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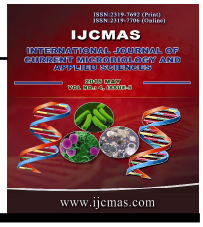

Original Research Article

Phytochemical analysis, in vitro evaluation of antioxidant and antimicrobial activity of methanolic leaf extract of *Vernonia amygdalina* (bitter leaf) against *Staphylococcus aureus and Pseudomonas aeruginosa*

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Keywords

Antimicrobial activity, in vitro antioxidant, minimum inhibitory concentration, minimum bacteriocidal concentration, *Pseudomonas aeruginosa, Staphylococcus aureus* and *Vernonia amygdalina*

A B S T R A C T

The study was conducted to determine the phytochemical analysis, *in vitro* evaluation of antioxidant and antimicrobial activity of methanolic leaf extract of *Vernonia amygdalina* (bitter leaf) against *Staphylococcus aureus* and *Pseudomonas aeruginosa.* The qualitative phytochemicals in the extract of V.A include tannin, alkaloids, glycosides, anthraquinone, flavonoids, phlobatanin, saponin etc. The amount of total proanthocyanidins, flavonoids, alkaloids, and total phenols present in the extract are 0.09 ±0.03 mg quercetin/g of dry plant material, 1.1%, 9.3% and 163.24 mg PE / g DW respectively. The obtained values for β-Carotene and lycopene were 0.49 ± 0.25 µg and 0.08 ±0.04 µg/g respectively. In the *in vitro* antioxidant assay, *V. amygdalina* was found to have DPPH (95.65 μg/mL) and FRAP (0.708) scavenging activity. Antibacterial activity of the extract by disk diffusion method was characterized by inhibition zones of 20 ± 2.2 mm for *S. aureus* and 23 ± 1.2 mm for *P. aeruginosa*. *S. aureus* was tested against 12 standard antimicrobial agents. Of these, pefloxacin, gentamicin, ampiclox, roceptin, zinnacef, amoxicillin, septrin, erythromycin, augumentin and ampicilin were resistance to the organism while ciprofloxacin and streptomycin were sensitive. *P. aeruginosa* was sensitive to pefloxacin, gentamicin, amoxicillin, ciprofloxacin, erythromycin, sparfloxacin and tarivid. Resistance to amplicox, roceptin, zinnacef, streptomycin, septrin, augumentin and ampicilin were also observed with the same organism. The minimum inhibitory concentration (MIC) for *P. aeruginosa* was 30 mg/ml while *S. aureus* has a value of 70 mg/ml. *V. amydalina* exhibited a minimum bacteriocidal concentration (MBC) of 60 mg/ml for *P. aeruginosa* and 140 mg/ml for *S. aureus.* The extract exhibited strong potency against these microorganisms with *P. aeruginosa* being the most susceptible. The results of this study support the use of *Vernonia amygdalina* as herbal remedies in Nigeria.

Introduction

Staphylococcus aureus is an important pathogen of humans and animals and is implicated in a wide variety of infections. It is a pathogen of greater concern because of its virulence (Chambers, 2005), its ability to cause a diverse array of life threatening infections and its ability to adapt to different environmental conditions (Lowy, 2003). *Pseudomonas aeruginosa* is a gram negative, rod shaped, a sporogenous, and monoflagellated bacterium that has incredible nutritional versatility.

Antibiotics are naturally occurring or synthetic organic compounds which inhibit or destroy selective bacteria, generally at low concentrations (Brooks *et al.*, 2007). In the last two decades, antibiotic resistance is emerging as a serious problem worldwide (Walsh, 2000 and Cohen, 2002). This has lead to the exploration for alternative, safe and efficient antimicrobial compounds from natural resources like plants. The increasing resistance of bacteria and fungi to currently marketed antimicrobial agents is becoming a world-wide medical problem (World Health Organization, 2004). In recent years many bacteria have developed antimicrobial drug resistance, these include but not limited to *Staphylococcus aureus* and most of the *Enterobacteriaceae*, such as *Klebsiella pneumonia* (WHO, 2004). Statistics indicate that more than 70% of the bacteria causing infections are resistant to at least one of the drugs most commonly used to treat them (WHO, 2004).

Medicinal plants have formed the basis of health care throughout the world since the earliest days of humanity and have remained relevant in both developing and the developed nations of the world for various chemotherapeutic purposes. Plants have ability to synthesize a wide variety of chemical compounds such as resins, alkaloids, glycoside, saponins, lactose and essential oils (Soraya, 2011). Many of these phytochemicals have beneficial effects on a long term human health and may be used to effectively treat human disease (Lai and Roy, 2004).

Oxidative stress leads to enhanced generation of reactive oxygen species (ROS) and has been implicated in the etiology of over one hundred human diseases including inflammation, metabolic disorders, cellular aging, atherosclerosis, heart disease, stroke, diabetes mellitus, cancer, malaria, rheumatoid arthritis, HIV / AIDS, Alzheimer's disease, ulcerative colitis and Parkinsons disease (Smith *et al*., 2000; Olukemi *et al*., 2005 ; Hyun *et al*., 2006 and Aliyu *et al*.,2008). Antioxidants are substance or molecules that are capable of neutralizing the harmful effects of the ROS through the andogenous enzymatic defense system. The antioxidant effect of plant is mainly due to phenolic components like flavonoids, phenolic acids and phenolic diterpenes (Shahidi *et al*., 1992). The antioxidants capacity of phenolic compounds is mainly due to their redox properties, which play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen and decomposing peroxides (Osawa, 1994).

Vernonia amygdalina (V.A) is a shrub that grows predominantly in the tropical Africa. Leaves from this plant serve as food vegetable and culinary herb in soup (Argheore *et al.*, 1998). *Vernonia amygdalina* commonly known as bitter leaf is a shrub or small tree of 2 – 5 m tall, belonging to the family *Asteraceae*. It has petiolate leaves of about 6 mm diameter and elliptic shape. The leaves are green with a characteristic odour and a bitter taste. The taxonomic classification of *Vernonia*

amygdalina is as follows: Kingdom: plantae, Division: Angiosperms, Order : Asterales, Family: Asteraceae, Genius: Vernonia, Species*: V. amygdalina*, Botanical Name: *Vernonia amygdalina.* It is commonly called "Bitter leaf" in English language, "Onugbu" in Igbo language, it is called "Etidot", in Efik, Ijaw and Ibibio, "Ewuro" in Yoruba language, "Oriwo" in Edo and "Chusa-doki" in Hausa (Egedigwe, 2010). In many parts of Nigeria, the plant has been domesticated. Studies on the nutritional composition of the bitter leaf are numerous (Nimenibo-Uadia, 2003). *V. amygdalina* has been found to be rich in minerals, especially phosphorus, calcium, potassium, magnesium, zinc, iron and some vitamins like vitamin A, C and E. VA extracts have been shown to exhibit profound ethnomedical and pharmacological properties viz, anti-diabetic (Momoh *et al*., 2014), antimalarial (Abort and Raserika, 2003), antihelminthic and antibiotic properties (Farombi, 2003). The present study investigated the *in vitro* antioxidant and antibacterial activity of methanolic leaf extract of *Vernonia amygdalina* against *Staphylococcus aureus* and *Pseudomonas aeruginosa*

Materials and Methods

Collection and identification of plant extract

The leaves of *Vernonia amygdalina* were obtained from Ikorodu in Lagos State, Nigeria. The plant was authenticated by Mrs Shokefun a botanist from Science Laboratory Technology Department (Environmental biology Unit), Lagos State Polytechnic, Ikorodu.

Preparation of methanolic leaf extract of *Vernonia amygdalina*

The leaves of *Vernonia amygdalina* were washed, air dried under shade in the Biochemistry Laboratory, pulverised to coarse power using blender. Extraction was carried out by dispersing 200g of the grounded *Vernonia amygdalina* plant material in 1L of 80% methanol and shaking was done with GFL shaker for 72 hours. This was followed by vacuum filtration and concentrated by rotary evaporator at a temperature not exceeding 40^{o}C. The concentrated extract was dried to complete dryness in an aerated oven at 40^{o}C for 48 hours. The extract was latter stored in a refrigerator at 4^{o}C.

Qualitative phytochemical analysis of *Vernonia amygdalina*

Phytochemical analysis for phytochemical constituents were carried out on the methanolic extract of *Vernonia amygdalina* using standard phytochemical procedures by Sofowora (1993), Harborne (1973), Trease and Evans (1989).

Quantitative phytochemical analysis of the methanolic leaf extract of *Vernonia amygdalina*

Determination of total proanthocyanidins content of the extract

Determination of proanthocyanidin was based on the procedure reported by Sun *et al*., (1998). A volume of 0.5 ml of 0.1 mg/ml of extract solution was mixed with 3 ml of 4% vanillin-methanol solution and 1.5 ml hydrochloric acid; the mixture was allowed to stand for 15 min. The absorbance was measured at 500 nm. Extract samples were evaluated at a final concentration of 0.1 mg/ml. Total proanthocyanidin content were expressed as catechin equivalents (mg/g) using the following equation based on the calibration curve:

$y = 0.5825x$, $R^2 = 0.9277$, where x was the absorbance and y is the catechin equivalent (mg/g).

Flavonoid content determination

One hundred millilitres of 80% aqueous methanol was used to repeatedly extract 1 g of the defatted sample at room temperature. The solution was then filtered through Whatman filter paper. The filtrate was evaporated to dryness in a crucible over a water bath and weighed to a constant weight using the method of Oseni et al (2014).

Alkaloid content determination

To about 1 g of the defatted sample of *Vernonia amygdalina*, 80 ml of 10% acetic acid in ethanol was added. The beaker was covered and then allowed to stand for 4 hours. The suspension was then filtered and the extract concentrated on a water bath to one-quarter of the original volume. Concentrated ammonium hydroxide was added drop wise to the extract until the precipitation was completed. The precipitate was collected and washed with dilute ammonium hydroxide and then filtered to obtain the alkaloid residue. This was dried and weighed using method of Oseni et al (2014).

Total phenolic content of the extract

The quantitative determination of total phenolic content using Folin-Ciocalteu (F-C) reagent involves oxidation in alkaline solution of phenols by the yellow molybdotungstophosphoric heteropolyanion reagent and colorimetric measurement of the resultant molybdotungstophosphate blue according to the method of Singleton and Rosi (1965) and modified by Dogÿan et al., (2005). The polyphenol fraction (extract corresponding to 1 g of dry plant material) was dissolved in 5 ml of double distilled water. An aliquot of 100 µl of this solution was diluted with double distilled water to 3 ml. Afterward, the obtained solution was added to 300 µl of double distilled water and 500 µl of the F-C reagent. After shaking, the mixture was incubated for 3 min at room temperature. Then 2000 µl of 20 % Na_2CO_3 solution was added. The volume obtained was mixed vigorously, and held for 60 min in the dark at ambient temperature. The absorbance of the solution was then measured at 650 nm against a blank in a spectrophotometer. The sample was analysed in triplicate and the average content was noted for each measurement. The total phenolic content, expressed as mg of pyrrocatechol equivalents (PE) per g of dry weight of plant material (mg PE / g DW), was calculated through the calibration curve obtained using the equation given bellow:

Absorbance = 0.0828 x C, R^2 = 0.9993

where C was the concentration in mg/l.

Determination of β-Carotene and lycopene content of the extract

β-Carotene and lycopene were determined by the method of Barros et al (2007). The dried extract of *Vernonia amygdalina* (100 mg) was vigorously shaken with acetone-hexane mixture (4:6, 10 mL) for 5 min and filtered through a disposable filter (0.45 µm, Millipore). The absorbance of the filtrate was measured at 453, 505, and 663 nm. Contents of β-carotene and lycopene were calculated according to the following equations:

β-carotene (mg/100 mL) = 0.216 (A663) − 0.304 (A505) + 0.452 (A453);

lycopene (mg/100 mL) = −0.0458 (A663) + 0.372 (A505) − 0.0806 (A453).

The assays were carried out in triplicates, the results were mean ± SD and expressed as µg of carotenoid/g of the extract.

In-vitro plant antioxidant assay of *Vernonia amygdalina*

DPPH Radical Scavenging Activity Assay.

1,1-Diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity was measured according to the method of Barros et al (2007) with slight modification on the basis of the method of Blois (1958). Different concentrations of ethanol dilutions of samples were mixed with 2.0 volume of 6.5 × 10^{-5}M solution of DPPH. The resulting solutions were thoroughly mixed and absorbance was measured at 517 nm after keeping the tubes in dark for 30 minutes. The scavenging activity was determined by comparing the absorbance with that of control containing equal volumes of DPPH solution and ethanol. The radical scavenging activity was obtained by the following equation:

$$\text{Radical scavening activity (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

The IC_{50} was defined as the concentration (in μg/mL) of the extract required to deplete the amount of DPPH radical by 50%. Gallic acid (GA) and butylated hydroxytoluene (BHT) were used as positive control

Ferric reducing antioxidant power assay (FRAP)

The reducing power assay was conducted as previously described by Wang et al. (2008) and Oyaizu (1986) with ascorbic acid (AA) and tert-butyl-4-hydroxyanisole (BHA) being used as the positive controls. In brief, 2.5 ml of individual deionized water diluted *Vernonia amygdalina* extract (ranged from 0.1 to 1 mg/ml) was sequentially mixed with equal volume of phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide (2.5 ml, 1% w/v). After incubation at 50°C for 20 min, 2.5 ml of trichloroacetic acid (10 % w/v) was then added to the mixture followed by centrifuging at 3000 rpm for 10 min. Consequently, 5 ml of the upper layer was mixed with 2.5 ml of distilled water and 0.5 ml of ferric chloride (0.1 % w/v). After 30 min of incubation at room temperature in the dark, absorbance of the resulting solution was measured at 700 nm using a spectrophotometer. The ferric reducing power capacities of the plant extracts and standard antioxidants were expressed graphically by plotting absorbance against concentration. Samples for the assay were prepared in triplicate

Preparation of methanolic extract impregnated paper discs

The methanolic extract impregnated paper discs were prepared as described by Ekundayo and Ezeogu (2006). Whatman No. 1 filter paper was cut into discs of 6 mm diameter using an office perforator. The discs were placed in glass Petri dish and sterilized in hot air oven at 160°C for 1 hour. Each disc was impregnated with 20 µl portion of stock solution of the methanolic extract of *Vernonia amygdalina* (100 mg/ml). The discs were dried in an incubator at 35-37°C for 2 hours.

Test organisms

The two bacterial strains used in this investigation were obtained from Microbiology Department, University of Lagos, Nigeria. The organisms are *Staphylococcus aureus*, a Gram-positive bacteria and *Pseudomonas aeruginosa* a Gram-negative bacteria. The microorganisms were maintained at 4°C on Nutrient Agar slant in the Department of Science Laboratory Technology (Microbiology Unit), School of Pure and

Applied Science, Lagos State Polytechnic, Ikorodu, Lagos, Nigeria and fresh subcultures were made before use.

Inoculum preparation

A loopful of isolated colonies was inoculated into 4 ml of peptone water, incubated at 37°C for 4 hours. This actively growing bacterial suspension was then adjusted with peptone water so as to obtain a turbidity visually comparable to that of 0.5 McFarland standard prepared by mixing 0.5 ml of 1.75% (w/v) barium chloride dehydrate ($BaCl_2$. $2H_2O$) with 99.5 ml of 1% (v/v) tetraoxosulphate (vi) acid (H_2SO_4). This turbidity is equivalent to approximately10^8 colony forming units per ml (CFU/ml).

Determination of antibacterial activity by disk diffusion method

Sensitivity of *Staphylococcus aureus* and *Pseudomonas aeruginosa* strains to methanolic leaf extracts of *Vernonia Amygdalina* were measured in terms of zone of inhibition using disk diffusion method as described by Kirby-Bauer diffusion technique (Cheesbrough, 2002). Here the discs were soaked in the leaf extract as described above before use.

The plates containing Muller Hinton were smear with 0.1ml of the inoculums using swab stick. This was followed by mounting of the impregnated paper disc. The inoculated *Staphylococcus aureus* and *Pseudomonas aeruginosa* isolates were incubated at 37°C for 24 hours and the diameter of any resultant zone of inhibition was measured. For each combination of extract and the bacterial strains, the experiment was performed in triplicate. The bacteria with a clear zone of inhibition of more than 17 mm were considered to be sensitive.

Antibiotic susceptibility testing

Susceptibility of organisms to different antibiotics were tested using disk diffusion method on freshly prepared Mueller Hinton agar and standardized by the method of National Committee for Clinical Laboratory Standard using some selected antibiotics namely: Amoxicillin (30ug), Ciprofloxacin (5ug), Gentamicin (10ug), Ampicillin (25ug), Streptomycin (25ug) etc. For each combination of the antibiotics and the bacterial strains, the experiment was performed in triplicate. The bacteria with a clear zone of inhibition of more than 17 mm were considered to be sensitive

Minimum inhibitory concentration (MIC)

MIC is defined as the lowest extract concentration that inhibited the growth of the test organisms as indicated by absence of visible turbidity in the tube compared with the control tubes. The minimum inhibitory concentration (MIC) of the extracts was determined using the tube serial dilution method. Extract (100 mg/ml) was dissolved in water and diluted with Nutrient broth in two fold serial dilutions in test tubes. An overnight broth culture of the test organism was adjusted to McFarland turbidity standard and 50 µl (0.05 ml) of the cell suspension added to each of the tubes. The tubes were incubated aerobically at 37°C for 18 hours.

Minimum bactericidal concentration (MBC)

The MBC of the methanolic leaf extract of *Vernonia amygdalina* was prepared by modification of the method of Spencer and Spencer (2004). Here 0.1ml, aliquots of samples taken from the non-turbid tubes of the MIC assay tube was sub-cultured onto Mueller Hinton agar plates. The resulting plates were then incubated aerobically at

37°C for 24 hrs. The lowest concentration of the extract at which no colonies of *Staphylococcus aureus* and *Pseudomonas aeruginosa* were seen was taken as the MBC. The results were compared with that of control using sterilized distilled water. The experiment was performed in triplicate. The MBC was taken as the concentration of the extract that did not show any growth on a new set of agar plates.

Data analysis

All analyses were carried out in triplicate and results expressed as mean ± SEM. The data analysis was done using the Graph Pad prism computer software version 6. Student's'-test and one-way analysis of variance (ANOVA) were used for comparison. A *P*-value < 0.05 was considered significant. The IC_{50} values were calculated using linear regression graphs.

Result and Discussion

Qualitative phytochemical analysis of the methanolic leaf extract of *Vernonia amygdalina*

The results obtained from the phytochemicals analysis of *Vernonia amygdalina leaf* extract showed the presence of some secondary metabolite like tannins, Phlobatanin, alkaloids, saponins, flavonoids, anthraquinone, glycoside, polyphenols, terpenoid etc (Table I). The presence of these secondary metabolites in this extract may be responsible for the anti-microbial activity of the extract.

Quantitative analysis of the phytochemical constituents of the methanolic leaf extract of *Vernonia amygdalina*

The results of table 2 below revealed the level of phenolic compounds in *Vernonia amygdalina*. The values are significantly lower when compared to the standard compounds used in this study.

Determination of β-Carotene and lycopene

β-Carotene and lycopene were found in small amounts. The obtained values for β-Carotene and lycopene were 0.49 ± 0.25 µg and 0.08 ±0.04 µg/g respectively.

Figure I below shows the DPPH radical scavenging activities of *Vernonia amygdalina.* Figure 2 below shows the reducing power activities of *Vernonia amygdalina.*

Vernonia amygdalina is widely consumed as food and used in traditional medicine practice because of its immense medicinal properties which exert bacteriostatic and bacteriocidal effect on some bacteria. The qualitative phytochemical screening revealed the presence of various secondary metabolites in the extracts examples are: tannins, phlobatanin, flavonoids, alkaloids, glycosides, terpenoids, saponin, polyphenol, cardenolide etc (Table 1). A study conducted by Oseni et al (2014) on *Vernonia amygdalina* (bitter leaves) revealed that the plant contained flavonoids, saponins, tannins, glycosides, phenolic, terpenes, anthracenosides, reducing sugar and alkaloids. Flavonoids have been reported to be synthesized by plants in response to microbial infections and are good antibacterial agents and tannins have been demonstrated to have antibacterial activities (Akiyama et al., 2001).

Some of the previously isolated constituents found in *Vernonia amygdalina* Del. include: sesquiterpene lactones (Cimanga *et al.,* 2004; Izevbigie, 2003 and Igile *et al.,* 1995), flavonoids like luteolin, luteolin 7-O-glucosides and luteolin 7-O-glucuronide (Igile *et al.,* 1994), steroid glycosides (Igile

et al.,1995; Jisaka *et al*., 1992 and Jisaka *et al*., 1993), and vernonioside A, B, A1, A2, A3, B2, B3 and A4 (Igile *et al.,* 1995; Jisaka *et al*., 1992; Jisaka *et al.,* 1993 and Ohigashi *et al*., 1991).

The amount of total proanthocyanidins, flavonoids, alkaloids, and total phenols present in the extract of V.A are 0.09 ± 0.03 mg quercetin/g of dry plant material, 1.1%, 9.3% and 163.24 mg PE / g DW respectively (Table 2). Strong antioxidant activities have been reported for flavonoids from V.A and, its saponins have been reported to elicit antitumoral activities in leukemia cells (Jisaka *et al.,* 1993). Antimicrobial activities in plants have been attributed to the presence of alkaloids, saponins, tannins, flavonoids and terpenes (Odugbemi, 2006).

The plant has been used as remedies for many infectious diseases, plants with secondary metabolites such as flavonoids, alkaloids, saponins, terpenoids, sesquiterpene lactones and steroids have been found to have antimicrobial properties *in vitro* (Cartagena *et al.,* 2008, Cowan, 2002 and Sibanda and Okoh, 2007).These phytochemicals were all found to be present in the methanolic leaf extract of *V. amygdalina*. β-Carotene and lycopene were found in small amounts. The obtained values for β-Carotene and lycopene were 0.49 ± 0.25 µg and 0.08±0.04 µg/g respectively. The presence of these compounds in the extract indicates the anti-oxidant potential of the plant.

Oxidative stress which is caused by insufficient capacity of biological system to neutralize excessive free radical product, has been associated with all kind of human diseases and aging (Jensen *et al*., 2008). In this study, *V. amygdalina* extract possessed *in vitro* antioxidant activity when tested with DPPH radical scavenging assay. DPPH free radical scavenging ability is one of the most popular methods utilized in screening for anti-oxidative activity. DPPH is a free radical compounds and has been widely used to test the free radical scavenging ability of various samples. The DPPH radical is employed as a substrate to evaluate antioxidant activity. IC_{50} expresses the amount of antioxidant required to decrease the DPPH radical concentration by 50% (Chanda *et al*., 2011). Basically, a higher DPPH radical scavenging activity is associated with a lower IC_{50} value. IC_{50} value was determined from plotted graph of scavenging activity against the different concentrations of *V. amygdalina* extracts, gallic acid (GA) and butylated hydroxytoluene (BHT). The highest percent DPPH radical-scavenging activities were observed in the GA, followed by BHT whilst the extract showed lowest scavenging activity. At a concentration range of 10–50 μg/mL, the IC_{50} of GA was 32.20 μg/mL that of BHT was 48.72 μg/mL and *V. amygdalina* has a value of 95.65 μg/mL (Figure 1).

This result suggested that the extract can scavenger DPPH radicals. The reducing capacity of an extract may be an important indicator of its potential antioxidative activity (Chanda *et al*., 2011). A higher absorbance indicates a higher ferric reducing power. Increase absorbance indicates an increase in reductive ability. Figure II presents the dose dependent ferric reducing powers of the extracts of *V. amygdalina*, BHA, and AA respectively. The extract of V.A has a reducing power value of 0.708 compared to the standard BHA and AA with reducing power of 1.36 and 0.9 respectively. This is an indication that the extract has a ferric reductive ability.

Resistance to antimicrobials is highly prevalent in bacterial isolates worldwide, particularly in developing countries.

Table.1 The qualitative phytochemical constituents of the methanolic leaf extract of *Vernonia amygdalina*

Phytoconstituent	**Qualitative abundance**
Tannins	+
Phlobatanin	+
Flavonoids	++
Alkaloids	+++
Glycosides	++
Cyanogenic glycosides	++
Anthraquinone	++
Terpenoids	+
Saponin	++
Polyphenols	++
Cardenolide	+

+ (Present in low concentration), ++ (present in moderate concentration) and .+++ (present in high concentration).

Table.2 Quantitative analysis of the phytochemical constituents of the methanolic leaf extract of *Vernonia amygdalina*

Phenolics	***Vernonia amygdalina* extract**
Total proanthocyanidins[b]	0.09±0.03
Flavonoids	1.1%
Alkaloids	9.3%
Total phenol[a]	163.24

Table.3 Zones of inhibition indicating the antimicrobial activity of the methanolic leaf extract of *Vernonia amygdalina* on the tested organisms

Test organisms	**Gram property**	**Conc. of the methanolic extract of VA**	**Diameter of zones of inhibition (mm)**
S. aureus	+ve	100 mg/ml	20 ± 2.2
P. aeruginosa	-ve	100 mg/ml	23 ± 1.2

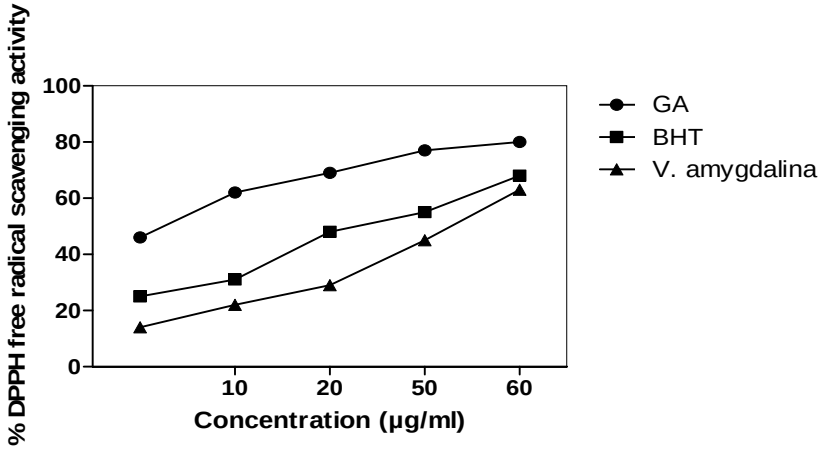

Figure.I DPPH radicals scavenging activities of the extracts of V. amygdalina, GA and BHT at different concentrations. Each value represents mean ± SD (n =6).

Table.4 Antimicrobial susceptibility pattern of standard antibiotics against *Staphylococcus aureus*

Antibiotic sensitive disc	**Concentration (µg)**	**Diameter of zone of inhibition (mm)**	**Interpretation**
Pefloxacin	10	14.8	R
Gentamicin	10	12.3	R
Ampiclox	30	10.6	R
Rocephin	25	9.2	R
Zinnacef	20	8.1	R
Amoxycilin	30	15.1	R
Ciprofloxacin	10	22.2	S
Streptomycin	30	18.3	S
Septrin	30	12.0	R
Erythromycin	10	17.2	R
Augmentin	30	14.6	R
Ampicilin	10	16.1	R

KEY: S = Sensitive (zone of bacterial inhibition is ≥ 18 mm) and R = Resistance (zone of bacterial inhibition is < 18 mm).

Table.5 Antimicrobial susceptibility pattern of standard antibiotics against *Pseudomonas aeruginosa*

Antibiotic sensitive disc	**Concentration (µg)**	**Diameter of zone of inhibition (mm)**	**Interpretation**
Pefloxacin	10	17.6	S
Gentamicin	10	18.1	S
Ampliclox	30	12.2	R
Rocephin	25	17.2	R
Zinnacef	20	5.6	R
Amoxycillin	30	20.1	S
Ciprofloxacin	10	18.1	S
Streptomycin	30	15.3	R
Septrin	30	15.2	R
Erythromycin	10	18.1	S
Augmentin	30	9.3	R
Ampicilin	10	15.2	R
Sparfloxacin	10	18.1	S
Tarivid	10	20.2	S

KEY: S = Sensitive (zone of bacterial inhibition is ≥ 18 mm) and R = Resistance (zone of bacterial inhibition is < 18 mm).

Table.6 MIC and MBC determination of *Vernonia amygdalina* against *Staphylococcus aureus* and *Pseudomonas aeruginosa*

Test organisms	MIC	MBC
Staphylococcus aureus	**70 mg/dl**	**140 mg/ml**
Pseudomonas aeruginosa	**30 mg/dl**	**60 mg/dl**

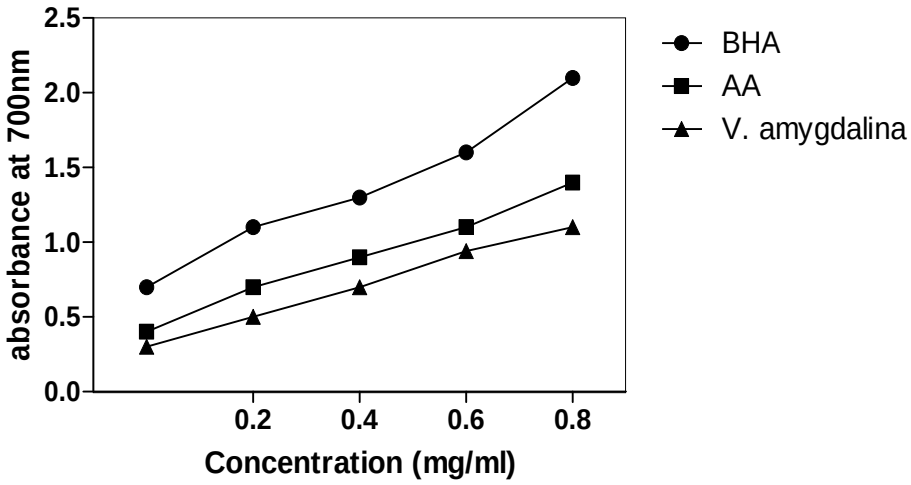

Figure.II Reducing power of BHA, AA and *V. amygdalina* at different concentrations. Each value represents mean ± SD (n =6).

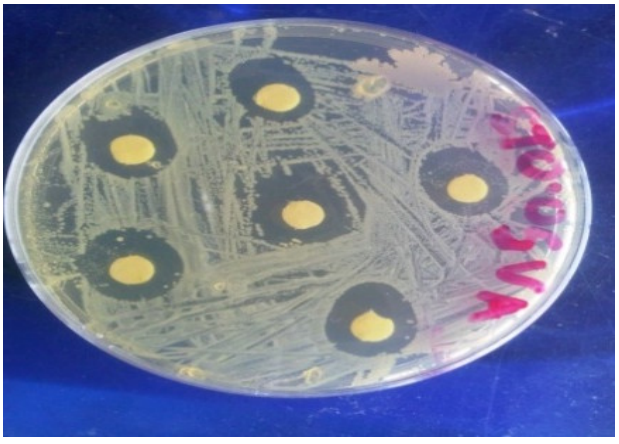

Figure.3 Agar plate showing zones of inhibition of the methanolic leaf extract of *Vernonia amygdalina against Staphylococcus aureus.*

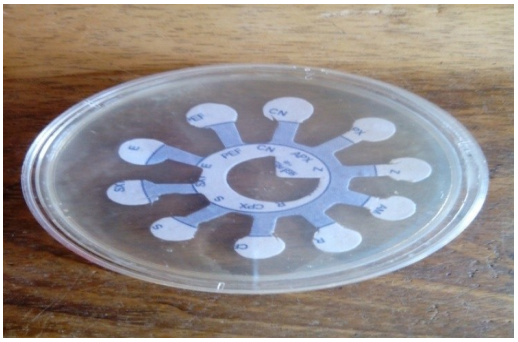

Figure.4 Sensitivity and resistance of *Staphylococcus aureus* strain to multi drug sensitive antibiotic disc

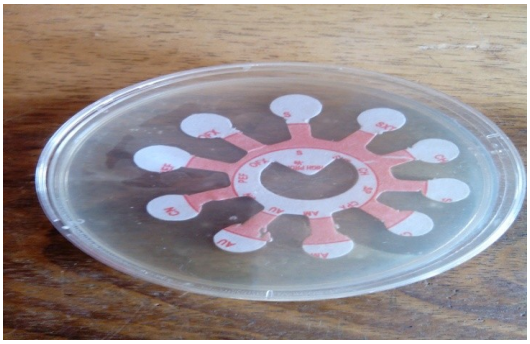

Figure.5 Sensitivity and resistance of *Pseudomonas aeruginosa* strain to multi drug sensitive antibiotic disc

The mean diameters of zones of inhibition for the methanolic leaf extract were 20 ± 2.2 mm against *S. aureus* and 23 ± 1.2 mm against *P. aeruginosa* (table 3).The activity of the extract significantly ($P<0.05$) showed antimicrobial activities against all the tested organisms used. The extract exhibited strong potency against the micro-organisms with *P. aeruginosa* being the most susceptible. Therefore, it appears that the methanolic leaf extract of *V. amygdalina* could present an important treatment option of infections caused by these organisms (*S. aureus* and *P. aeruginosa*). Figure III shows the zone of inhibition of V.A against *S. aureus*. The clear zone of inhibition is an indication that the extract has antimicrobial activities. Figure IV and V show the sensitivity and resistance of *S. aureus* and *P. aeruginosa* strains to standard multi drug antibiotic sensitivity discs.

Twelve different antimicrobial standard antibiotic agents were used to test the susceptibility against *S. aureus.* The organism was resistant to the first line antibiotics that are commonly prescribed by physicians. The organism (*S. aureus*) was found to be resistance to pefloxacin, gentamicin, ampiclox, roceptin, zinnacef, amoxicillin, septrin, erythromycin, augumentin and ampicilin. It was also observed that the bacterium was too sensitive to ciprofloxacin and streptomycin (table 4). In table 5, fourteen antibiotic sensitive discs were used to test the susceptibility against *P. aeruginosa.* The organism was sensitive to pefloxacin,

gentamicin, amoxicillin, ciprofloxacin, erythromycin, sparfloxacin and tarivid. *P. aeruginosa* was resistance to amplicox, roceptin, zinnacef, streptomycin, septrin, augumentin and ampicilin.

The zone of inhibition that is ≥ 18mm is considered to be sensitive while those that are < 18mm are considered to be resistance. Low MIC indicates the minimum inhibitory concentration required to inhibits the growth of the test organism, which translate to high potency of the extract. The MIC of 30 mg/ml was exhibited by the extract on *P. aeruginosa* while 70 mg/ml was observed in *S. aureus*. Similar report revealed the antibacterial potency of active leaf extract of *V. amygdalina* with MICs of 22.5 to 26.0 mg/ml and 19.8 to 26.4 mg/ml for the Gram-negative and Gram positive isolates tested (Iwalokun *et al*., 2003). *V. amydalina* exhibited a minimum bacteriocidal concentration (MBC) of 60 mg/ml for *P. aeruginosa* and 140 mg/ml for *S. aureus.* The extract exhibited strong potency against these microorganisms with *P. aeruginosa* being the most susceptible. The results of this study support the use of the extract as herbal remedies in Nigeria.

In conclusion, this study showed that methanolic leaves extract of *Vernonia amygdalina* exerted significant ($P<0.05$) antimicrobial activities against *Staphylococcus aureus* and *Pseudomonas aeruginosa* and might be source of active antimicrobial agents for the development of drugs for the treatment of these microorganisms.

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