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Reginald Chukwuemeka Njokuocha
Department of Plant Science and Biotechnology, University of Nigeria, Nsukka, Enugu State, Nigeria

Corresponding Author:
Reginald Chukwuemeka Njokuocha
Department of Plant Science and Biotechnology, University of Nigeria, Nsukka, Enugu State, Nigeria

Evaluation of *in vitro* antimicrobial properties of tannins isolated from the leaves of *Vitex doniana* Sweet. (Verbenaceae)

Reginald Chukwuemeka Njokuocha

Abstract
Introduction: *Vitex donniana* Sweet is a medicinal plant used in traditional medicine in Nigeria for the treatment of many infections and ailments. The therapeutic effects have been attributed to high content of phenolic compounds such as tannins.
Objective: To evaluate the antimicrobial effect of the tannins leaf extract against isolates of *Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli Salmonela typhi* and *Candida albicans.*
Materials and Methods: The tannins content of the leaves was extracted by solvent extraction procedure and tannins confirmed using lead sub-acetate, bromine water and ferric chloride tests. Two-fold serial dilutions of 5mg/ml, 2.5mg/ml, 1.25mg/ml, 0.625mg/ml and 0.3125mg/ml were made. The antimicrobial assay was determined using the agar well diffusion method. The inhibitory effect was determined through diameter of inhibition zone.
Results: The results showed that the minimum inhibitory effect against all the microbial isolates occurred at 2.5mg/ml concentration. The inhibitory effect was found to be dose dependent. The most sensitive microorganisms were *Salmonella typhi* and *Candida albicans* at concentration of 0.3125mg/ml and the most resistant microbial isolate was *Staphilococcus aureus.*
Conclusion: The results showed that the tannins leaf extract of *Vitex doniana* has remarkable antimicrobial property. This confirms that the tannin constituent of the plant plays important role in its antimicrobial property.

Keywords: Antimicrobial, tannins, microbials, serial dilutions, inhibitory effect

Introduction
Plants contain numerous secondary metabolites such as tannins, most of which exert not only antimicrobial activity, but promote healing of wounds, formation of new tissues as well as used in the treatment of ulcers and diabetes among other ailments [1, 2]. The antibacterial and fungal activities exhibited by the seed and fruit extracts of pomegranate (*Punica granata* L.) were attributed to phenols, tannins and flavonoid which constituted the major phytochemical constituents of the plant materials [3]. Tannins are water-soluble natural polyphenolic compounds and are among the most occurring phytochemical constituents commonly found in plants. They have been reported to constitute about 20-60% of the composition of bark and leaves of many woody plant species [4].
Apart from the use of tannins in the leather and wine industries, tannin rich extracts have been reported to have antiviral potential, antibacterial and antiparasitic effects [5-9]. Numerous studies have shown that naturally derived tannins from plants have antimicrobial activity against many genera of microorganisms belonging to fungi, yeast and bacteria [10]. They have also been reported to be bacteriostatic or bactericidal against some micro-organisms [11]. Many bioactive properties and antibacterial enhancing principles have been associated with tannins and flavonoids of plants [6]. Tannic acid has been reported as a possible useful adjuvant agent for the treatment of *Staphylococcus aureus* skin infections in addition to β-lactam antibiotics at least under *in vivo* conditions without blood [12]. Antimicrobial activity has been demonstrated by tannins extracted from the leaves of *Psidium guajava* against bacterial (*Pseudomonas aureginosa, Escherichia coli, Staphylococcus aureus*) and fungal (*Candida albicans, Aspergillus niger*) isolates [13]. The tannins isolated from *Dichostachys cinerea* showed remarkable bactericidal activity against bacterial pathogens [14].
Studies indicate that source and concentration are important factors that influence antimicrobial activity of tannins. In their study Min *et al.* [6] found that purified tannin extracts from tannin-containing perennial plants exhibited a range of antimicrobial activity against isolates of *Klebsiella pneumonia, Staphylococcus aureus* and *Escherichia coli.* The susceptibility of microbial pathogens to tannin rich plant extracts depends on many variables

such as the nature and thickness of the cell wall/cell membrane of the organism [15], as well as the source, composition and concentration of the tannins [6].

Vitex doniana Sweet. commonly known as black plum is widely distributed in south western and middle belt of Nigeria. It is a medium sized deciduous tree belonging to the Family Verbenaceae and is found in coastal, woodlands, savanna, riverine and lowland forests as well as deciduous woodlands extending as high as in upland grassland [16]. It grows up to 15-20m, with dense crown and the bark is light grey with vertical fissures. Leaves are long stalked with 5-7 leaf-lets usually broadest at the tip. Flowers white, tinged purple, borne on short, stout axillary cymes on a long stalk with the calyx and pedicels densely hairy. The fruit is oblong to ellipsoidal, up to 2.5cm long, green when young and turns black on ripening [17]. According to Agbede and Ibitoye [18], the fruit is rich in nitrogen, calcium, magnesium, sodium, phosphorus, potassium and has a ratio of K/Na of 8.8 as well as tannins. The fruits are also rich in vitamins A, B, C and E. The fruits and the young tender leaves are consumed as edible vegetables. The stem bark, root and leaves are used in folklore medicine for the treatment of gastroenteritis, wound, diarrhea, dysentery, malaria, gonorrhea, leprosy, kidney issues, liver disease, as antihemorrhage after child birth and jaundice among others [19, 20].

The leaf extract of *Vitex doniana* not only demonstrated antimicrobial activity against spectrum of bacterial and fungal pathogens [21, 22], the phytochemical constituents have revealed the presence of bioactive compounds such as alkaloids, tannins, flavonoids, saponins and anthraquinone which have been reported to have antimicrobial activities [23, 24]. The aqueous and methanolic extracts of leaves, root and stem-bark of *Vitex doniana* have shown antimalarial properties against chloroquine resistant *Plasmodium berghei* (NK 65) [20]. To my knowledge and literature search, there are no previous reports about antimicrobial activity of tannins extracted from the leaves of *Vitex doniana*. Therefore, the aim of this study is to assess the *in vitro* antibacterial and antifungal activities of tannins leaf extract of *Vitex doniana* against *Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli Salmonela typhi* and *Candida albicans*.

Materials and Methods

The twigs of *Vitex doniana* were collected from Nkpologu along Adada River, Uzo-Uwani Local Government Area, Enugu State, Nigeria and the plant was authenticated in the Herbarium Unit, Department of Botany, University of Nigeria, Nsukka. The leaves were dried properly for two weeks and pulverized into powder with an electric mill (Thomas Wiley Laboratory Mill, Model 4) in the Department of Crop Science, University of Nigeria, Nsukka.

Method of extraction of tannins

Two hundred grams of the pulverized leaves were soaked in 10 ml of petroleum ether for 24 hrs. The mixture was filtered with Whatman No. 1 filter paper and the residue washed again with five 20 ml portions of petroleum ether. Ten percent lead acetate was added to the filtrate and heated in a water bath to about 75 ^{0}C and left until precipitates containing lead acetate coagulates appeared. It was then centrifuged at 2000 revolutions per minute and the filtrate discarded. Five drops of sulphuric acid were added to the precipitate and mixed thoroughly and 30 ml of water was added, stirred and centrifuged to obtain tannin [25].

Confirmation tests for tannins

One mg/ml of the isolated tannins was dissolved in 5 ml of ethanol and the solution was used for the following tests [26]:

1. **Lead sub-acatate test:** Three drops of lead acetate solution were added to 1 ml of the test solution and observed for formation in precipitate.
2. Bromine water tes**t:** Half (0.5 ml) a ml of bromine water was added to 1 ml of the test solution and observed for formation of precipitate.
3. **Ferric chloride test:** Two% Ferric chloride was added to 1 ml of the test solution and observed for colour formation.

Microorganisms and culture medium

Four clinical bacterial isolates and one fungal isolate used for the study were obtained from Medical Laboratory Clinic, Faculty of Veterinary Medicine, University of Nigeria, Nsukka. The bacterial isolates were *Pseudomonas aeruginosa, Staphylococcus aureus, Salmonella typhi* and *Escherichia coli*, while the fungal isolate was *Candida albicans*. The microorganisms were cultured on nutrient agar medium at 4 ^{0}C. The culture was prepared by aseptically streaking a loop full from suspension of each of the isolated microorganisms on the surface of solid agar media in different petri dishes and incubated at 37 ^{0}C for 24 hrs. A colony was aseptically transferred from the growth medium and incubated in a prepared double strength nutrient broth in bijou bottles and incubated at 37 ^{0}C for 24 hrs. The content was then used to flood the surface of solid nutrient agar slant in test tube bottles. Each microorganism was bulked in sterilized test tube and made up to 10 ml with sterile normal saline and stored at 4 ^{0}C until needed [26].

Antimicrobial assay

Agar well diffusion method was used for determination of the antimicrobial activity [27]. Standard solutions of the extracts were prepared with Dimethylsulfoxide (DMSO). Two-fold serial dilutions of the extracts were prepared to obtain 5.0 mg/ml, 2.5 mg/ml, 1.25 mg/ml, 0.625 mg/ml and 0.3125 mg/ml [28]. All the microorganisms were suspended in a normal sterile saline solution to a concentration of 1.0 x 10^5 cfu /ml. Ten milliliter of molten nutrient agar at 40 ^{0}C was inoculated with 0.1 ml of each of the suspension of microorganism and poured into sterile petri dishes; mixed thoroughly and allowed to set. Five wells (7 mm in diameter) were bored on each of the seeded agar medium with sterile cork borer and 0.5 ml of each of the extract delusions were transferred into the cups. The inoculated petri dishes were incubated at 37 ^{0}C for 24 hrs. The tests were replicated three times. Positive control was done with micro ring GBMTS-NEG standard sensitivity disc containing eight drugs: Augmentin, Tetracycline, Amoxicillin, Co-trimoxazole, Nitrofurantoin, Gentamycin, Nalidixic acid and Ofloxacin. Antimicrobial activity was evaluated by measuring the diameter of inhibition zone (DIZ) of tested microorganisms.

Results and Discussion

The antimicrobial assay of tannins leaf extracts of *Vitex doniana* as confirmed in Table 1 showed considerable spectrum of bio-activity against all the test pathogens, although there were variations in the effect elicited by the different concentrations. This compares with the effects of standard antibiotics especially that of ofloxacin against all the bacterial isolates. The results showed that inhibitory activity was highest against *Salmonella typhi* and *Candida albicans* at

concentration of 0.3125 mg/ml and moderately active against other bacteria isolates (Table 1). This antimicrobial activity exhibited by the tannins extract has been attributed to is astringent property and formation of complexation with metal ions accounting for its toxicity on membrane of the microorganisms [12]. The bioactive property shown by the tannins extract is comparable to the findings of Ngoupayo *et al*. [11] in which condensed tannin extract from the stem bark of *Erythrophleum guineensis* exhibited bactericidal and fungicidal effects against microbial isolates known to cause food poisoning and acute infectious diarrhea especially *Staphilococcus aureus, Candida albicans* and *Candida parapsilosis*.

Also, Zhu *et al.* [10] evaluating the activity of tannins on *Penicillium digitatum* on stored *Citrus* fruit revealed that the tannins not only inhibited the mycelial growth and spore germination, it reduced the disease severity of green mold of the Citrus by 70%. In another study on the antimicrobial activities of hydrolysable and condensed tannins extracted from the bark of *Rhizophora apiculata*, the findings showed that condensed tannins had outstanding inhibitory effect on the bacterial and yeast isolates, but not on the fungi [29]. It was suggested that the hydrolysable tannins must have affected certain stages in the biosynthesis during the syntheses of cell wall and cell membrane leading to abnormalities and changes in permeability of the cell membranes of the microbial pathogens [19, 30]. These reports can be strongly related to the findings in this study in which the bacterial and fungal isolates; *Candida albicans* and *Salmonella typhi* were highly susceptible to the different concentrations of the tannins extract with ZID in the range of 7.1 ±0.96 – 18.1 ± 0.58 mg/ml for *Salmonella typhi* and ZID of 8.1 ± 0.06 – 21.0 ± 0.36 mm and MIC 0.3125 – 5 mg/ml for *Candida albicans* in increasing order of concentration (Tables 2 and 3).

Table 1: Results of the confirmatory test of tannins extracted from the leaves of *Vitex doniana*

Type of test	Procedure	Results	Reference
Lead sub-acetate test	3 drops of lead acetate solution + 1 ml of test solution	A gelatinous precipitate formed	Harbone [26] and Maker and Goochild [25]
Bromine water test	0.5 ml of Bromine water + 1 ml of test solution	A pale brown precipitate formed	,,
Ferric chloride test	2% of Ferric chloride + 1 ml of test solution	A blue precipitate which turns green on addition of more Ferric chloride	,,

The inhibitory effect of the tannins extract was also found to be dose dependent with 2.5 mg/ml and 5.0 mg/ml concentrations having remarkable effects on all the bacteria and fungus isolates (Table 2). This agrees with the findings that source and concentration of tannins are important factors that influence antimicrobial activity [6, 14]. Quite a number of studies have shown that tannin rich extracts of plants have demonstrated notable antimicrobial activity against diversity of microorganisms [23, 24, 31]. Evaluation of the antibacterial activities of tannin extracts of roots of *Dichrostachys cinerea* [14], and leaves of *Anacardium humile* [32] have shown wide range of antimicrobial activities against a number of clinical pathogens. In comparable studies on the antimicrobial properties of tannin extracts of plants, Ukoha *et al*. [33] working on pods of *Samanaea saman* and Dahham *et al*. [3] on seeds and fruits of *Punica granatum* demonstrated the ability of tannins to show inhibitory activity on both clinical isolates of bacteria and fungi in their investigations. This compares favourably with the findings of this work in which the tannin extract from *Vitex doniana* showed inhibitory activity against the bacterial and fungal isolates.

Table 2: Effects of different dilutions of tannins extract from *Vitex doniana* leaves on the bacterial and fungal cultures

Concentration (mg/ml)	Mean of diameter of inhibition zone (mm ± SD)				
	Pseudomonas aeruginosa	***Staphylococcus aureus***	***Escherichia coli***	***Salmonella typhi***	***Candida albicans***
0.3125	0	0	0	7.1 ± 0.96	8.1 ± 0.06
0.625	0	0	0	8.1 ± 0.95	12.1 ± 0.72
1.25	8.1 ± 0.53	0	10.1 ± 0.39	12 ± 0.67	19.0 ± 0.54
2.5	18.1 ± 0.88	16.3 ± 0.85	12.1 ± 0.58	13.0 ± 0.83	20.1 ± 0.31
5.0	20.0 ± 0.40	21.4 ± 0.26	19.1 ± 0.47	18.1 ± 0.58	21.0 ± 0.36
Control					
Augmentin	0	0	0	0	0
Tetracycline	0	0	0	11	0
Amoxicillin	0	0	0	0	0
Co-trimoxazole	0	0	18	0	0
Nitrofurantoin	0	23	20	8	0
Gentamicin	20	0	0	22	0
Nalidixic acid	0	0	0	27	0
Ofloxacin	28	15	24	38	0

Table 3: Minimum inhibitory concentrations (MIC) and diameter of inhibition zone of tannins extract from *Vitex doniana* leaves

Microorganism	MIC (mg/ml)	DIZ (mm ± SD)
Pseudomonas aeruginosa	1.25	8.1 ± 0.53
Staphylococcus aureus	2.5	16.3 ± 0.85
Escherichia coli	1.25	10.1 ± 0.39
Salmonella typhi	0.3125	7.1 ± 0.96
Candida albicans	0.3125	8.1 ± 0.06

Of the bacterial isolates in the study, the gram-negative bacteria; *Salmonella typhi, Pseudomonas aeruginosa* and *Escherichia coli* were more susceptible to the tannins extract. However, this result contrasts with that of Maistta *et al*. [34]

where the aqueous and ethanolic extract of gallotannins (especially 1-O-galloyl-β-D-glucose) profiled from *Cytinus hypocistis* and *Cytinus ruber* showed strong inhibitory activity against three gram-positive bacteria (*Staphylococcus aureus, Staphylococcus epidermis, Enterococcus faecium*), but not active against gram-negative strains (*Pseaudomonas aeruginosa, Klebsiella pneumoniae*). The variation in inhibitory activity against the gram negative and gram-positive bacterial isolates may be due to differences in their cell surface structures.

It has been reported that the outer membrane of gram-negative bacteria acts as a protective barrier against hydrophobic compounds [35]. Such preventive barrier may be responsible for the differences observed in the effects of the tannins extract on bacterial isolates. The fungal pathogen, *Candida albicans* was susceptible to all the concentrations of the tannins extract. Comparative reports have been reported of the antifungal effects of tannins extract from other plants [3, 36, 37]. Several explanations have been given on the mode of action of plant extracts on bacterial pathogens. It has been reported that plant extracts significantly affect the cell membrane of Gram-positive and Gram-negative bacteria by causing a decline in their internal pH and membrane hyperpolarization [38]. The inhibitory effects of the plant extracts have also been attributed to the tannin content of the plant extracts that permit them to react with protein of microbial cell membrane and mitochondria upsetting their structures and changing their permeability [39, 40]. Such inhibition has also been linked to the destabilization of cytoplasm and plasma membranes, inhibition of extracellular microbial enzymes and metabolisms leading to deprivation of the substrate required for microbial growth [35, 41].

With respect to the mechanism of action of plant tannins, it forms complexation with proteins and other macromolecules through hydrogen and hydrophobic interactions as well as by covalent bonding [6, 42]. Also, the action of tannin can be linked to the damage of the cell wall and plasma membrane leading to leakage of the intracellular contents. The consequent effect of such antimicrobial interaction will be associated with the capacity of tannins to deactivate microbial adhesions, enzymes, cell envelop transport proteins and mineral uptake [43] as well as precipitation of proteins and chelating to mineral elements making them biologically unavailable to the microorganisms [10]. With respect to fungal inhibition, tannins notably minimizes fungal enzymatic activities of cellulases and pectinases and through that means reduce their concentration and rate of spread of infection [44].

Conclusion

This study has demonstrated that the tannins leaf extract showed inhibitory effect against all the microbial isolates at 2.5mg/ml and that the most susceptible microbial isolates were *Salmonella typhi* and *Candida albicans*. The study therefore, suggests that the tannins isolated from the leaves of *Vitex doniana* have remarkable antimicrobial toxicity. Hence, the plant is a potential source of antimicrobial agents needed for drug development and health care delivery.

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