ORIGINAL PAPER

https://dx.doi.org/10.4314/njpr.v18i1.4

Nig. J. Pharm. Res. 2022, 18(1) pp 35-41
ISSN 0189-8434 e-ISSN 2635-3555
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Available online at http://www.nigjpharmres.com

Bioassay-Guided Isolation of an Antibacterial Flavone and Cytotoxicity of the Leaf Extracts of *Vitex rivularis* Gurke (Lamiaceae)

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of article.

Abstract

Background:
The global challenge of resistance to several antibacterial agents has necessitated the continuous search for new antibacterial molecules. Natural products have proven to be a major source of new therapies for infections.

Objectives:
This study aimed at investigating the antibacterial and cytotoxicity activities of *Vitex rivularis* leaf extract and isolate its bioactive principle based on its local application in the treatment of bacterial infections.

Materials and Methods:
Methanol extract was tested against *Escherichia coli* ATCC 25922, *Bacillus subtilis* ATCC 6638, *Staphylococcus aureus* ATCC 59232, *Proteus mirabilis* ATCC 15290 and *Pseudomonas aeruginosa* ATCC 15442. Cytotoxicity activity of the crude extract was done using Brine Shrimp Lethality Assay (BSLA). Crude methanolic extract was subjected to column chromatography on silica gel and that afforded eight fractions (F1 – F8). The pooled fractions were monitored for antimicrobial activity against *Escherichia coli* and *Bacillus subtilis*. Minimum inhibitory concentration (MIC) of the crude and the most active fraction were determined by agar-dilution method using Gentamicin as the positive control. Fraction 7 was purified by recrystallisation in ethyl acetate to obtain Compound F7. The isolated bioactive compound was characterized by spectroscopic data (IR, ^{1}H and ^{13}C NMR, COSY, HMQC).

Results:
The MIC of the extract and isolated compound, F7, were between 0.25-0.50 mg/ml on *E. coli* and *B. subtilis*. Cytotoxicity activity showed LC_{50} of leaf extract to be 168.66µg/ml. The structure of the isolated bioactive compound was confirmed to be 5-hydroxy-3',4',6,7-tetramethoxyflavone

Conclusion:
The observed antibacterial activity of *Vitex rivularis* leaf extract justified the traditional use of the plant's leaf in the treatment of bacterial infections by local users. The isolated compound can serve as a lead compound in the development of new antimicrobials agents.

Keywords*: Vitex rivularis*, Antibacterial, Cytotoxicity, 5-hydroxy-3',4',6,7-tetramethoxyflavone

INTRODUCTION

Antimicrobial resistance is responsible for a lot of therapeutic drug failure with consequent morbidity and mortality worldwide (Okeke *et al.*, 2007). Considering the rate at which pathogenic and virulent microbes develop resistance to the existing conventional antimicrobial agents, it is imperative to search for new compounds with improved activity to replace those that have become inactive (Cowan, 1999; Newman *et al*., 2003). Plants have been utilized as medicines for thousands of years, therefore the search for new antimicrobial agents using ethnomedicinal approach can yield interesting chemical agents for direct therapeutic use or as lead compounds to develop new and effective antimicrobials. Many medicinal plants of ethnobotanical source have been screened for antimicrobial activities and found effective both in *in-vitro* and *in-vivo* assays against microbial pathogens, including multidrug resistant (MDR) strains (Dzotam *et al.*, 2015).

Towards drug discovery, after confirming the antimicrobial activity of a plant extract, it is very important to isolate and characterize the active compound(s). Bioassay-guided methods are effective in direct isolation of target chemical compounds from plant extracts and fractions obtained from chromatographic or partition separations. However, the process is cumbersome and tedious, requiring many spectroscopic methods including Infrared (IR), Ultraviolet (UV), Nuclear Magnetic Resonance (NMR) and Mass Spectrometric (MS) techniques to elucidate the structure of an isolated compound. Toxicity profiles of many herbal medications are not known, and the assumption that they are generally safe for use needs scientific support. Brine Shrimp Lethality assay has been used worldwide to screen medicinal plants for acute toxicity (Rahaman *et al*., 2008; Idowu *et al*., 2010) with appreciable correlation with cell-line and animal model methods (Sahgal *et al*., 2010).

Vitex rivularis Gurke (Lamiaceae), called "Ori-nla" in Yoruba land (Nigeria) is indigenous to West African coast and are found in deciduous forest in Nigeria extending from Liberia to Zaire (Agbafor and Nwachukwu, 2011). It was reported to be used locally as a substitute for *Vitex doniana* which is used traditionally in the treatment of cough, skin infections, gastro-intestinal tract ailments and other bacterial infections (Anita and Sharma, 2013). The genus Vitex has produced many bioactive compounds with various pharmacological actions (Rani and Sharma, 2013). The various bioactive constituents of V. agnus-cactus (Aranda, 1999) were reported to have antimicrobial and cytotoxic activities. The plant was reported to be used locally in the treatment of bacterial infections and its stem bark used in the treatment of paralysis, epilepsy, convulsions, spasm as well as sedatives (Burkill, 1985; Chandramu *et al*, 2003; Manjunatha, 2007). The antifungal activity of *Vitex rivularis* seed oil has also been reported (Chawla *et al*, 1992; Cabral *et al*., 2009). This current study investigated the antibacterial and cytotoxicity activities of *Vitex rivularis* leaf extract.

METHODOLOGY

Preparation of Plant Material

Fresh leaf of *Vitex rivularis* was collected from Okomu Forest Reserve in Edo State, Nigeria (latitude 6º N and 6º10'N, and longitudes 5ºE and 5º30'E). Identification and authentication of the plant was done at Forestry Research Institute of Nigeria (FRIN), Ibadan, Nigeria with the deposited voucher specimen number FHI – 109899. The air-dried leaves were pulverized and extracted with distilled methanol using Soxhlet apparatus.

Test Organisms

Bacillus subtilis (ATCC 6638), *Pseudomonas aeruginosa* (ATCC 15442), *Proteus mirabilis* (ATCC 15290), *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 25922) were obtained from the Department of Pharmaceutical Microbiology, University of Ibadan. All bacteria were cultured on nutrient agar (No 2) and nutrient broth (pH 7.4) (Oxoid) and were maintained on agar slope before testing. Brine shrimp (Artemia salina, Sander®) eggs were purchased from Great Salt Lake Company, USA.

Chromatographic Fractionation and Isolation of compound

Five gram of the extract of *Vitex rivularis* was subjected to chromatographic separation by the use of Silica gel 60-120 mesh (Oxford), precoated aluminum sheet silica gel $60F_{254}$ (Oxford), and a sintered glass filter column (porosity 3, diameter 1 x 3). A dried slurry of the extract was made by weighing 5g of the extract into 5mls of acetone and 5g of silica gel. These was mixed and allowed to dry before loading it on the silica gel already packed to a column. A gradient solvent mixture of hexane, ethyl acetate and methanol in varying proportions were used as eluent. The loaded extract (5g) gave 226 fractions (30ml each) which

were monitored by TLC and viewed under UV lamp (254nm and 365nm) and vanillin-sulphuric acid spray. Fractions with similar R_f values were pooled together to give 8 fractions. Fraction 7 was further purified by recrystallisation in ethyl acetate to obtain Compound F7. The isolated bioactive compound was subjected to spectroscopic analysis for structural determination (Tokarek *et al*., 2016). All column fractions were tested for antibacterial activity using the typed strains of *Escherichia coli, Bacillus subtilis, Staphylococcus aureus, Proteus mirabilis* and *Pseudomonas aeruginosa* as test organisms.

Spectroscopic Analysis of the bioactive Compound [F7]

1D and 2D NMR data were obtained by dissolving the sample in deuterated chloroform. The spectra were obtained on Brooker NMR system at the Pharmaceutical Science Department, University of the Sciences, Philadelphia, United States of America. Melting point was determined on Barnstead Electrothermal IA9000 at Chemistry Department and Infrared spectra obtained on Perkin Elmer Spectrum BX FTIR at the Multidisciplinary Central Research Laboratory, University of Ibadan, Nigeria

Determination of Antibacterial Activity

The antibacterial activity of the extract and fractions was determined using agar-well diffusion technique of Perez *et al.* (1990) with slight modification. Nutrient agar or *Salmonella shigella* (SS) agar plates were seeded with 100µL of an overnight culture of each of the bacterial isolate (equivalent to 1.0 x 10^8 cfu/mL according to the 0.5 McFarland standard). The seeded plates were allowed to set and a standard cork borer of 8.0 mm diameter was used to cut uniform wells on the surface of the agar. The wells were then filled with 100µL of each extract at concentrations of 100, 50, 25, 12.5 mg/ml and fraction at 20mg/ml (fractions) respectively in dimethyl sulfoxide (DMSO). Control wells containing antibiotic gentamicin (Sigma) at 10 µg/mL and DMSO were used as positive and negative control respectively. A pre-incubation period of 60 min was allowed for diffusion of extracts and test solutions before incubation at 37ºC for 24 hours, after which the diameter of zone of inhibition were measured. Each test was carried out in triplicate.

Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) was determined using the agar dilution method of Andrew (2001), with slight modifications. The extract at 20 mg/mL was serially diluted in test tubes to give a final concentration in the range of 20.0, 10.0, 5.0, 2.5, 1.25, 0.625 mg/mL respectively. One milliliter of each dilution of the extract with 9 mL of Mueller Hinton agar, poured into 10 cm diameter petri dishes and allowed to set. After allowing the agar to dry for about 30 minutes, each plate was inoculated with 1:100 dilutions of overnight broth cultures of each test organisms (containing 1.0 x 10^8 cfu/mL according to turbidity equivalent to 0.5 McFarland standards) and incubated for 24 hours at 37ºC. Nutrient agar plates with extract but without an organism and one containing only the organism without extract served as positive and negative control respectively. Each test was carried out in triplicate. The plates were then examined for the presence of growth after the incubation period. The least concentration that gave no visible colonies of the test organism was taken as the MIC of the extract.

Determination of cytotoxicity (Brine-Shrimp Lethality Assay)

Cytotoxicity test was carried out according to Meyer, *et al.,* (1982) and Rahman *et al*., (2008) as follows. Eggs of *Artemia salina* were hatched in sea water (collected from Badagry Beach, Lagos) for 48-72 hours. The nauplii (10 per tube) were put into tubes containing the extracts dissolved in DMSO to produce three different concentrations (1000, 100 and 10ppm). The assay was done in triplicate and vincristine sulphate and DMSO were used as positive and negative controls, respectively. The number of nauplii alive after 24 hours were used to estimate the number of dead naupli, from where the median Lethal Concentration (LC_{50}) was then calculated graphically using AAT Bioquest program (2020)

RESULTS AND DISCUSSION

Antibacterial screening of the methanolic extract (Table 1) and fractions (Table 2) of *Vitex rivularis leaf* demonstrated potent broad spectrum antibacterial activity on common pathogenic bacteria like *Escherichia coli, Bacillus subtilis, Staphylococcus aureus, Proteus mirabilis* and *Pseudomonas aeruginosa* (Table 1). Both the Gram positive and Gram negative organisms were susceptible to the extract. The diameters of zone of inhibition for the crude extract were between 12.0 -18.0 mm on the tested bacteria at 12.5-100 mg/ml. However, the extract was not active against *Staphylococcus aureus*

at 12.5mg/ml and neither was the control gentamycin showing the resistance status of the organism.

Table 1: Results of the antibacterial activity of the crude extract.

Concentration (mg/mL)	Zone of inhibition (mm)				
	Bacillus subtilis	*Escherichia coli*	*Staphylococcus aureus*	*Proteus mirabilis*	*Pseudomonas aeruginosa*
100	16.0	17.0	15.0	16.0	18.0
50	15.0	17.0	13.0	14.0	16.0
25	14.0	13.0	12.0	13.0	14.0
12.5	13.0	12.0	---	12.0	13.0
Gentamicin (10 µg/mL)	13.0	17.0	---	16.0	14.0

The column fractions (FR1 – FR8) were tested for antibacterial activity on *E. coli* and *B. subtilis.* From the result (Table 2), FR2 and FR8 did not show any activity on the bacteria while FR1 was only active on *E. coli*. FR3, FR4, FR5, FR6 and FR7 (representing the intermediate polar fractions) showed significant activity on the bacteria with FR7 having the most consistent highest activity. This showed that the major antibacterial constituents of the plant are of intermediate polarity.

Table 2: Results of the antibacterial activity of the pooled fractions

Fractions (50mg/ml)	Zone of inhibition (mm)	
	E. coli	*B. subtilis*
FR 1	11	---
FR 2	---	---
FR 3	10	12
FR 4	10	11
FR 5	10	10
FR 6	11	10
FR 7	12	12
FR 8	---	---
Gentamicin (10µg/ml)	15	14
DMSO	---	---

The Minimum Inhibitory Concentration (MIC) of the crude extract on *E. coli* is 0.25mg/ml and 0.5mg/ml on *B. subtilis*. For FR7, the MIC on the two bacterial species is 0.5mg/ml (Table 3).

Table 4 shows the result of Brine shrimp lethality assay (BSLA) and the graphical calculation of LC_{50} (Fig. 1) which was found to be 168.66 µg/ml. This assay has been found useful as a simple, convenient and fast method of screening many medicinal plant extracts for cytotoxic properties (Quazi *et al*., 2017). The value represents an intermediate cytotoxicity when compared with Vincristine sulphate standard of 0.9 µg/ml. For an extract to be considered as having significant cytotoxic action that is suitable for anticancer use, the LC_{50} should be less than 100 µg/ml while values between 500-1000 µg/ml are considered non-toxic and as such may lack antitumor constituents (Rahman *et al*., 2008). According to El-Sayed *et al*. (2011), *Vitex trifolia* from Egypt gave LC_{50} of 173 µg/ml which is comparable with our result. Therefore *V. rivularis* leaf extract is non-toxic as an herbal medicine but may possess moderately cytotoxic constituents; which can be established by appropriate cell line or animal model studies.

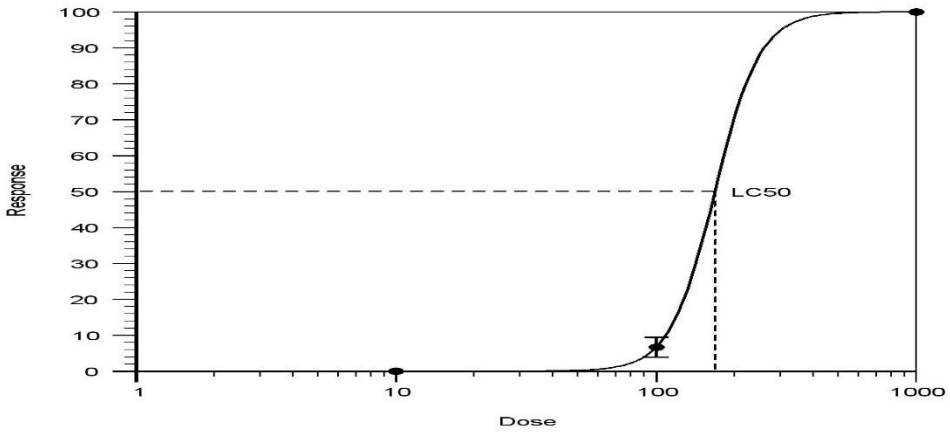
Table 3: Results of the minimum inhibitory concentration (MIC) of extract and fraction 7

Conc. (mg/ml)	Crude extract		Fraction 7	
	E. coli	*B. subtilis*	*E. coli*	*B. subtilis*
0.20	----	---	---	---
0.10	---	---	---	---
0.50	---	---	---	---
0.25	---	+	+	+
0.125	+	+	+	+
0.0625	+	+	+	+
MIC	**0.25 mg/mL**	**0.5 mg/mL**	**0.5 mg/mL**	**0.5mg/mL**

Key: + = growth of bacteria; --- = no growth of bacteria

Table 4. Brine Shrimp Lethality (BSL) assay of Extracts of *Vitex rivularis* leaves

Extracts/ Concentration	1000 ppm		100 ppm		10 ppm		LC_{50} µg/mL
	0 min	24 hrs	0 min	24 hrs	0 min	24 hrs	
A	10	0	10	9	10	10	168.66
B	10	0	10	10	10	10	
C	10	0	10	9	10	10	



LC_{50} = 168.660

$$y = 0.000 + \frac{(100.013 + 0.000)}{1 + \left(\frac{x}{168.660}\right)^{-5.049}}$$

Fig. 1: Graphical plot to calculate LC_{50} in Brine Shrimp Lethality Assay
AAT Bioquest Inc Quest GraphTM LC_{50} Calculator. Retrieved from https://www.aatbio.com/tools/LC50 calculator.

Structural elucidation of compound F7

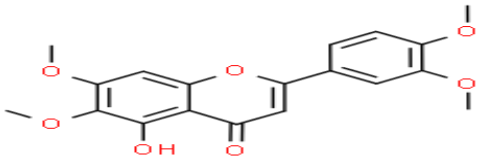
Column chromatographic separation led to the isolation of compound **F7** as a yellow amorphous solid powder with the melting point of 185^0C. The compound showed characteristic spectra pattern that clearly indicated it has the basic flavonoid skeleton with distinct methoxy signals between 55 to 60 ppm. It also clearly showed the characteristic C-5 NMR peak of 12ppm observed in flavonoid based compound. This is due to the presence of hydrogen bonding between carbonyl at the C-5 position and the hydroxyl at the C-6 position. Methoxy signals were observed between 3.6 to 3.8ppm in the ^{1}H NMR spectra and clear ^{13}C NMR peaks between 55 to 60ppm. The compound was isolated in the aglycone form due to the absence of clear glycosidic signals between 4 to 5 ppm. Comparative spectra data to a previously isolated compound confirmed compound F7 to be 5-hydroxy-3',4',6,7-tetramethoxyflavone (Azizu *et al.*, 2010; Kabouche., *et al.*, 2011). Although closely related flavones were reported in *Vitex negundo* and *Vitex trifolia* (Thuy *et al.*, 1998; Kuruüzüm-Uz, 2003; Kiuchi, 2004; Rani and Sharma, 2013;), this compound is reported for the first time in *Vitex rivularis*; The isolated compound which was purified by recrystallisation of Fraction 7 is the major compound in the Fraction; hence it could be the main compound responsible for the antimicrobial activity of *Vitex rivularis* extract. The potential of related compounds in the treatment of inflammations, cardiovascular ailments, microbial infections, and ulcers have been reported in phytomedicine research (Hernandez, 1999; Li *et al*., 2005; Li *et al*., 2007).

Spectra data of Compound F1

IR: 3384 cm^{-1} [broad - OH], 2925cm^{-1} [C-H], 1703 cm^{-1} [C=O], 1609cm^{-1} [aromatic C=C].

^{13}C NMR (125 MHz, CDCl3): 56.0 (4-OMe), 56.1 (3-OMe), 57.5 (7-OMe), 61.0 (6-OMe), 90.0 (C-8), 103.5 (C-3), 105.5 (C-10), 112.0 (C-2), 115.0 (C-5), 120.0 (C-6), 124.0 (C-1), 133.0 (C-6), 149.0(C-4), 150.4 (C-3), 152 (C-5), 154 (C-9), 155.0 (C-7), 163.0 (C-2), 182.0 (C-4)

^{1}H NMR (500 MHz, $CDCl_3$): 3.95 (3H, s, OCH3), 3.96 (3H, s, OCH3), 4.00 (3H, s, OCH3), 4.00 (3H, s, OCH3), 6.59 (1H, s, H-8), 6.60 (1H, s, H-3), 7.0 (1H, H-5), 7.35 (1H, d, J = 2, H-2), 7.54 (1H, H-6).



5-hydroxy-3',4',6,7-tetramethoxyflavone

CONCLUSION

The study confirmed the antibacterial potential and cytotoxic properties of *Vitex rivularis* leaves. The antibacterial activity justified the traditional uses of the plant's leaf in the treatment of bacterial infections as reported by the local users. 5-hydroxy-3',4',6,7-tetramethoxyflavone was isolated and characterized as one of the antibacterial principles in *Vitex rivularis* leaf extract, this could serve as a lead compound towards the development of a new drug candidate in the treatment of chronic infections. The result of the lethality assay also indicated the relative safety of *Vitex rivularis* extract.

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Conflict of Interest: None declared

Received: June 30, 2021

Accepted: January 31, 2022