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Antimicrobial Activity of Leaf Extract – Fractions of *Vernonia calvoana* against Selected Stock Cultures in Microbiology Laboratory, Cross River University of Technology, Calabar

B.U. Ati[1*], I.A. Iwara[1], A.O. Bassey[2], G.O Igile[1], E.E. Duke[2] and P.E. Ebong[1]

[1]Department of Biochemistry, Faculty of Basic Medical Sciences, University of Calabar, Calabar, Nigeria
[2]Department of Medical Laboratory Science, College of Health Technology, Calabar, Nigeria
**Corresponding author*

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A B S T R A C T

This study aims to determine antimicrobial activity of the leaf extract – fractions of *Vernonia calvoana* against selected stock cultures in Microbiology Laboratory, Cross River University of Technology, Calabar. Agar disk diffusion method was used for screening of extract – fractions against *Escherichia coli, Staphyloccus aureus, Salmonella typhi, Candida albicans, Aspergillus flavus, Pseudomonas aeruginosa* and *Helicobacter pylori*. Out of the seven, three of the microorganisms were used to determine the minimal inhibitory concentration (MIC) owing to their profound inhibition. The crude extract, 30% and 100% methanol fractions were assayed for antimicrobial activities. The crude extract exhibited the highest antimicrobial potency against S. aureus, E. coli, and P. aeruginosa with inhibition zone diameter ranged from 20 - 22mm. The inhibitory effect of 100% methanol fraction was recorded with inhibitory zone diameter ranged from 12 – 19mm; and the 30% methanol fraction slightly inhibited with zone diameter ranged from 10 – 15mm against the test microorganisms. *Vernonia calvoana* plant showed antimicrobial potency against the selected stock cultures and probably could be used to control infections associated with these organisms.

Introduction

The use of plants and plant products as medicines could be traced as far back as the beginning of human civilization (Saranraj, 2014). Plants are important source of drugs; especially in traditional medicine (Bako *et al.*, 2005). It is a common practice in Nigeria and most parts of the world to use plant in form of crude extracts, decoction, infusion or tincture to treat common infection and chronic condition (Odeja *et al.*, 2015).

It is estimated that, plant materials are present in, or have provided models for 50% Western drugs (Robbers *et al.*, 1996). Many commercially proven drugs used in modern medicine were initially used in crude form in traditional or folk healing practices, or for other purposes that suggested potentially useful biological activity (Robbers *et al.*, 1996). The primary benefits of using plant derived medicines are that they are relatively safer than synthetic alternatives, offering

profound therapeutic benefits and more affordable treatment (Iwu *et al.,* 1999). Plants containing protoberberines and related alkaloids, and garcinia biflavonones used in traditional African system of medicine, have been found to be active against a wide variety of micro-organisms (Iwu *et al.,* 1999).

Much of the exploration and utilization of natural products as antimicrobials arise from microbial sources. The discovery of penicillin led to later discoveries of antibiotics such as streptomycin, aureomycin, and clindamycin (Trease, 1972). Though most of the clinically used antibiotics are produce by soil micro-organisms or fungi, higher plants have also been a source of antibiotics (Trease, 1972). Plant based antimicrobials represent a vast untapped source for medicines. Continuous and further exploration of plant antimicrobials is of the increase. They are effective in the treatment of infectious diseases while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials (Iwu *et al.,* 1999) and the use of phytochemical products and plant extracts as resistance-modifying agents (RMAs) represents an increasingly active research topic (Abreu *et al.,* 2012).

Vernonia (Asteraceae) is the largest genus in the tribe Vernoniae, with close to 1000 species (Keeley, 1979). Vernonia species grow in a wide range of habitats of broad ecological diversity and climatic conditions including tropical forest, marshes and wet areas, dry plains; tropical savannahs dry sites and even frosty regions of eastern part of North America (Keeley, 1979). The genus is morphologically made up of annuals, herbaceous perennials lianas, shrubs, and trees. The genus Vernonia is known for having several species with food, medicinal and industrial uses (Iwara *et al.,* 2015). *Vernonia amygdalina* is the most studied member of the Vernonia genus as well as one of the most studied plants in Africa (Ijeh and Ejike, 2011). The report on antimicrobial activities of *V. amygdalina* against oral microbes by Anibijuwon *et al.,* (2015) has been published; Ijeh and Ejike (2011) reported on its antibacterial potency. The antifungal of *V. amygdalina* was also reported by Erasto *et al* (2006) while Atangwo *et al.,* 2009a worked on its antidiabetic properties; and hypoglycemic action (Ebong *et al.,* 2006).

Despite the widespread use of plants of the Vernonia genus in food and medicine, and a comprehensive review on the nutritional and health of Vernonia genus by Toyang and Verpoorte (Ngeh and Rob, 2013) in human and animal subjects, *Vernonia calvoana* Hook. F. (V.C) is amongst the less known species of the genus. *Vernonia calvoana* also known as "Ekeke" is a grean-leafy vegetables found in Yakurr LGA of Cross River State, Nigeria. The hypoglycemic and hypolipidemic potentials have been reported (Iwara *et al.,* 2015). Also the plant has been evaluated for its Phytochemical, Proximate and Nutrient Composition (Igile *et al.,* 2013). There is need, therefore, for the plant antimicrobial activities to be scientifically determined.

Materials and Methods

Plant Materials

Vernonial calvoana green-leafy vegetable was purchased from Ugep in Yakurr Local Government Area of Cross River State of Nigeria. The plant sample was identified by Mr. Aboh, Andrew Ahieta, a Botanist in the Department of Botany, University of Calabar, Nigeria.

Preparation of Plant Extracts

The fresh leaves were washed and air-dried at a room temperature (27+2oC). The dry leaves were blended with the use of a manual hand blender, Made in Colombia (Medellin-Colombia) into powder. The blended leaves (310.9g) was soaked in 1200ml of a solution of equal volume of methanol and dichloromethane (1:1). The mixture was put in a thermostatic water bath at a temperature of 50oC for 30 min and removed and kept overnight for thorough extraction of the plants active components. It was then filtered with a chess cloth material and later with Whatman No. 1 filtered paper to obtain a homogenous filtrate. The filtrate was evaporated to dryness using water bath at a temperature of 50oC. The crude extract was then refrigerated at 2-8oC for further use.

Preparation of Fractions

The crude extract (5.0g) was dissolved in 5 milimeters of methanol and eluted using column chromatography on silica gel (50 – 100 mesh) with methanol gradient. Two fractions were collected – at 30% methanol and 100% methanol elusion. These two fractions were labeled, then evaporated using water bath (37+ 2oC) to remove the solvent (methanol). The crude extract was therefore labeled (A), 30% methanol (B) and 100% methanol (C); these were used for antimicrobial analysis.

Determination of Antimicrobial Activities

The following microorganisms were employed in the screening studies: Escherichia coli, Staphyloccus aureus, Salmonella typhi, Pseudomonas aeruginosa, Helicobacter pylori, Candida albicans, Aspergillus flavus all from stock cultures of Cross River University of Technology Microbiology Department who in turn obtained them from National Veterinary Research Institute (NVRI), Vom, Plateau state, Nigeria. Two methods were employed for the determination of antimicrobial activities; an agar paper disc diffusion method and determination of Minimal inhibitory concentration (MIC).

Agar Paper Disc Diffusion Method

Antimicrobial activity of the *V. calvoana* plant was determined by the agar paper disc diffusion method according to Rubio *et al*. (2003) with slight modifications. A suspension of each microorganism (1 ml) was carefully mixed in a tube with the prepared Peptone water and then pipetted into the appropriately labeled Petri dishes. 30.4g of Mueller Hinton agar was weighed and dissolved in 840ml of distilled water. The solution was swirled until it completely mixed; and autoclaved at 121oC for 60 minutes then, allowed cooling. The agar was later, asceptically dispensed into sterile Petri dishes containing the test microorganisms. The crude extract, 30% methanol and 100% methanol fractions, each was dissolved in dimethylsuloxide (DMSO) to make a serial dilution with the following concentrations: 25, 50 and 100(mg/ml). Sterile filter-paper discs (Whatman no. 1, 6 mm diameter) were impregnated with the different concentrations and placed on the inoculated plates. These plates were incubated at 37ºC for 24 hours. The diameters of the inhibition zones were measured in millimeters (diameter of inhibition zone plus diameter of the disc were measured). The dimethylsulfoxide (DMSO) was used as a control.

Minimum Inhibitory Concentration

The Minimal Inhibitory Concentration (MIC) was considered as the lowest concentration of *V. calvoana* able to inhibit the growth of test micro-organisms after 24

hours incubation. The following microorganisms were used owing to their significant zones of inhibition: *C. albicans*, *S. aureus* and *E. coli*. The MIC of the crude extract, 30% methanol and 100% methanol of the plant were determined by dissolving each of the samples in 1ml of DMSO to make serially diluted solutions of 3.13, 6.25, 12.5, 25, 50, and 100 (mg/ml) in sterile test tubes. The suspension of each microorganism (4 drops) was added to the broth dilutions followed by 4.5ml of prepared peptone water. The test tubes were incubated at 37°C for 24 hours and were examined for micro-organism growth inhibition. The lowest concentration capable of preventing growth of the microorganisms was recorded; and the microorganism was said to be sensitive at that concentration.

Results and Discussion

The result obtained from this study indicates that, the crude extract, 30% methanol and 100% methanol fractions of *V. calvoana* showed antimicrobial potency on selected stock cultures (microorganisms) in Microbiology Laboratory, Cross River University of Technology, Calabar. The antimicrobial activity of *V. calvoana* was determined by measuring the zones of inhibitions on the agar plates. Table 1, 2 and 3 show the result of the antimicrobial activities on the selected microorganisms while Table 4 shows the minimum inhibitory concentrations of the plant against three selected microorganisms owing to their wider zones of inhibition by the plant extract and fractions.

Table 1 shows the crude extract; at a concentration of 100mg/ml has the highest zones of inhibition of 22mm, 21mm and 20mm against *S. aureus*, *P. aeruginosa* and *C. albicans* respectively. At the concentration, *H. pylori*, and *A. flavus* were sensitive with the same inhibitory zones of 11mm; 14mm and 12mm were recorded against *E. coli* and S. typhi. The concentration of the extract at 50mg/ml also show large inhibition zones against *A. flavus* and *S. aureus* with clearance of 18mm and 17mm. *H. pylori*, *C. albicans*, *E. coli* and S. typhi were sensitive with zones diameters of 14mm, 12mm, 12mm and 11mm respectively while *P. aeruginosa* was not sensitive at this concentration. At the concentration of 25mg/ml, *E. coli* was highly sensitive to the crude extract of *V. calvoana* with inhibition zone diameter of 21mm while. *H. pylori* and *S. aureus* have the same zones of inhibition of 15mma. Also, *P. aeruginosa* and *A. flavus* were sensitive with the same inhibition zones of 12mm while S. typhi was inhibited with zone diameter of 10mm of the 25mg/ml of the crude extract concentration. The control sample at Fig 1, shows activity against *S. aureus* and *E. coli* with inhibition zones of 13mm and 8mm respectively. *H. pylori* and *P. aeruginosa* were sensitive showing the same zones of 10mm while the rest of the test microorganisms were insensitive to the control (DMSO).

Table 2 shows the 30% methanol fraction; slightly inhibited with zone diameter of 10mm, against *H. pylori* and *E. coli* at a concentration of 100mg/ml. At the same concentration, *P. aeruginosa*, *C, ablbicans*, *A. flavus and S. aureus* were sensitive with the clearance zones of 13mm, 14mm, 12mm and 15mm - which was the highest with this fraction (30% methanol) while S. typhi was not sensitive. The concentration of 50mg/ml of 30% methanol also show slightly inhibition zones against *H. pylori*, *P. aeruginosa*, *C. albicans A. flavus*, *S. aureus*, *E. coli* and S. typhi with clearance of 7mm, 12mm, 9mm, 10mm, 8mm and 14 mm accordingly while S. typhi was not inhibited. The concentration of 25mg/ml inhibited *P.*

aeruginosa and *S. aureus* with the same zones of inhibition of 10mm. *C. albicans*, *A. flavus* and *E. coli* were recorded with 13mm 8mm and 11mm; while *H. pylori* and S. typhi were insensitive. The highest zone of inhibition recorded for the control sample was 9mm against *C. albicans*; followed by 8mm against *P. aeruginosa* and 7mm against *H. pylori* while the remaining four microorganisms were not sensitive to the control.

Table 3 shows the 100% methanol; at a concentration of 100mg/ml has the highest zones of inhibition of 19mm and 18mm against *H. pylori* and *E. coli*. 14mm zone of inhibition was measured the same against *C. albicans* and *S. aureus*. S. typhi was sensitive with zone inhibition of 10mm. *P. aeruginosa* and *A. flavus* were not inhibited by the 100mg/ml concentration. The concentration (50mg/ml) of the 100% methanol fraction of *V. calvoana*, also, exhibited inhibition zone diameter of 19mm against *C. albicans*. *H. pylori*, *S. aureus* and *E. coli* were sensitive with the same zones inhibition of 11mm; while *P. aeruginosa* and *A. flavus* were not sensitive to 100% methanol fraction. The concentration (25mg/ml) of the 100% methanol fraction show inhibition diameter of 12mm against *C. albicans*, *S. aureus* and S. typhi. *H. pylori*has the highest of inhibition zone of 17mm at this concentration. 11mm was recorded aginst *E. coli* while *P. aeruginosa* and *A. flavus* were not inhibited. The control has the highest inhibition zone of 10mm against *E. coli* and 8mm against *C. albicans* and *S. aureus*. The rest of the test microorganisms were not sensitive to the control (DMSO).

Table 4 shows the minimum inhibitory concentration (MIC) of 12.5mg/ml against *S. aureus* with the crude extract of *V. calvoana*. The MIC of 30% methanol fraction was recorded against *C. albicans* at 3.13mg/ml while 50mg/ml concentration of 100% methanol fraction was recorded against *E. coli*.

Table.1 The Antimicrobial Activity of Crude Extract of *V. calvoana* against Selected Microorganism

O	**Zones of Inhibition (mm)**			
	Concentrations of Crude extract			
	25mg/ml	50mg/ml	100mg/ml	Control
H. pylori	15	14	11	10
P. aeruginosa	12	-	21	10
C. albicans	16	12	20	-
A. flavus	12	18	11	-
S. aureus	15	17	22	13
E. coli	21	12	14	8
S. typhi	10	11	12	-

Key: - = Not sensitive, Control = DMSO

Table.2 The Antimicrobial Activity of 30% Methanol Fraction of *V. calvoana* against Selected Microorganism

Test microorganism	Zones of Inhibition (mm)			
	Concentrations of Crude extract			
	25mg/ml	50mg/ml	100mg/ml	Control
H. pylori	-	7	10	7
P. aeruginosa	10	12	13	8
C. albicans	13	9	14	9
A. flavus	8	10	12	-
S. aureus	10	8	15	-
E. coli	11	14	10	-
S. typhi	-	-	-	-

Key: - = Not sensitive, Control = DMSO

Table.3 The Antimicrobial Activity of 100% Methanol Fraction of *V. calvoana* against Selected Microoagnism

Test microorganism	Zones of Inhibition (mm)			
	Concentrations of Crude extract			
	25mg/ml	50mg/ml	100mg/ml	Control
H. pylori	17	11	18	-
P. aeruginosa	-	-	-	-
C. albicans	12	19	14	8
A. flavus	-	-	-	-
S. aureus	12	11	14	8
E. coli	11	11	19	10
S. typhi	12	11	10	-

Key: - = Not sensitive, Control = DMSO

Table.4 Minimum Inhibitory Concentration (MIC)

Test Microorganism	*V. calvoana*	MIC (mg/ml)
S. aureus	A	12.5
C. albicans	B	3.13
E. coli	C	50

Key: MIC = Minimum Inhibitory Concentration, A = Crude extract
B = 30% Methanol fraction, C = 100% Methanol fraction

This study shows that the crude extract of *V. calvoana* at 100mg/ml concentration exhibited the highest antimicrobial activity against *S. aureus*. This correlates with the report of Anibijuwon *et al.*, 2012 showing that, the cold aqueous extract had a higher activity from *V. amygdalina* against *S. aureus*. There is also an agreement of result

with the findings of Shelly (2016), reporting that, the methanolic extract of Solanum xanthocarpum was found to be most effective against *S. aureus* at (18mm at 100%). This proves the antimicrobial efficacy of medicinal plants against microbes. The crude extract, 30% methanol and 100% methanol of *V. calvoana*, at 100mg/ml concentration of each, exhibited the highest inhibition zones than the 50mg/ml and 25mg/ml concentrations. This agrees with the work of Alozie and Sonye (2015), where the results obtained suggest that the aqueous extracts of Moringa oleifera require higher concentrations to inhibit growth of bacteria isolates. One of the factors that affects microbial susceptibility is the concentration of the activity component; the more the concentration the higher the activity of the chemical substance (Alozie and Sonye, 2015).

However, the large zone of inhibition recorded for crude extract against *E. coli* in Table 1, *C. albicans* and *H. pylori* (Table. 3) at 25mg/ml concentration and 100% methanol respectively disagrees with the work of Alozie and Sonye (2015) but concords with the report of Odeja *et al* (2015) in the hydrogen peroxide scavenging potency of methanolic extract of Senna occidentalis, that, the lowest concentration showed the highest percentage inhibition value. There is a characteristic increase in inhibition as the concentration decreases (Odeja *et al.,* 2015).

The resistance of *P. aeruginosa* at 50mg/ml concentration of the crude extract (Table 1) could be attributed to - Tsun-Thai (2013) report that the insensitivity of this gram-negative bacteria (*P. aeruginosa*) against antimicrobial agents due to the permeability barrier posed by the outer membrane of the bacteria and efficient multidrug efflux pumps traversing the bacteria membranes (Li and Nikaido, 2009). Table 2 reveals also that, at 25mg/ml concentration of the 30% methanol, *H. pylori* was not inhibited, likewise, S. typhi was insensitive to all the concentrations. Table 3 shows that both *A. flavus* and *P. aeruginosa* were completely insensitive to all the concentrations of 100% methanol.

According to Mesaros *et al* (2007), *P. aeruginosa* shows a remarkable capacity to resist antibiotics, either intrinsically (because of constitutive expression of B-lactamases and efflux pumps, combined with low permeability of the outer membrane) or following acquisition of resistance genes (e.g genes for B-lactamases, or enzymes inactivating aminoglycosides or modifying their targets), over-expression of efflux pumps, decreased expression of porins, or mutations in quinolone targets. Worryingly, these mechanisms are often present simultaneously, thereby conferring multiresistant phenotypes. In their view, Susceptibility testing is therefore crucial in clinical practice (Mesaros *et al.,* 2007).

The dimethylsulfoxide (DMSO) solvent used for making different concentrations of the *V. calvoana* showed a less or no antimicrobial activities against most of the test microorganisms. The development of resistance to chemotherapeutical agents by the microorganisms has appeared to be a continuous process since the discovery of antibiotics. So every antibiotic has certain life span regarding its efficacy (Alam, 2009). The MIC (Table 4) shows that *C. albicans* and *S. aureus* were sensitive at concentrations as low as 3.13mg/ml and 12.5mg/ml for 30% methanol and crude extract respectively while the 100% methanol was active against *E. coli* at MIC of 50mg/ml.

Vernonia calvoana leaf – extract and fractions poses antimicrobial potency against the test microorganisms. The crude extract is best used for antimicrobial activities than the methanol fractions of the plant. And the plant deserves further investigation for clinical application in the treatment of ailments associated with these microorganisms.

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