculated into 4 ml of Muellar Hinton Broth (Oxoid) (MHB) and incubated with shaking for 2-8 h at 35^0C until the turbidity of the suspension reached or exceeded that of a 0.5 McFarland turbidity standard (approximately $1.5x10^8$ CFU/ml). When required, the turbidity was adjusted to match the McFarland standard by diluting with sterile physiological saline (Oxoid Unipath Ltd.,Hampshire, England; 0.85% w/v Sodium Chloride). The suspensions were further diluted with saline as required.

Plant extracts: The plant materials were obtained from BDCP. They were air-dried at room temperature (28±2^{0}C) and then pulverized in a mill (Christy Lab Mill, Christy and Norris Ltd., Process Engineers, Chelmsford, England). The stem bark extract was prepared by maceration in water as well as in analytical grade ethanol, methanol and acetone. Approximately 50 g of the pulverized plant material were sequentially macerated at room temperature in 300 ml of aqueous (cold and hot) solvent for 18 h in each case and then ethanol, methanol and acetone for 72 h respectively. The macerate was first passed through a double-layered muslin cloth. The supernatant was then filtered through a Whatman No. 1 filter paper before concentrating the filtrate to dryness in a Gallenkamp Rotary Evaporator (England) at 40^{0}C. The residue, a reddish brown, flaky substance with a waxy polish smell was preserved aseptically in an amber bottle at 4^{0}C until required for further use (Gupta *et al*., 1996).

Phytochemical screening: Phytochemical analyses for the major active compounds in the ethanol, methanol, acetone and aqueous extracts of the plant material were carried out according to the methods of Harborne (1973).

Broth microdilution tests: The broth microdilution method followed that approved by the Clinical and Laboratory Standards Institute-CLSI (formerly NCCLS). Polystyrene microtitre trays (Falcon; Becton Dickinson and Co., Lincoln Park, N.J.) with multiple wells were filled with 0.1ml of two-fold serial dilutions of the extracts in 1% dimethylsulfoxide (DMSO) (BDH, Milan Italy) (Hili *et al*.,1997; Nostro *et al*., 2000), a dispersing solvent to achieve a decreasing concentration range of 100-6.25 mg/ml. The inocula suspensions were standardized so that the final concentration of test bacteria was 5x10^6 CFU/ml (i.e. 5x10^5 CFU/0.1ml well); while the yeast was 5x10^5 (5x10^4 CFU/0.1ml well). The diluted inocula were kept in an inocula reservoir. Using a multipoint replicator, (Mast Laboratories Ltd., Liverpool, U.K) the panel was inoculated by submersing the tips of the replicator into the suspension before transferring to the wells containing the extract dilutions. A growth control well (broth plus inoculum) and a sterility control well (broth only) were inoculated on each panel. A loopful of each inoculum suspension was subcultured onto the surface of sterile predried Muellar-Hinton Agar (MHA) plate as a purity check to make certain that the inocula were not contaminated. The inoculated panels and purity plates were incubated at 35^{0}C for 24h for bacteria and 25^{0}C for 48h for the yeast in ambient air incubator. The purity plates were examined to ensure that a pure suspension was tested (any mixed culture required repeat testing). The plant dilution/inoculum mixture formed a brownish suspension which made visual identification of MIC endpoints by turbidity development impossible. Nevertheless, provided there was satisfactory growth in the growth control, no growth in the sterility control and single colony type on the purity plate, the different dilutions were plated out onto MHA agar plates to determine the minimum bactericidal concentration and minimum inhibitory concentration.

Minimum bactericidal concentration (MBC): MBC was determined by performing a colony count on the actual test inoculum by spot inoculating 10 µl of respective dilutions from each well onto MHA medium to determine if the initial inoculum was killed or merely inhibited from multiplying in the broth. The growth control was also sub cultured as purity check. Following 24-48 h incubation, the number of colonies appearing on the subcultured plates was counted and the endpoints defined as follows: the lowest concentration that maintained or reduced inoculum viability was the MIC whereas the MBC was the concentration in which less than 0.1% of the initial inoculum survived. Tests were performed in triplicate (Hammer *et al*., 1996).

Results

Yield of extracts: Table 1 shows the percentage yield of the different extracts after they had been concentrated to dryness. Ethanol, methanol and acetone had the highest yields in that order. Cold water extract had the least yield.

Table 1: Yield of ethanol, methanol, Acetone cold and Hot water extracts of *D. oliveri*

Extraction Solvent	Yield (g)	Yield (%age)
Ethanol	2.8 ± 0.37	5.6
Methanol	2.7 ± 0.68	5.4
Acetone	2.4 ± 0.12	4.8
Cold water	1.60 ± 0.42	3.2
Hot water	1.75 ± 0.17	3.5

Phytochemical screening: The phytochemical analyses of the plant material showed the presence of many phytocompounds known to have activity against diverse genera of microorganisms (Table 2). Steroids and flavonoids were not detected in the methanol and acetone extracts while glycosides were not present in the coldwater extract. Generally, the phytoconstituents were higher in the aqueous extracts than the solvent extracts. Saponins, tannins, protein and alkaloids were present in all the extracts analyzed.

Table 2: Phytochemical constituents of the extracts of *D. oliveri*

Constituent	Ethanol	Methanol	Acetone	ColdH_2O	HotH_2O
Saponins	+	+	+	++	++
Glycosides	+	+	+	-	++
Carbohydrates	+++	++	++	+	-
Tannins	+	+	+	++	+
Protein	++	+	+	++	++
Steroids	+	-	-	++	++
Alkaloids	+	+	+	++	+
Flavonoids	+	-	-	++	++

Key: *- : absent; ++: average, +++: High, +: low*

Minimum inhibitory concentration (MIC): The MIC of the extracts is presented in Table 3. Ethanol, methanol and acetone extracts were active against the test microorganisms at the lowest concentration

Table 3: Minimum inhibitory concentration of the extracts against the seven test microorganisms

Test Organisms	Extracts (mg/ml)				
	Ethanol	Methanol	Acetone	Cold water	Hot water
Staphylococcus aureus	6.25	6.25	6.25	25	25
Bacillus subtilis	6.25	6.25	6.25	50	25
Escherichia coli	12.5	6.25	6.25	50	25
Klebsiella pneumoniae	6.25	6.25	6.25	100	12.5
Pseudomonas aeruginosa	6.25	6.25	6.25	50	50
Enterococcus faecalis	6.25	6.25	6.25	_	12.5
Candida albicans	6.25	6.25	6.25	25	50

Table 4: Minimum bactericidal concentrations (MBC) for the range of plant extracts

Test Organisms	Extracts (mg/ml)				
	Ethanol	Methanol	Acetone	Cold water	Hot water
Staphylococcus aureus	12.5	25	50	100	50
Bacillus subtilis	12.5	25	25	100	50
Escherichia coli	25	50	50	>100	100
Klebsiella pneumoniae	25	50	25	>100	100
Pseudomonas aeruginosa	25	25	50	>100	100
Enterococcus faecalis	6.25	25	12.5	_	100
Candida albicans	12.5	12.5	25	100	50

assayed. The MIC of both aqueous extracts (cold and hot) are comparable except that *E. faecalis* was not sensitive to any concentration of the cold water extract while *K. pneumoniae* was more susceptible to the hot water extract (12.5 mg/ml) than the cold water (100 mg/ml).

Minimum bactericidal concentration (MBC): Ethanol extracts displayed the strongest antibacterial effect of the five extracts assayed (Table 4); cold water extract was the least active. Most of the organisms showed very high sensitivity to low concentrations of ethanol extract in a manner only comparable to much higher concentrations of other extracts. Among the organisms challenged, *S. aureus, B. subtilis, E. faecalis* and *C. albicans* were more sensitive to ethanol extract than were *E. coli*, *K. pneumoniae and P. aeruginosa. E. faecalis* was most sensitive at 6.25 mg/ml. The activities of methanol and acetone extracts were comparable to that recorded in respect of ethanol except that the sensitivity of *K. pneumoniae* and *P. aeruginosa* were identical to the Gram positive organisms and the yeast. *S. aureus, P. aeruginosa and E. coli* were sensitive to the same concentration of acetone extract (50 mg/ml).

The concentration at which the aqueous extracts showed bactericidal effect were higher than the concentrations recorded for the other extracts. At a concentration of 100 mg/ml, the cold water extract killed all the organisms except *E. coli, K. pneumoniae* and *P. aeruginosa* in which bactericidal concentrations were out of the range tested. *E. faecalis* was not sensitive to any of the concentrations of cold water extract. The cold water extract was less active than the hot water extract in all the organisms tested. The bactericidal concentration of the Gram positive bacteria and yeast were identical (50 mg/ml). Gram negative bacteria were killed at 100 mg/ml.

Discussion

The yield of the plant material varied with the solvents used (Table 1). The low yield reported may be due to the maceration method applied in the extraction. Maceration is known to give lower yield of extracts than soxhlet extraction (Ibrahim *et al*., 1997). The higher recoverable yield of ethanol, methanol and acetone may be related to the longer duration of maceration than the aqueous extract. Maceration in organic solvents was carried out over a period of 72 h while the aqueous extraction was terminated after 18 h to forestall onset of microbial decomposition. The yield of the hot water extract was slightly higher than the recorded value obtained from the cold water because secondary plant metabolites are known to be more soluble and dissoluble in hot water than cold water (Okoli *et al*., 2002).

The photochemical analyses confirmed the presence of secondary plant metabolites (Table 2). The concentration of each compound also varied with the extracting solvent. Moreso, the antimicrobial property of plant extracts is closely associated with the presence, absence or interrelationships which occur among phytocompounds (Maffei-facino *et al*., 1990; Hili *et al*., 1997). It is not clear why the aqueous extract performed poorly in the assays despite having extracted the most phytoconstituents compared to other solvents used. It might be that the active principles were more soluble in the organic solvents than the aqueous solvents (Nkere and Iroegbu, 2005) or that the compounds tested were not the ones directly responsible for the reported antimicrobial activity. Oleoresin was not tested.

The ethanol, methanol and acetone extracts were found to exhibit stronger antimicrobial property than the aqueous extracts (Table 3). Even among the solvent extracts, ethanol performed better than methanol and acetone. The concentration at which ethanol showed bactericidal action suggests that there could be basis for use of the product in the treatment of gastro intestinal infection as is traditionally practiced.

Determination of bactericidal concentration is a more sensitive technique for evaluating the antimicrobial property of extracts than the agar well technique (Smith-Palmer *et al*., 1998). The results

of the bactericidal concentrations showed that the Gram positive bacteria and yeast were more sensitive to inhibition than the three Gram negative organisms. The high sensitivity of the Gram positive bacteria to the extracts is shown by a bactericidal concentration of 12.5-25 mg/ml (Table 4). In contrast, concentration of between 25-50 mg/ml would be required to achieve similar inhibition against the Gram negative bacteria. This variation in sensitivity between Gram negative and Gram positive organisms to inhibition by plant extracts have been reported (Tomas-Barberan *et al*., 1990*;* Nostro *et al*., 2000). The exact reason for such variation in sensitivity between Gram positive and Gram negative bacteria is still very much speculative. The most plausible scientific explanation presently ascribes the difference to structural dissimilarities relating to bacterial surface membranes. Gram negative bacteria have an outer phospholipid membrane carrying lipolysaccharide components which makes the cell wall impermeable to lipophilic solvents, while porins constitute a barrier to the hydrophilic solutes (Nikaido and Vaara, 1985). Despite the difference in membrane permeability, the ethanol extract was active against the Gram negative bacteria and had a broader spectrum of activity against all the organisms tested than other extracts. The relatively higher activity spectrum of the ethanol extract is significant because traditional administration of *D. oliveri* and most herbal preparations is often as decoction of locally distilled gin commonly referred to as '*Kai-kai'.* The antifungal properties of the plant assayed were previously unknown. However, according to Tomas-Baberan *et al.* (1990), interactions between lipophilic phytocompounds and certain membrane constituents might be responsible for toxicity of the extract on the only fungal organism tested.

The wide and varied medicinal properties of *D. oliveri* is thought to be attributable to the latex called Oleoresin (Basille *et al*.,1988; Raffaulf,1992; Fleury,1997; Jegede *et al*., 2006; Ahmadu *et al*., 2007). It might also be connected to the antimicrobial activity reported in this study. Establishing the contribution of this component to the overall antimicrobial activity of the extract would require further investigation. Results obtained however might be considered sufficient for further studies directed toward extracting, and purifying oleoresin, and then evaluating its antimicrobial properties as well as possible interactions with other phytocompounds.

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