Evaluation of Anti-Microbial Activity and Proximate Composition of Alkaloids from *Vitex doniana* Seed

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(Received: November 12, 2020; Accepted: May 22, 2021; Published (web): June 20, 2021)

ABSTRACT: The study investigated the *in vitro* anti-microbial activity of crude alkaloids from the seeds of *Vitex doniana* against some selected bacteria and fungal strains. Agar well diffusion method was used to test the anti-microbial activity using ciprofloxacin and ketoconazole as controls. Phytochemical and proximate analyses were carried out by standard methods. The crude extract showed the presence of alkaloids, tannins and resins in abundance. Proximate analysis indicated fat/oil (11±0.73 %), protein (0.26±0.02 %), moisture (0.21±0.01 %), ash (5.0±0.22 %) and carbohydrate (6.42±0.28 %). The antimicrobial study indicated that the crude extract was more effective against *Pseudomonas aeruginosa* (MIC 3.84 mg/ml) and *Salmonella typhi* (MIC 3.84 mg/ml) and other Gram-positive bacteria. The crude alkaloids generally showed lower activity in case of Gram-negative (MIC *Salmonella typhi* 4.20 mg/ml) than in Gram-positive bacteria (MIC *Staphylococcus aureus* 2.52 mg/ml). Surprisingly, the crude alkaloids from the seeds, in addition to improved activity against all the bacteria strains, showed significant activity against *Candida albicans* (MIC 1.18 mg/mL. *V. doniana* seed extract was found to be potent against some clinical strains of both Gram-positive and negative bacteria but not fungi; however, its alkaloids has promising antifungal activity.

Key words: Vitex doniana seed, proximate, anti-microbial, alkaloids, phytochemicals

INTRODUCTION

Phytochemicals derived from plants have shown great potential in the treatment of intractable infectious diseases including opportunistic infections.[1] Certain medicinal plants containing protoberberine and related alkaloids, picralima type indole alkaloids and garcinia of flavanones used in the traditional African system of medicine, have also been found to be active against a wide variety of microorganisms.[1] In the management of diseases, plant materials are known to be present in more than 50 % of western drugs, have provided defense against pathogens or template for synthetic drugs.[2,3] *Vitex doniana* (Verbenaceae), commonly called black plum, is a savanna plant species in wooded grasslands and can also be found along forest edges.[4] The seeds appear singly and traditionally, fresh fruits cannot be stored for long periods. *V. doniana* is used in the treatment of conjunctivitis, skin rashes due to measles, chickenpox, respiratory infections and abdominal disorder and diarrhea.[5-7] Its antioxidant and anti-microbial activities against the methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant Enterococcus (VRE) and multidrug-resistant *Burkholderia cepacia* and *Pseudomonas aeruginosa* are known.[8,9] Several other activities of the stem bark, leaves and fruits of black plum have been documented, however, the enormous phytochemical constituents in *V. doniana* are still underutilized.[10-12] Available data, to the best of our knowledge, showed that the seeds of *V.*

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Dhaka Univ. J. Pharm. Sci. **20**(1): 81-86, 2021 (June)
DOI: https://doi.org/10.3329/dujps.v20i1.54035

doniana is underutilized in folk medicine due to scanty information about its ethnopharmacological relevance, phytochemical and nutritional composition as well as proximate content. To further understand the relevance of this plant in folk medicine, the present study investigated the phytochemical and proximate compositions and then evaluated the anti-microbial activity of the extract and alkaloidal constituents of *V. doniana* seeds.

MATERIALS AND METHODS

Plant material. Ripe *V. doniana* seeds were collected from Nike forest Enugu, Nigeria and identified and authenticated by Mr. Alfred Ozioko at the Department of Plant Science and Biotechnology, University of Nigeria Nsukka. A voucher specimen (UNH 356a) was prepared and stored at the herbarium of the Institute. The seeds were washed thoroughly with distilled water and allowed to dry at room temperature. The dried seeds were pulverized into coarse material.

Extraction. The coarsely pulverized seeds (500 g) was macerated in (1 L) of methanol for 48 h with intermittent agitation. The mixture was filtered and the filtrate was allowed to evaporated *in-vacuo*. The extract (18.8 g) was stored in an airtight container and left at -4ºC until used.

Extraction of alkaloids. An aliquot (5 g) of the extract was dissolved in methanol-water (1:9, 500 ml). It was then rendered alkaline (pH 12) with 1 % v/v of NH_4OH solution. The mixture was successively partitioned with dichloromethane (3 x 500 ml) in a separatory funnel as previously described.[13] The combined dichloromethane soluble extract were evaporated in a rotary thin-film evaporator to obtain the crude alkaloid. The presence of alkaloid-rich extract was confirmed by colour intensity in dragendorff reagent.

Test microorganisms. The test microorganisms were cultures of *Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus subtilis, Enterococcus faecalis, Salmonella typhi, Candida albicans* and *Aspergillus nigger*. They were all of the wild type strains. These were obtained from the Microbiology Unit, Department of Pharmaceutical Microbiology and Biotechnology of the University of Nigeria Nsukka.

Growth media. Molten Meuller Hilton agar (MHA), saboroud dextrose agar (SDA), mannitol salt agar (MSA), nutrient agar and nutrient broth used were prepared according to the manufacturers' directives after which the planted media were tested for sterility at a temperature of 30 °C.

Preparation and standardization of inocula. The test microorganisms were sub-cultured from nutrient agar slants into the sterile nutrient broth. They were incubated at 37 °C for 18 h. Using 4 ml of sterile normal saline, each of the test organisms was standardized to 0.5 McFarland's turbidity standard which is equivalent to $1.5x10^8$ cfu/ml. This was used in the seeding of organisms on the plates.

Sensitivity test of microorganisms. The sensitivity of test microorganisms to the extract and alkaloids of *V. doniana* was determined using the agar well diffusion methods.[14] A 100 mg of extract was solubilized in 1 ml of dimethyl sulphoxide (DMSO). Sterile petri dishes containing 25 ml of solidified MHA and SDA media were appropriately labeled with the test isolates. Each plate was seeded with the corresponding standardized test organism. Using a sterile 6 mm cork borer, five (5) wells were bored in the seeded plates and 100 µL of each of the five concentrations (100.0, 50.0, 25.0, 12.5, and 6.25 mg/ml) of the extract was introduced (dropwise) into the respective wells. The plates were left for 20 minutes to allow for diffusion of extracts before they were incubated at 37°C for 24 h for bacteria and 25°C for 48 h for fungi. Then the inhibition zone diameters (IZD) of the test strains sensitive to the extracts were measured. Similar procedures were carried out using standard drugs (ciprofloxacin and ketoconazole) at concentration (10-0.3125 mg/ml), alkaloids (50-1.5625 mg/ml) and dimethyl sulphoxide (100 µl) in place of the plant crude extract.

Determination of minimum inhibitory concentration (MIC). The MICs of the crude

extract, alkaloids and the control agents determined using the agar well diffusion method were obtained by a plot of the logarithm of the concentration of the extracts/controls against the squared IZD (mm^2). The antilogarithm of the intercept (I) on the log concentration-axis was used to calculate the MICs.[14]

Phytochemical analysis of the extract. The qualitative phytochemical composition of the extract was determined using standard methods.[1,8]

Proximate analysis. The proximate analysis was carried on the different seed harvested at various time intervals to determine the effect of time of harvest on the nutrient composition of seeds. The harvested seeds were air-dried in the greenhouse for 21 days, ground into a powder and stored in an airtight container till the required analysis. Proximate composition of seeds was carried out to determine the ash content, total carbohydrate, crude protein content, crude fat content, crude fiber and moisture content. The analysis was carried out using the standard method of the Association of Official Analytical Chemist.[15]

RESULTS and DISCUSSION

The results of the phytochemical analysis showed the presence of secondary metabolites. The crude extract showed the presence of alkaloids, tannins and resins in high concentration (Table 1). The carbohydrates, reducing sugar, flavonoids, proteins, oil and terpenoids were found in medium concentration while glycosides, saponins, steroids and acidic compounds were observed in relatively smaller concentration. The proximate studies indicated the presence of fat/oil, protein, moisture, carbohydrate and ash with a high percentage of fat and oil. Fibre was, however, absent in the extract (Table 2) and the proximate composition was found to be independent of season or time of collection.

Table 1. Phytochemical components of *V. doniana* extract.

S/No	Phytochemicals	Relative abundance
1	Carbohydrates	++
2	Reducing sugars	++
3	Alkaloids	+++
4	Saponins	+
5	Tannins	+++
6	Flavonoids	++
7	Oil	++
8	Terpenoids	++
9	Glycosides	+
10	Steroids	+
11	Acidic compounds	+

+ Low, ++ Medium, +++ High

Table 2. Proximate composition of *V. doniana* seed.

	Protein	Fibre	Moisture	Ash	Fat/oil	Soluble sugar	Carbohydrate
aVD	0.26±0.02	0.00	0.21±0.01	5.00±0.22	11.0±0.73	6.42±0.28	Nd
bVDr	7.28	6.73	48.77	5.70	3.0	Nd	28.95
cVDr	0.85±0.09	11.48±0.55	39.42±0.72	3.41±0.09	2.44±0.06	13.55±0.49	29.57±0.67
cVDw	2.32±0.22	11.82±0.25	38.16±0.71	3.63±0.08	2.46±0.09	9.88±0.12	31.71±0.81
cVKr	0.87±0.05	10.42±0.47	40.56±0.77	3.40±0.13	2.35±0.06	14.45±0.50	28.04±0.56
cVKw	2.24±0.28	10.35±0.33	40.86±0.39	3.34±0.41	2.07±0.10	10.44±0.61	31.56±0.55
cVFr	0.98±0.06	12.38±0.43	37.74±0.76	3.66±0.26	2.66±0.09	15.39±0.44	27.28±0.35
cVFw	2.78±0.22	12.39±0.53	38.46±0.92	3.31±0,52	2.35±0.09	9.53±0.07	31.30±0.40
dVDr	8.24±0.24	0.58±0.08	16.66±1.06	11.5±1.1	34.62±0.56	Nd	28.4±1.06
eVPvl	13.73	28	16.85	8.00	7.00	Nd	26.43
fVNl	16.94	33.18	16.95	9.37	1.8	Nd	21.76
gVNl	12.2-15.2	25.5-30.5	15-18.7	7.5-8.5	5.0-9.0	Nd	7.5-10.57
hVNl	0.87±0.01	11.61±0.14	53.45±0.15	8.07±0.17	7.05±0.03	Nd	18.95±0.05
iVDl	8.75	15.58	8.04	7.92	5.10	Nd	70.20
jVPl	3.4±0.07	60±0.4	240±20	5.3±1.0	0.44±0.05	Nd	30±0.3

VD (*V. doniana*), VK (*V. kiniensis*), VF (*V. fischerii*), VPv (*V. parviflora*), VN (*V. negundo*), VP (*V. payos*) a(this study), b[12], c[16], d[17], e[18], f[19], g[20], h[21], i[22], j[23], r (ripe fruits), w (raw fruits), Nd (no data). L (leaves). Values are expressed as mean ± SD (an= 3, others = not stated).

The results of the susceptibility test of the extract and alkaloids of *V. doniana* are shown in Table 3. The extracts, at varying concentrations, inhibited the growth of *B. subtilis, S. aureus, E. faecalis, P. aeruginosa* and *S. typhi*. The extract has no activity against the strains of *C. albicans* and *A. niger*. The alkaloids showed improved activity against all the bacteria tested and *C. albicans.* The minimum inhibitory concentrations (MICs) of the crude extract and standard drugs against some clinical strains are shown in Table 4 which was obtained from the graphs of log concentration of extract/drugs against squared IZD. The MIC values indicated that both the extract and alkaloids were less active against all the microorganisms tested compared to standard drugs. The order of sensitivity of the microorganisms to the extract and alkaloids showed that *B. subtilis* and *S. typhi* were respectively the least active while no activity was observed against *A. niger* in both cases.

Table 3. Susceptibility test of extract and alkaloids of *V. doniana*.

Microorganism / IZD (mm)	Extract (mg/ml)						Alkaloids (mg/ml)						Controls (mg/ml)					
	100	50	25	12.5	6.25	3.13	50	25	12.5	6.25	3.13	1.56	10.0	5.0	2.5	1.25	0.63	0.31
B. subtilis	17.7	7.7	7.0	6.0	6.0	6.0	16.5	12.4	10.2	9.0	8.5	7.8	36.3	30.3	28.0	26.3	22.3	17.3
S. aureus	16.0	9.0	8.3	7.7	6.6	6.0	14.4	10.0	9.0	8.4	7.2	6.0	32.7	28.0	23.7	20.0	6.0	6.0
E. faecalis	21.3	17	12.3	6.0	6.0	6.0	14.0	11.0	9.8	9.2	7.8	6.2	28.0	24.0	21.7	19.3	16.0	6.0
P. aeruginosa	10.6	8.3	7.7	7.3	6.0	6.0	18.4	14.4	12.0	11.0	8.0	7.5	36.3	31.0	26.3	21.0	6.0	6.0
S. typhi	10.6	8.3	7.7	7.3	6.0	6.0	25.3	11.0	10.0	9.5	8.0	6.5	26.0	23.0	17.7	15.3	6.0	6.0
C. albicans	6.0	6.0	6.0	6.0	6.0	6.0	10.8	8.3	7.0	6.0	6.0	6.0	36.7	31.0	26.3	23.0	19.0	16.3
A. niger	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	22.7	19.7	18.7	17.3	14.0	12.0

IZD of 6.0 means no inhibition; IZD (mm) includes the diameter of hole borer (6 mm); ciprofloxacin and ketoconazole served as standards for antibacterial and antifungal tests, respectively.

Table 4. MIC of extract and alkaloids of *V. doniana* against some microorganisms.

Microorganism	Extract		Crude alkaloids		Controls	
	Log I	MIC (mg/ml)	Log I	MIC (mg/ml)	Log I	MIC (mg/ml)
B. subtilis	1.4495	28.15	0.1641	1.46	-0.9245	0.12
S. aureus	0.9493	8.90	0.4476	2.52	-0.4000	0.40
E. faecalis	1.1073	12.80	0.0226	1.05	-0.6467	0.22
P. aeruginosa	0.5841	3.84	0.1693	1.48	-0.3936	0.40
S. typhi	0.5841	3.84	0.6228	4.20	-0.3976	0.40
C. albicans	-	-	0.0723	1.18	-0.6903	0.20
A. niger	-	-	-	-	-1.0578	0.09

Log I=c from the regression y=mx+c of log concentration against IZD^2 of Table 3 (Figures not shown for brevity).

Susceptibility tests are used to describe the array of microorganisms in which particular agents can inhibit their growth, reduce their virulence, or eliminate from the host system. The extracts of *V. doniana* inhibited the growth of both Gram-positive and Gram-negative bacteria as shown by their various zones of inhibition diameters (IZDs) and MICs. However, the extract could not inhibit the growth of fungi used in our study. This showed a broad spectrum of activity against bacterial pathogens which was concentration-dependent. The alkaloids, however, showed a surprisingly strong activity against *C. albicans.*

The minimum inhibition concentration of the extract against bacteria showed varied activities which were lowest against *B. subtilis* and highest against both *S. typhi* and *P. aeruginosa*. The alkaloids showed broad spectrum of activity against Gram-negative and Gram-positive pathogens as well as *C. albicans*. The slight differences in the antimicrobial activity of the *V. doniana* alkaloids against Gram-negative and positive bacteria could be explained based on differences in the structural architect of bacteria. Gram-positive bacteria possess a thicker peptidoglycan layer and lipoteichoic acid barrier unlike Gram-negative which have thin peptidoglycan with an outer membrane containing liposaccharides acting as endotoxins.[24] The lipoteichoic acid, acting as exotoxins to invading agents, could have neutralized the alkaloids which are basic thus reducing the antimicrobial activities. The thick peptidoglycan layer could also block penetration thereby making resistance to physical disruption by external agents higher.[25] The high activity of the extract against *S. typhi* (a gastrointestinal pathogen) also supports the use of the fruit and seed as food in Africa. Previous studies had demonstrated the antimicrobial activities of alkaloids of plant origin, in some cases, the antimicrobial activity was attributed to *Vitex* species.[26,27] These phytochemical constituents of plants are known to exhibit medicinal activity as well as physiological activity.[27] The improved activity in crude alkaloids could be attributed to the presence of inhibiting or complexing substances present in the extract which probably were removed during alkaloid extraction.

The results obtained from the proximate analysis of *V. doniana* established that it can be ranked as oil-rich seeds due to their relatively high oil content when compared with the other components of the seeds. The low moisture content of the seeds would hinder the growth of micro-organisms and the storage life would be high.[28] The proximate content of the seeds did not compare favourably with other species of *Vitex*, however, its consumption as food could be implicated in certain cardiovascular disorders such as atherosclerosis, cancer and aging because a diet providing 1-2 % of its caloric of energy from fat is said to be sufficient to human.[16-23,29] The crude fibre content of zero (0.0 %) for the seeds cannot be compared to other *Vitex* species that could be beneficial in some disease conditions such as obesity, diabetes, cancer and gastrointestinal disorders.[12,30] The variation in composition, even within the same morphological parts of the same *Vitex* species could be attributed to the geographical location of the plants.

CONCLUSION

V. doniana seed extract proved to be potent against some Gram-positive and negative microorganisms and *C. albicans* but not *A. niger*. Most importantly, the susceptibility patterns of the tested microorganisms showed the presence of broad-spectrum antimicrobial agents. This research has shown that the seed, seldom used in herbal practices, has medicinal properties when consumed as food due to its proximate and phytochemical composition.

DECLARATION

We (authors) declare that the work described in this manuscript was carried out by us and the contents of the paper have not been published before or submitted elsewhere for publication. Theophils Udeani and Chigozie Okwuosa developed the concept and supervised the research. Linus Ugwu conducted the benchwork. Charles Nnadi collected and analyzed the data. All the authors were involved in the preparation of the manuscript for publication.

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