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RESEARCH ARTICLE

ANTIBACTERIAL ACTIVITY OF VERNONIA AMYGDALINA EXTRACTS AGAINST MULTI-DRUG RESISTANT GRAM-POSITIVE AND GRAM-NEGATIVE BACTERIA

Adebesin IO[1], Sule IO[2], Ogunniyi ED[3], Amoka, SO[4]

[1,2,3,4]Department of Microbiology, Faculty of Life Sciences, University of Ilorin, PMB 1515, Nigeria

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Abstract: The incidence of antibiotic resistance is one of the challenges in the treatment of different ailments. Hence, this study focused on the use of *V. amygdalina* (ethanolic and methanolic) extracts on some selected bacterial isolates. Different concentrations of the extracts were prepared and the residues were re-suspended in the solvents. The zone of inhibition, minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and antibiotic susceptibility patterns were determined according to standard methods. The ethanolic extract appeared to produce better antibacterial effect than methanolic extract. *K. pneumoniae* and *S. marcescens* were inhibited by ethanolic extract of 12.5-200mg/ml used. However, only *S. aureus* was inhibited by all methanolic extract concentrations used. Most of the isolates (83.3%) had lower MIC in ethanolic extract when compared with methanolic extract. The MIC of the isolates in the ethanolic and methanolic extracts ranged from 12.5-100mg/ml and 25-100mg/ml respectively. Two of the isolates, *B. cereus* and *K. pneumoniae* did not cease to grow on MBC plates even at the highest concentration of 200mg/ml used. All the isolates showed multiple antibiotic resistance patterns with AMR index range of 0.63-1.0. All the isolates were resistant to augmentin, cefexime, cefuroxime, ceftazidime and nitrofurantoin. The order of susceptibility of the isolates to the antibiotics were gentamicin (83.3%) > ofloxacin (66.7%) > ciprofloxacin (33.3%). It is concluded in this study that the extracts of *V. amygdalina* inhibited 83% of the isolates at lower concentration of 25mg/ml, were bactericidal at 100-200mg/ml for 66.7% of the isolates and produced higher inhibition zone in comparison with the standard antibiotics on the isolates.

KEYWORDS: Antibacterial activity, *Vernonia amygdalina*, Antibiotics, Inhibition

INTRODUCTION:

Before scientists' do well in researches for drugs that can cure human and other animals' diseases, the traditional means of preventing, treating and curing diseases involve the use of plant parts like stem, root, leaves, bark, seed and flower into concoction has been in practice.

They did this without knowing the components of the plants that work against these pathogenic microorganisms. Medicinal plants are known to contain substances which could be used for diseases treatment purpose and production of synthetic drugs. These substances are called phytochemical.

Corresponding Author:
ADEBESIN, Ibraheem Olakunle,
Department of Microbiology, Faculty of Life Sciences, University of Ilorin, PMB 1515, Nigeria

The medicinal value of these plants lies in their phytochemical constituents such as alkaloids, tannin, flavonoids, saponins and phenolic compound. Many plants have been screened to have medicinal importance, some of which include: *Carthamus tinctorius* (safflower), *Corchorous olitorius* (okro), *Azadirachta indica* (neem), *Allium sativum* (garlic), *Ocimum gratissimum* (scent leaf) and *Zingiber officinale* (ginger). These plants have been used traditionally in the treatment of ailments such as stomach disorder, fever symptoms and cough. [1,2] Upon the discovery of antibiotics and their use for diseases treatment in human and livestock, there was initial believe in medical and pharmaceutical sciences that this discovery would bring total eradication to infectious disease. [3] The indiscriminate use of antibiotics has become the major set-back and factor for the emergence, dissemination and spread of several groups of multi-drug resistant strains. [4] Nowadays, there is renewed interest in searching for new sources of drug from plants with novel targets and mode of actions due to the slow pace in the development of new antibiotics. [5,6] Plants have the major advantage of being effective, accessible and cheaper alternative source of drugs.

In Nigeria, *V. amygdalina* is one of the plants used in homes for treating diseases such as diarrhea, wound, sore throat, deworming. *V. amygdalina* belongs to the family of Asteraceae growing in tropical Africa with the height of 2-5m and petiole leaf of about 6mm in diameter. [7] The bitterness of the plant which is characterized by after taste sweetness is due to the presence of saponins, tannin, glycoside and alkaloid. [8] It has been reported to have therapeutic abilities like antihelmitic, hypoglycemic, antitumorgenic, hypolipidermic, [9] active anticancer, antibacterial, antimalarial, antiparastic agent [10], relieve inflammation and decrease depression symptoms. [11] The leaf extracts of this plant have been used against gastro-intestinal parasitic infections. [12] This study was done to determine the antibacterial activities of the ethanolic and methanolic extracts of *V. amygdalina* on some selected bacterial isolates. The resistance or susceptibility of the isolates to some standard antibiotics was also determined.

MATERIALS AND METHODS:

Collection of Plant Materials

Fresh leaves of *Vernonia amygdalina* were collected from Ipata market, Ilorin, Kwara State, Nigeria. Identification and authentication of the leaves were carried out in the herbarium section of Department of Plant Biology, University of Ilorin; Ilorin, Nigeria. The leaves were air dried and grinded by use of blender.

Preparation of Crude Extracts

One hundred gram (100g) of the grinded *V. amygdalina* leaves was soaked in 500ml each of ethanol and methanol for 24hours separately. The extracts were sieved to remove debris. The filtrates were then filtered through Whattman No 11 filter paper. The solvent in the filtrate was evaporated in a rotary evaporator to get the plant extract residue. [13]

Preparation of Concentration of Plant Extract

One gram (1g) each of *V. amygdalina* extracts was added to 5ml of ethanol and methanol separately to give a concentration of 200mg/ml. Other lower concentrations of 100, 50, 25 and 12.5mg/ml were prepared by double fold dilution method. [14]

Collection and Maintenance of the Test Organisms

Pure cultures of bacterial isolates of *S. aureus, K. pneumoniae, P. aeruginosa, E. coli, B. cereus* and *S. marcescens* were obtained from the Department of Microbiology, University of Ilorin and University of Ilorin Teaching Hospital, Nigeria and were re-identified by PCR.

Determination of Zone of Inhibition of the Extracts on the Isolates

The isolates were standardized using 0.5 McFarland standard. [8] The antibacterial activity of leaf extracts on the Isolates was determined by agar well diffusion method. The standardized culture was streaked on sterile solidified Mueller Hinton agar (MHA). Then, 2 wells were bored on each MHA plates with cock borer of 4mm in diameter. From the 200mg/ml concentrated extract, 0.1ml extract was transferred into the wells using sterile pipette. This process was repeated for other concentrations and extract with each concentration in duplicates. The wells were sufficiently spaced to prevent the resulting zones of inhibition from overlapping. The plates were incubated at 37^0C for 24hr. After incubation, the antibacterial activity of the plant extract was determined by measuring the zone of inhibition with meter rule in millimeter. [15]

Antibiotics Susceptibility Testing of the Test Bacterial Isolates

Standardized bacterial culture (0.5 McFarland) was used to streak MHA plates. Multiple antibiotic discs was gently placed on the plate using sterile forceps. The diameter of clear zone of inhibition in the immediate vicinity of each disc was measured in millimeter after incubation at 37°C for 24hours and interpreted as resistant, intermediate or susceptible. [16]

Determination of Minimum Inhibitory Concentration (MIC) of the Extracts

MIC of the ethanolic and methanolic extracts were determined for each of the test isolates at varying concentrations of 12.5, 25, 50, 100 and 200mg/ml by broth dilution method. The standardized culture (0.1ml) was inoculated into sterile 5ml nutrient broth in test tubes containing 0.5ml of different concentrations of the extracts. Control test tube containing only the 5ml nutrient broth and 0.5ml of the extract was made. The tubes were incubated at 37°C for 24hours and examined for bacterial growth by comparing its turbidity with the control. The tube with the lowest concentration of extract that inhibited particular bacterial growth after incubation was recorded as the MIC of that bacteria. [17]

Determination of Minimum Bactericidal Concentration (MBC) of the Extracts

Among the tubes used for MIC, the tubes without visible growth were used to determine MBC. The culture (0.1ml) was pipetted from the MIC tubes onto solidified sterile nutrient agar plates, spread and incubated at 37°C for 24hours. After incubation, the tube with minimum concentration where there was no bacterial growth on the plates was recorded as the MBC. [17]

RESULTS:

Inhibition of Bacterial isolates by *V. amygdalina* Ethanolic Extract.

The zone of inhibition produced by ethanolic extract ranged from 20-33mm against *S. aureus*, 14-37mm against *K. pneumoniae*, 19-34mm against *P. aeruginosa*, 15-34mm against *E. coli,* 20-30mm against *S. marcescens* and 20-32mm against *B. cereus* (Table 1).

Table 1: Inhibition of Bacterial Isolates by *V. amygdalina* Ethanolic Leaf Extract

	Zone of inhibition (mm)				
Test isolates	200mg/ml	100mg/ml	50mg/ml	25mg/ml	12.5mg/ml
S. aureus	33	28	23	20	-
K. pneumoniae	37	34	26	20	14
P. aeruginosa	34	28	25	19	-
E. coli	34	30	26	17	15
S. marcescens	*30*	27	24	20	-
B. cereus	32	28	20	-	-

- = no inhibition

Inhibition of Bacterial Isolates by *V. amygdalina* Methanolic Extract

The antibacterial activity produced by methanol is lower when compared to ethanol. *S. aureus* was the most susceptible and least on *E. coli*. The zone of inhibition produced range from 15-32mm on *S. aureus*, 11-24mm on *K. pneumoniae*, 14-28mm on *P. aeruginosa*, 13-22mm on *E.coli*, 15-26mm on *S. marcescens* and 16-30mm on *B. cereus* (Table 2).

Table 2: Inhibition of Bacterial Isolates by *V. amygdalina* Methanolic Leaf Extract

	Zone of inhibition (mm)				
Test isolates	200mg/ml	100mg/ml	50mg/ml	25mg/ml	12.5mg/ml
S. aureus	32	29	25	20	15
K. pneumoniae	24	20	17	11	-
P. aeruginosa	28	22	16	14	-
E. coli	22	18	15	13	-
S. marcescens	26	22	20	15	-
B. cereus	30	25	21	16	-

- = no inhibition

MIC of the Extracts on Test Isolates

The ethanolic extract had the least MIC of 12.5mg/ml on *S. aureus, S. marcescens* and *B. cereus* while it was 25mg/ml for *K. pneumoniae* and *E. coli.* The extract had the highest MIC of 100mg/ml on *P. aeruginosa* (Table 3). The methanolic extract had the least MIC of 25mg/ml on *S. aureus,* 50mg/ml for *P. aeruginosa, E. coli* and *S. marcescens*, and 100mg/ml for *K. pneumoniae and B. cereus* (Table 3).

Table 3: Minimum Inhibitory Concentration (MIC) of *V. amygdalina* Leaf Extracts.

MIC (mg/ml)		
Test Isolates	Ethanol	Methanol
S. aureus	12.5	25
K. pneumoniae	25	100
P. aeruginosa	100	50
E. coli	25	50
S. marcescens	12.5	50
B. cereus	12.5	100

MBC of the extracts on Test Isolates

The MBC of *V. amygdalina* ethanolic extract on *B. cereus* was above the limit of 200mg/ml used in this study. For the rest of the isolates, the MBC ranged between 50-200mg/ml (Table 4). The methanolic extract was unable to result in the death of *K. pneumoniae* even at highest concentration of 200mg/ml used in this study. MBC, however, ranged from 50-200mg/ml for the other isolates (Table 4).

Table 4: Minimum Bactericidal Concentration (MBC) of *V. amygdalina* Leaf Extracts.

MBC (mg/ml)		
Test Isolates	Ethanol	Methanol
S. aureus	200	100
K. pneumoniae	200	ND
P. aeruginosa	100	100
E. coli	50	100
S. marcescens	100	50
B. cereus	ND	200

ND = MBC not detected even at 200mg/ml

Antibiotic Susceptibility Patterns

The antibiotic susceptibility test revealed that all the bacterial isolates were resistant to augmentin, cefexime, cefuroxime, nitrofurantoin and ceftazidime. Most of the isolates (83%) were susceptible to gentamicin, 66.7% susceptible to ofloxacin and 33.3% to ciprofloxacin (Table 5). The MAR index of the isolates ranged from 0.63-1.0 (Table 5).

Table 5: Susceptibility of Bacterial Isolates to Standard Antibiotics

	Zone of Inhibition (mm)								
Test Isolates	AUG	OFL	CXM	GEN	CRX	CAZ	NIT	CPR	MAR index
S. aureus	R	R	15(S)	R	R	R	R	R	0.88
K. pneumoniae	R	R	R	R	R	R	R	R	1
P. aeruginosa	R	20 (S)	R	15(S)	R	R	R	R	0.75
E. coli	R	20 (S)	R	14(S)	R	R	R	R	0.75
S. marcescens	R	23 (S)	R	18(S)	R	10(R)	24 (S)		0.63
B. cereus	R	20 (S)	R	20(S)	R	R	R	22 (S)	0.63

AUG=Augmentin (30µg), OFL= Ofloxacin (50µg), CXM= Cefexime (5µg), GEN= Gentamicin (10µg), CRX= Cefuroxime (30µg), CAZ= Ceftazidime (30µg), CPR= Ciprofloxacin (5µg), NIT= Nitrofurantoin (300µg), R= Resistant, S= Susceptible, MAR= Multiple antibiotics resistance.

DISCUSSION:

Bacterial infections that were known to be easily controlled when good hygiene is practiced and antibiotics are used are becoming difficult in treating due to the increase in resistance to various antibiotics and the speed at which the resistant genes are transferred.

In this study, the antibacterial activity was found to be dependent on the solvents used for the phytochemical's extraction, the extract concentrations and the isolates involved. The ethanolic extract was observed to have more antibacterial activity (zone of inhibition) than methanolic extract. This can be linked to ethanol being better extraction agent than methanol. The determination of antibacterial activity of *V. amygdalina* ethanolic and methanolic leaf extracts by agar well diffusion method showed that the leaf extract possessed antibacterial potential against Gram positive and Gram-negative bacteria. Hence, *V. amygdalina* can be used as broad-spectrum phytochemical agent. *K. pneumoniae* was the most susceptible to the ethanolic extract followed by *E. coli, P. aeruginosa, S. aureus, S. marcescens* and lastly *B. cereus*. The ethanolic extract was more active on Gram negative than Gram positive while methanolic extract was more active on Gram positive than Gram negative. This susceptibility can be used to explain the extracts' usage for the treatment of diseases such as wound, cough, dysentery, diarrhea, sore throat in native medicine [6]. This difference in the bacterial group behavior can be attributed to difference in affinity to the bioactive components of the leaf and bacterial cell wall composition. Lack of inhibition to *B. cereus* and high MBC concentration may be attributed to the fact that this bacterium can produce endospores under unfavorable conditions. Hence, enhancing its survival. Higher concentration of the extracts appeared to be able to produce large zone of inhibition than low concentration.

In a similar study by Uzoigwe and Agura, *V. amygdalina* ethanolic extract was found to be more effective against *K. pneumoniae* than other pathogens which is in agreement with our study's result. [18] The outcome of this study showed that *V. amygdalina* leaf extract possessed antibacterial potential against *S. aureus, P. aeruginosa E. coli, K. pneumoniae, B. cereus* and *S. marcescens* as being reported. [6, 8, 10, 19] This finding agreed with the reports from Preethi et al. and Bukar et al. who concluded that even at lower concentration, the leaf extracts still possess antimicrobial effects on clinical isolates [6, 20]. This higher activity showed by ethanol can also be attributed to the reason of being used as liquid for herbs preparation and usage by local herbalists. [21] Also, Tula et al. and Bukar et al. reported *V. amygdalina* ethanolic leaf extract as having higher antibacterial effect than methanolic leaf extract against tested pathogens. [5, 6] Hence, extracts from these leaves could be used for the treatment of diseases caused by pathogens.

The MIC which showed similar pattern of inhibition when compared with agar diffusion method. The

MIC ranged from 25-100mg/ml in methanolic extract and 12.5 to 100mg/ml on ethanolic extract. The MIC and MBC values of *S. aureus, P. aeruginosa* and *K. pneumoniae* in this study follow the same pattern as reported by Evbuomwan et al [10].

The MBC results clearly indicate that some of the isolates need higher concentration than the range used in this study for them to be killed by the phytochemicals. In this study, *K. pneumoniae* and *B. cereus* showed that they need above 200mg/ml. The MBC of both extracts was found in the range of 50-200 mg/ml (Table 4). The need for higher values of methanolic and ethanolic extract is not surprising since some of the isolates are endospore former (*B. cereus*) and capsulated bacteria (*K. pneumoniae*). This capsule and endospore offer them protection against direct contact with the phytochemicals. Organisms such as *S. aureus, E. coli* and *P. aeruginosa* have been reported to possess plasmid as part of their resistance factors to antibiotics and phytochemicals.

V. amygdalina extracts were compared with commercial antibiotics for their inhibitory potential (Table 5). When compared, the extracts were found to possess better antibacterial effect than these commercial antibiotic discs. Hence, this bioactive component could be used for the next-line drug development. All the bacteria were found to be resistant to augmentin, cefexime, cefuroxime, nitrofurantoin and ceftazidime with varying degrees of susceptibility to ofloxacin, gentamicin and ciprofloxacin by *P. aeruginosa, E. coli, S. marcescens, B. cereus* and *S. aureus.* Among the Isolates, 83.3%, 66.7% and 33.3% were susceptible to gentamicin, ofloxacin and ciprofloxacin respectively. *K. pneumoniae* was found to be resistant to all the antibiotics (100%), followed by *S. aureus* 88%, *P. aeruginosa* and *E. coli* 75%, *B. cereus* and *S. macerscens* 63%. All the isolates were resistant to at least 5 antibiotics in this study with MAR index of at least 0.63 which is far higher than 0.2 for an organism to be classified to as possessing multiple antibiotics resistant pattern .

CONCLUSION:

This study shows that both ethanolic and methanolic extracts of *V. amygdalina* inhibited all the test isolates especially at 50-200mg/ml when compared with standard antibiotics and can be suitable replacement. Furthermore, ethanol was seen to be a better extraction solvent than methanol.

RECOMMENDATION:

It is recommended that extract of *V. amygdalina* leaves can be used to treat infections caused by *S. aureus, P. aeruginosa, E. coli, K. pneumoniae, B. cereus* and *S. marcescens.*

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