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PHYTOCHEMICAL SCREENING AND ANTIBACTERIAL ACTIVITY OF CRUDE EXTRACTS OF BITTER LEAVES (*Vernonia amygdalina*) AND BOUNDARY TREE (*Newbouldia laevis*)

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ABSTRACT: *Vernonia amygdalina and Newbouldia laevis* plants belong to the families of *Astaraceae* and *Bignoniaceae.* The leaves of *Vernonia amygdalina and Newbouldia laevis* were extracted using ethanol, n-hexane and water and the extracts were screened for phytochemical components. The phytoconstituents of the plants contained are Saponins, Tannins, Flavones, Glycosides, Alkaloids, Quinolines and Anthraquinones. Ethanol, n-hexane and water extracts of the leaves were tested for antibacterial activity against clinical isolates obtained from the National Agency for Food and Drugs Administration and Control (NAFDAC), Agulu Branch, Anambra State Nigeria using agar diffusion assay and were found to show inhibitory activity against *Escherichia coli, Streptococcus pneumonia and Staphylococcus aureus.* The sensitivity test results showed highest activity of 80mm zone of inhibition in 100mg/ml of ethanol extract against *Streptococcus pneumonia.* These extracts showed zone of inhibition higher than the selected antibiotics used as control. Therefore, the bioactive of *Vernonia amygdalina and Newbouldia laevis* leaves can serve as a lead for the development of new pharmaceutical therapeutic needs. That shows that the plants extracts can be used for the treatment of various bacterial infections.

Key Words: Phytochemical, Antimicrobial activity, Bioactive, *Vernonia amygdalina, Newbouldia laevis.*

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INTRODUCTION: Plants have long served mankind as source of food, shelter and medical agents. The medicinal plants have been used for many years in daily life to treat diseases all over the world (Ates and Erzdogrul, 2003). Due to a steady rise in antibiotic resistance of bacteria due to indiscriminate use of commercial drugs (Ahmed *et al.*, 1998) and these urgently calls for

the discovery of alternative therapeutic agents by using medicinal plants, which involve the use of plant extracts or their active substance. Secondary plant metabolites (phytochemicals) have been extensively investigated as the sources of medicinal agents (Krishnaraju *et al.,* 2005). The medicinal value of plants lies in some chemical substances that produce definite physiological action on the human body and the most important of these bioactive compounds of plants are alkaloids, flavones, tannins and phenolic compounds, they have defence mechanism and protection from various diseases (Chidambara *et al.,* 2003). Knowledge of the chemical constituent of plant is very important and not only for the discovery of drugs and other therapeutic agents but also in disclosing new sources of such economic materials as tannins, oils, gums and precursors for the synthesis of complex chemical substances (Mojab *et ai,.* 2003).

Thus the search for newer sources of antibiotics is a global challenge preoccupying research institutions, pharmaceutical companies and academia (Latha *et ai.,* 2006). However, the past record of rapid, widespread and emergence of resistance to newly introduced antibiotics and the emergence of previously uncommon infections is now one of the medical problem posed by the high cost, adulteration and increasing toxic side effects of these synthetic drugs coupled with their inadequacy in diseases treatment found more especially in developing countries should also be emphasized.

Many plants possess antimicrobial activities and are used for the treatment of different diseases (Arora *et ai.,* 1999). The use of plant as source of remedies for the treatment of many diseases dated back to prehistory and people of all continents have this old tradition.

MATERIALS AND METHODS

Plant Material Collection and Identification

Fresh bitter leave and boundary tree were obtained from a farmland in Agulu Anambra state, during the month of July, 2020. The leaves of plants were identified by Mrs. Onwunyili Amakaat of the department of Pharmacognosis and medicinal plants, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Anambra state, Nigeria with a voucher specimen number for *Vernonia amygdalina* PCG/474/A/020 and Newbouldia laevis PCG/474/A/035 deposited in a Herbarium.

Sources and Maintenance of Test Organisms

Standard clinical isolates of Gram negative bacterium (*Escherichia coli*) and gram positive bacteria (*Streptococcus pneumonia* and *Staphylococcus aureus*) were obtained from National Agency for Food and Drug Administration and Control (NAFDAC)**.** The bacteria were maintained on nutrient Agar at 4℃.

Processing of Plant Samples

The fresh plants samples are hand-plucked and properly washed with tap water and air dried under shade for two weeks. The dried samples were then pulverized in heavy duty blender and stored in sterile polythene bag for analysis. Whatman number 1 filter paper was used to filter the extracts and filtrates were concentrated using rotary evaporator. The crude extracts were partitioned into water, ethanol and n-hexane fractions by liquid-liquid extraction.

Extraction of Plants Materials

Water, Ethanol, n-Hexane Extracts

The leaves of *Vernonia amygdalina and Newbouldia laevis* plants 1g each was extracted with 100ml each of water, ethanol and n-hexane using rotary evaporator at 90℃, 68.3℃, and 58.7℃. The crude extracts were obtained and were used for preliminary qualitative test for phytochemicals and antibacterial evaluation.

Sterility Test for Extract

One loopful of water, ethanol and n-hexane extract were inoculated onto Nutrient Agar and Sabouraud Dextrose Agar and incubated for 24-28hrs. Absence of visible growth indicated.

Test Organisms Authentication

The isolates were identified by standard microbiological procedures as described by Cheesbrough (2004).

Cultural Method

From the stock culture, a loopful of *Eschirichia coli* was streaked on Eosine Methylene Blue Agar (EMBA), *Streptococcus pneumonia* and *Staphylococcus aureus* on Mannitol Salt Agar (MSA).

Phytochemical Screening

Test for Saponins

2cm^3 of each filtrate was diluted with 5cm^3 of distilled water and vigorously shaken, then allow to stand for 30mins. Persistent frothing indicated the presence of saponins. To the foam, 3 drops of olives oil were added and shaken vigorously. Formation of an emulsion was considered as a confirmation for saponins (Gariapathi, *et al.,* 2011).

Test for Tannins

Each extracts (0.5g) was boiled in 100cm^3 of water in a test tube and then filtered. A few drops of 0.1% ferric chloride were added and observed for brownish-green or a blue-black colouration (Sofowora, 1984).

Test for Flavones

Sodium Hydroxide test: each filtrate (2cm^3) was acidified with 1% HCL followed by drops of 20% of NAOH canary yellow colour indicates presence of flavonoids (Gariapathy *et al.,* 2011).

Test for Alkaloids

Mayer's test: five (5) drops of Mayor's reagent were added into 2cm^3 of each extract in a test tube. Appearance of off-white to dirty brown colour was taken as indication for the presence alkaloids.

Wanger's Test: five (5) drops of wanger's reagent were added to 2cm^3 of each extract in test tube. Formation of reddish brown colour was taken as indication for the presence of alkaloids.

Test for Purine

Test the cardiac Glycosides.

Each extracts (0.5g) was dissolved in 2cm^3 of glacial acetic acid containing one drop of ferric chloride solution and shaken vigorously, then, 1cm^3 of concentrated sulphuric acid was added and carefully shaken. A positive test was indicated by a blue layer at the interface (Harbone, J. 1999).

Test for Quinoline

Test for Anthraquinone

The extract (0.5g) was boiled with 10cm^3 of sulphuric acid (H_2SO_4) and then filtered using Whiteman filter paper No 1. The filtrate was shaken with 5cm^3 of chloroform. The chloroform layer was pipette into another test tube and 1cm^3 of 10% of dilute ammonia was added. The resulting solution was observed for colour changes (Trease and Evans, 1989).

Test for Antibacterial Activity

Bacteria culture used in this study were obtained from the Microbiology Laboratory Unit at NAFDAC zonal laboratory Agulu, Nigeria. Clinical bacterial cultures used in this study were *Escherichia coli, Streptococcus pneumonia* and *Staphylococcus aureus,* all the cultures were grown in Mueller-Hinton Agar. The inoculum was used for antibacterial assay.

Antibacterial Assay

Agar diffusion assay

Antibacterial activity was evaluated using the agar diffusion assay on Mueller Hinton Agar (MHA). The media was prepared according to manufacturer's specifications.

Media Preparation

The sterilized media was allowed to cool to 45℃ and poured aseptically into appropriate number of labelled sterile Petri dishes and allow to gel. Prior to analysis, the test organisms from the agar slants were sub cultured into Tryptic Soy Broth and incubated at 37℃ for 18-24hrs. The extract and fractions (ethanol, n-hexane, water extracts and fractions) of leaves of *Vernonia amygdalina* and *Newbouldia laevis* were tested for antimicrobial activities by agar well diffusion assay. Bacteria isolated were prepared to match

0.5 McFarland standards. The procedure was the same for all test organisms. Using a sterile cork borer of 8mm diameter, two holes were made, in each of culture plates, each of the two holes were filled with a concentration 1g/100ml of ethanol, n-hexane and water extracts of plants. The cultures were then incubated at 37℃ for 24hrs. The clear zones of incubation were observed after the incubation period (Cheesbrough, 2002). Diameters of the zones of growth inhibition were measured in millimetre for each concentration of the extract and fractions used, using a meter rule. The entire test was conducted in duplicate and the mean values of zone of incubation calculated. Commercial antibiotics (Azithromycin 100mg/ml) and solvents were used as positive and negative controls respectively to determine the sensitivity of the isolates.

Determination of Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentrations of the extracts against tested microorganisms were determined by broth dilution method. Tryptic Soy Broth was prepared according to manufacturer's specifications. A two-fold serial dilution (10^{-1} – 10^{-6}) was done by transferring 0.5ml sterilised media into sterile test tubes aseptically, then 0.5ml of the water, and ethanol and 10^{-2} n-hexane extracts of each samples (each at 100mg) were serially transferred from the first tube to the last tube using pipette with sterilized tips to obtain various concentration of 50, 25, 12.5, 6.25, 3.125, 1.5625mg/ml of the different plant extracts. 0.5ml of 18-24hrs old standardized culture of each isolate was also used. Each was inoculated into the various test tubes accordingly to further obtain various concentrations of 25, 12.5, 6.25, 3.125, 1.5625, 0.78125mg/ml. Three control tubes were maintained for each test batch, (1) containing growth medium alone, (2) extract without inoculums, (3) containing growth medium and inoculums. All tubes were covered with non-absorbent cotton wool and incubated at 37℃ for 24hrs. The minimum inhibitory concentrations (MICs) were read.

Determination of Minimum Bactericidal Concentration (MBC)

The minimum bactericidal concentrations were determined by first selecting tubes that showed no growth during MIC determination; a loopful from each tube was subcultured onto extract free agar plates, incubated for another 24hrs at 37℃. The minimum bactericidal concentration was considered as the lowest concentration that could not produce a single bacterial colony (Richard, *et ai.,* 2004).

RESULTS

The result of the qualitative phytochemical screening revealed the presence of alkaloids, Saponins, Tannins, Flavones, glycosides, quinolines and anthraquinones (table 1). This report revealed that the water, ethanol and n-hexame crude extracts contain alkaloids, flavones and quinoline while all solvents showed the presence of alkaloid.

Antibacterial sensitivity tests revealed that the ethanol extracts of *Newbouldia laevis* had higher inhibitory zones followed by water extracts of *Vernonia amygdalina* and n-hexane extracts. These observed differences between these plant extracts could be as a result of insolubility of the bioactive compounds in water and n-hexane. Ethanol extracts inhibited the growths of all the test organisms (*E. Coli, Streptococcus pneumonia and Staphylococcus aureus*) while water extracts did not inhibit the growth of some test organisms (*E. Coli, Staphylococcus aureus*), n-hexane extracts did not inhibit only the growth of *Staphylococcus aureus*. But ethanol extracts of *Newbouldia laevis* plant had a greater zone of inhibition and this may be due to ability of ethanol to extract bioactive compounds like Saponins, Tannins, Flavones, Glycosides, Alkaloids and Quinolines.

The MIC of *V. amygdalina* and *N. laevis* were ranged between 6.25-25mg/ml. MIC for *V. amygdalina* varied between 12.5-25mg/ml for

ethanol=25mg/ml, water=12.5mg/ml, n-hexane=25mg/ml. *N. laevis* has its MIC ethanol=12.5mg/ml, n-hexane=25mg/ml.

The MIC of the extracts of both plants against the tested organisms ranged between 6.25-25mg/ml while the minimum lethal concentration of the different extracts against the tested organisms ranged between 6.25-1.5625mg/ml.

Table 1: Preliminary phytochemical screening of ethanol, water and n-hexane extracts of *V. amygdalina* and *N. laevis.*

Phytochemicals	*V. amygdalina*			*N. laevis.*		
	Ethanol	Water	n-hexane	Ethanol	Water	n-hexane
Saponins	+++	+++	-	-	+++	-
Tannins	+++	-	+++	+++	-	-
Flavones	+++	+++	-	-	+++	+++
Glyosides	+++	-	-	+++	-	-
Alkaloid (Wagner)	-	-	-	+++	-	+++
Alkaloid (Mayer)	+++	-	-	-	-	-
Alkaloid (Indole)	-	+++	-	+++	++	++
Alkaloid (Morphine)	+++	+++	+++	+++	+++	+++
Purine	-	-	-	-	-	-
Quinolone	++	-	+++	+++	+++	+++
Anthraquinone	-	-	-	-	-	-

Keys: - is absent, + is present, ++ is significant, +++ is very significent.

Antibacterial Activities

The antimicrobial activities of different solvent extracts of *V. amygdalin* leave against *Escherichia coli, Streptococcus pneumonia* and *Staphylococcus aureus* are presented in Table 2 based on Agar diffusion assay.

Table 2: Zone of Incubation Results (mm)

Sample Extracts	Conc. (Mg/ml)	*E. coli* (mm)	*Streptococcus pneumonia* (mm)	*Staphylococcus aureus* (mm)
Ethanols extract	100	30	47	40
	50	28	41	32
	25	20	38	30
N-hexane extract	100	50	45	-
	50	45	40	-
	25	40	33	-
Water extract	100	-	62	-
	50	-	58	-
	25	-	44	-

Results obtained are mean duplicate values.

Key: - indicate no zone of inhibition.

Antimicrobial Activities of Different Solvent Extracts of *Newbouldia laevis* against *Escherichia coli, Streptococcus pneumonia* and *Staphylococcus aureus* based on Agar Diffusion Assay.

Table 3: Zone of Incubation Results (mm)

Sample Extracts	Conc. (Mg/ml)	*E. coli* (mm)	*Streptococcus pneumonia* (mm)	*Staphylococcus aureus* (mm)
Ethanol extract	100	-	80	-
50	-	75	-	
25	-	70	-	
O-hexane extract	100	-	52	-s
50	-	50	-	
25	-	48	-	
Water extract	100	-	-	-
50		-	-	-
25		-	-	-

Results obtained are mean duplicate values.

Key: - indicate no zone of inhibition.

The Minimum Inhibitory Concentration (MIC) and Minimum Lethal Concentration (MLC)

The results of MIC and MLC on *Eschericia coli, Strptococcus pneumonia* and *Staphylococcus aureus* are shown in the tables below.

Table 4: Minimum Inhibitory Concentration (MIC) and Minimum Lethal Concentration (MLC) Profile of the extract of *V. amygdalina* against tested Organisms.

Test Organisms	Ethanol extract (mg/ml)		n-hexane (mg/ml)		water extract (mg/ml)	
	MIC	MLC	MIC	MLC	MIC	MLC
E. Coli	12.5	-	25.0	-	-	-
S. Pneumonia	25.0	-	12.5	-	25.0	-
S. aureus	12.0	3.13	-	-	-	-

Keys: - indicates no antimicrobial activity, MIC=Minimum inhibitory Concentration,
MLC=Minimum Lethal Concentration.

Table 5: Minimum Inhibitory Concentration (MIC) and Minimum Lethal Concentration (MLC) Profile of the extract of *N. laevis* against tested Organisms.

Test Organisms	Ethanol extract (mg/ml)		n-hexane (mg/ml)		water extract (mg/ml)					
			MIC	MLC		MIC	MLC		MIC	MLC
E. Coli			-	-		-	-		-	-

S. Pneumonia	25.0	-	12.5	-	-	-		
S. aureus	-	-	-	-	-	-		

Keys: - indicates no antimicrobial activity, MIC=Minimum inhibitory Concentration, MLC=Minimum Lethal Concentration.

DISCUSSIONS

The result of phytochemical screening in table 1 which indicate that the leaves of the plants are very rich in many secondary metabolites. Tannins were known for their oxidation inhibiting activity which had been known for long time (Calvi *et al.,* 1995). These are in line with the earlier report by (Gill, 1992) who said that herbs that contain tannin as their main components are astringent in nature and are used for treating intestinal disorder. The presence of tannins in the plants could be responsible for its traditional use in treating intestinal disorder such as diarrhoea, dysentery and inflammatory condition of the digestive tract (Burkill, 1985). Tannins are widely used in herbal medicine to treat wounds and to arrest bleeding that is probably the reason why the plants is used in treating skin irritation (Nguyi, 1988). FLavonoids are phenolic compounds that protect plant from the damaging effect caused by ultra-violet light and microbial infection (Allan and Miller, 1996). Flavones also posses many pharmacological properties such as antioxidant activities and anti-inflamatory activities. The potent antioxidant activity of flavones; their ability to scavenge hydroxyl radicals' superoxide anions and lipidperoxide radicals may be the most important function of flavones (Alan and Miller, 1996). These could be the reason why the plant can withstand many climatic conditions and also its use for pharmacological purposes (Linuma *et al.,* 1994). Saponins are well known for their antioxidant properties (Beak *et al.,* 1996). Saponins exhibit cytotoxic effect and growth inhibition against a variety of cells, making them have anti-infamatory and anti-cancer properties (Akindahusi and Salawu, 2005). They have a property of precipitating and coagulating red blood cells (Okwu and Josiah, 2006). Saponins have been reported to have antibacterial, anti-fungal and antiviral activities (Fingen *et al.,* 2005). The presence of saponins in the plant could be responsible for their traditional use in the treatment of skin diseases and venereal diseases.

Cardiac-glycosides are non-reducing substances that are composed of sugar and non sugar parts. They are used therapeutically mainly in the treatment of cardiac failure due to their anti-arrythmic effects (Harbone, 1984). Also the presence of Cardiac-glycosides could be responsible for the traditional use of *N. laevis* as a laxative. Alkaloids are naturally occurring organic bases. Table 1 shows their presence in both plants parts. Alkaloids are used as anaesthetic agent.

The result of antimicrobial activity shows that the inhibitory activities exhibited by the extracts agree with the report of (Gill, 1992), all of whom linked antimicrobial properties of plants to the presence of bioactive secondary metabolites. It was demonstrated that the activity of the extracts was concentration dependent. An increase in the concentration of the fraction yielded higher activity as shown by the diameter zone of inhibition (tables 2 and 3). Highest activities were observed with the standard antibiotic azithomycin (control) probably because the antibiotic was in the pure form (Prescott *et al.,* 2002). The fact that organism may need higher concentration of extracts to exhibit or kill them may be due to their cell wall components (Banso, 2009). The results in tables 4 and 5 indicate that the minimum inhibitory concentration (MIC) of the plant extracts of *V.*

amygdalina and *N. laevis* range between 6.25mg/ml-25mg/ml. The effect of the plant fractions on the MIC ffor the test microorganism is in line with the report that microorganisms varied widely in the degree oftheir susceptibility (Aboaba and Efuwape, 2001).

The extracts finally prevent the growth and killed the organism completely at the MLC between 1-53mg/ml-6.25mg/ml. The MIC and MLC are normally used to evaluate the efficacy of the agent and show that the fraction can inhibit the growth of some microorganism. Several studies had documented the scientific basis that bioactive constituents inhibit the growth of various microorganisms at different concentrations (Okeke, 2005). The activity of the plants extracts against the gram positive and gram negative bacteria is an indication of the presence of broad spectrum antibiotic compound in the plants (Okwu and Josiah, 2006). The large zone of inhibition indicates the potency of the active principle of the plants in tables 2 and 3. The large zone of inhibition exhibited by the extracts and fractions of *V. amygdalina* and *N. laevis* on *Escherichia coli, Streptococcus pneumonia* and *Staphylococcus aureus*, suggest that the plant can be used in the treatment of infections commonly associated with the bacteria. The tables 2 and 3 show that the ethanol extracts exhibited higher activity on the test organisms.

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