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Phytochemical and Antibacterial Activities of *Vernonia Amygdalina* Leaves (Bitter Leaf) on two Drug Resistant Bacteria

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Abstract: *This present study was aimed to investigate the phytochemical and in-vitro antimicrobial activities of aqueous and ethanolic extracts of Vernoniaamygdalina (bitter leaf) on Staphylococcus aureus and Escherichia coli. The phytochemical constituents of this medicinal plant was carried out using standard methods. Agar well diffusion method was used to determine the antibacterial activity of the plant extracts. The Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the plant extracts on the test isolates were determined by the macro broth dilution technique. Phytochemical analysis showed the presence of alkaloids, tannins, cardiac glycosides and saponins in all plant extracts. The hot ethanolic extract of Vernonia amygdalina showed antimicrobial activity with the mean inhibitory zone diameter of 8.0 – 19.0 mm against Staphylococcus aureus and 7.0 – 20.0 mm against Escherichia coli. The minimum inhibitory concentration (MIC) ranged between 25 and 150 mg/ml while the minimum bactericidal concentration (MBC) range between 25 and 100mg/ml. In conclusion, Vernonia amygdalina has potential bioactive phytochemicals that are responsible for its antibacterial activities. It has also proven that bitter leaf extract is a more antibacterial substance than conventionally used antibiotics.*

Keywords: *Ethanolic, Aqueous, Minimum inhibitory concentration, Minimum Bactericidal Concentration, Staphylococcus aureus, Escherichia coli.*

1. INTRODUCTION

Before scientists made inroads into the research of drugs that cure human infections, traditional means of treating diseases involved using concoctions from plants, either in single form or in mixtures. This they did without knowing that these agents were used against some pathogenic microorganisms [1]. Plants have been found useful to man, not only as food or as sources of raw materials for industrial purposes, but also as sources of medicaments [2]. Limited knowledge about the practices of the use of plants for medication (i.e. herbal medicine and lack of scientific studies of plants) have led to the neglect of novel bioactive components on the field that may bring about remarkable result in the treatment of infectious diseases, with little or no side effects [3].

Medicinal plants are known to contain in one or more of its organ substances that can be used for therapeutic purposes or as precursor for the synthesis of useful drugs [1]. Many of such plants known to be used primitively to alleviate symptoms of illnesses have been screened to have medicinal importance, a good example of which is *Vernoniaamygdalina* (Bitter leaf). This planthas been reportedly used in the traditional treatment ofailments such as diabetes, malaria, stomach disorder, fever symptoms and cough etc [4].

Vernonia amygdalina commonly called bitter leaf in English, “oriwo” in Edo, “ewuro” in Yoruba, “shikawa” in Hausa, and “olubu” in Igbo [5]. *Vernonia amygdalina*, a perennial shrub of 2-5m in height that grows throughout tropical Africa. The plant is scientifically classified as belonging to the Kingdom Plantae. It is an angiosperm, of the order *Asterales*, of the family *Asteraceae*, genus *Vernonia*, and *s*pecies *amygdalina*. The leaves are green and have a characteristic odor and bitter taste [6]. In many parts of West Africa, the plant has been domesticated. *Vernonia amygdalina* is drought tolerant (though it grows better in a humid environment). The plant has also been shown to contain

appreciable quantities of ascorbic acid and caroteinoids [6]. The extracts displayed potent antimicrobial activity against some pathogenic organisms [7]. The bitter taste is due to anti-nutritional factors such as alkaloids, saponins, tannins, and glycosides [8]. The leaves are used as green leafy vegetable and may be consumed either as a vegetable (leaves are macerated in soups) or aqueous extracts used as tonics for the treatment of various illnesses [8]. It is effective against amoebic dysentery [9], gastrointestinal disorders and has antimicrobial and antiparasitic activities [5]. The leaves are widely used for fevers and are known as a quinine–substitute in Nigeria and some other African countries [10].

The search for newer sources of antibiotics is a global challenge pre-occupying research institutions, pharmaceutical companies and academia, since many infectious agents are becoming resistant to synthetic drugs [11]. The situation has further been complicated with the rapid development of multi-drug resistance by the microorganism to the antimicrobial agents available. Over the past 20 years, there has been an increased interest in the development of resistance of pathogens against antibiotics caused by the indiscriminate use of modern antibiotics [12]. This study was therefore aimed to evaluate the Phytochemical properties and antibacterial efficacy of *Vernonia amygdalina* (bitter leaf) on two drug resistant bacteria.

2. Materials and Methods

2.1. Sample Collection

The leaves of *Vernonia amygdalina* were purchased from Eke Agbani market in Enugu State, Nigeria while the preparation was done at the Microbiology laboratory of Nnamdi Azikiwe University, Awka Anambra State. The leaveswere washed and placed into a neatly washed and dried tray. The leaves were then sun dried and crushed to coarse powder using a neatly washed local mortar and pestle. The powdered forms of the leaves were placed in different containers and were properly labeled and stored. It was authenticated by a Botanist, Mr. Tochukwu Egboka of Nnamdi Azikiwe University Herbarium.

2.2. Test Organisms

Bacterial cultures of *Escherichia coli and Staphylococcus aureus* obtained from the laboratory section of the Department of Microbiology, Nnamdi Azikiwe University, Anambra State, Nigeria; were used as antimicrobial test organisms. Their identity was confirmed using cultural, morphological and biochemical test as previously described [13].

The bacterial isolates were maintained on nutrient agar slants at 4°C.

2.3. Biochemical Identification of the Test Organism

2.2.1 Escherichia coli

The *E. coli* was placed on Eosine Methylene Blue agar for 18 hours. Colonies with green metallic sheen were observed which indicate a positive result for *E. coli* [14].

2.2.2 Staphylococcus aureus

The *S. aureus* was placed on Mannitol Salt Agar (MSA) for 18 hours. Smooth circular colonies with yellow colour indicate a positive result for *S. aureus* [14].

2.4. Standardization of the Tests Organism

The test organisms (*E coli and S aureus*) were standardized by the use of 24 hours old broth cultures prepared by inoculating the test organism into 5 ml of nutrient broth and the culture was adjusted to obtain 0.5 McFarland turbidity equivalent standards [13].

2.5. Preparation of Plant Material and Plant Extracts

Four different extracts namely cold ethanol, hot ethanol (80°C); and aqueous extracts i.e. cold water and hot water (80°C) were used for plant.

2.6. Preparation of Aqueous Extract

Ten grams of dried grinded leave powder was dissolved in 100 ml of distilled water for 24 hours. The mixture was filtered using Whatman's filter paper No. 1 to obtain solution free of solids. The filtrate was concentrated by drying at 37°C and stored at 4°C.

2.7. Preparation of Ethanolic Extract

Ten grams of dried grinded leave powder was dissolved in 100 ml of 95% ethanol for 24 hours. The mixture was filtered using Whatman's filter paper No. 1 to obtain solution free of solids. The filtrate was placed into evaporator to drive-off the solvent and stored at 4°C.

2.8. Extract Dilution

After preparation of the extract as described, the hot and cold aqueous and the ethanolic extract were reconstituted using sterile distilled H_20 to obtain concentrations of 200, 150, 100, 50, 12.5, 6.25 and 3.13 mg/ml.

2.9. Sterility Test of Leave Extract

The leave extracts (aqueous and ethanolic) was tested for growth of contaminants. One milliliter (1ml) of standard leave extract was inoculated aseptically unto Nutrient Agar and incubated at 37°C for 24hrs. The plates were observed for any sign of visible growth. No growth on the plates indicated/signified that the extracts were sterile.

2.10. Qualitative Phytochemical Screening of Seed Garcinia Kola

The analysis determines the biologically active compounds that are present in the leaves of *Vernonia amygdalina:* examples Alkaloids, saponins, tannins, flavonoids, glycosides, phlobatannin and anthraquinones. This was done following standard methods of [15].

2.11. Test for the Presence of Alkaloids

One gram (1g) of the ground sample was boiled in 2ml of hydrochloric acid in a water bath for 5 minutes. The mixture was allowed to cool and filtered. 1ml portion of the filtrate was treated with drops of mayers reagent. A creamy white precipitate indicates the presence of alkaloids.

2.12. Test for the Presence of Glycosides

Two grams (2g) of the sample was mixed to 10ml of distilled water and heated for 5minutes in a water bath. It was filtered using Whattman's filter paper. 2mls of the filtrate was added to 0.2ml of fehling solution A and B it turns alkaline and heated in water for 5minutes. A light blue coloration was seen which indicates the presence of glycoside.

2.13. Test for the Presence of Tannins

Half gram (0.5g) of the grounded sample was boiled in 20ml of distilled water in a test tube and then filtered. A few drops of 0.1% ferric acid were added and a brownish green colouration was recorded which shows the presence of tannins.

2.14. Test for the Presence of Phlobatannins

An aqueous extract of the plant sample was boiled with 15ml aqueous hydrochloric acid and deposition of a red precipitate was seen which shows the presence of phlobatannins.

2.15. Test for the Presence of Saponins

Two grams (2g) of the sample was boiled in 20ml of distilled water in a water bath and filtered. 10ml of the filtrate was added to 5ml of distilled water and shaken vigorously for persistent froth.

2.16. Test for the Presence of Flavonoids

Five milligram (5mls) of 10% dilute ammonia solution was added to a portion of the aqueous filtrate of the plant extract, followed, by addition of concentrated H_2SO_4. A yellow coloration observed in the extract indicates the presence of flavonoids.

2.17. Test for the Presence of Anthraquinones

Half gram (0.5g) of the plant extract is shaken with 10ml of benzene and filtered. 5ml of 10% ammonia is mixed to the filtrate. The mixture is shaken and the presence of pink, red or violet color shows the presence of anthraquinones.

2.18. Alkaloid Determination

Five grams (5g) of the sample was measured into 250ml beaker and 200ml of 10% acetic acid in ethanol was mixed and covered and stood for 4hours. This was filtered and the extract was

concentrated on a water bath to one quarter of the original volume. Concentrated ammonium hydroxide was mixed drop wise to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitate collected and washed with dilute ammonia hydroxide and then filtered. The residue is the alkaloid, which was dried and weighed.

2.19. Tannin Determination

Half gram (0.5g) of the sample was measured into 50ml distilled water and shaken for 1 hour in a mechanical shaker. This was filtered into a 50ml volumetric flask and made up to the mark. Then 5ml of the filtered was pippetted out into a test tube and mixed with 2ml of 0.1m FeCl3 in 0.1m HCl and 0.008m potassium ferrocyanide. The absorbance was measured at 120nm within 10min.

2.20. Saponin determination

Twenty grams (20g) of grounded sample was put into conical flask and 100ml of 20% aqueous ethanol was added. The sample was heated over a hot water bath for 4 hours with continues stirring at about 5500 c. The mixture was filtered and the residue re-extracted with another 200ml 20% ethanol. The combined extracts were reduced to 40ml over water bath at about 9000 c. The concentrate was transferred into a 250ml separatory funnel and 20ml of diethyl ether was added and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated. 60ml of n-butanol was added to the extracts and washed twice with 10ml of 55% aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation, the extracts were dried in the oven to a constant weight, and percentage saponin content determined.

2.21. Flavonoid Determination

Ten gram (10g) of the plant sample was extracted repeatedly with 100ml of 80% aqueous methanol at room temperature. The whole solution was filtered through Whattman's filter paper. The filtrate was later transferred into a crucible and evaporated to dryness over water bath and weighed to a constant weight.

2.22. Antibacterial Assay

The antibacterial assay of the plant leave extracts were carried out on the test isolates using Agar-well diffusion Technique. The isolates were inoculated on the surface of freshly gelled sterile nutrient agar plates by streaking using sterilized swab stick. Wells were aseptically bored on each agar plate using a sterile cork borer (6mm) and wells were properly labeled. Fixed volumes (0.1 ml) of different concentrations of the extracts (aqueous and ethanolic) were then introduced into the wells in the plates respectively. The last two wells were used as positive control well (filed with Gentamicin) and a negative control well (filled with sterile water) respectively. The plates were allowed on the bench for 40 minutes for pre-diffusion of the extract to occur and then incubated at $37^{o}C$ for 24 hours. The resulting zone diameter of inhibition was measured using a transparent ruler calibrated in millimetres. The readings were taken to be the zone diameter of inhibition of the bacterial isolate in question at that particular concentration [13].

2.23. Minimum Inhibitory Concentration (MIC)

The MIC of the potent extracts was determined according to the macro broth dilution technique. Standardized suspensions of the test organism were inoculated into a series of sterile tubes of nutrient broth containing two-fold dilutions of leaf extracts and incubated at $37^{o}C$ for 24 hours. The MICs were read as the least concentration that inhibited the growth of the test organisms [13]. The lowest or least concentration of the extract that shows no growth in the test tubes is the MIC of the extract tested.

2.24. Minimum Bactericidal Concentration (MBC)

The MBCs were determined by first selecting tubes that showed no growth during MIC determination; a loopful from each tube was sub-cultured onto already gelled nutrient agar plates using spread plate technique and incubated for 24 hours at $37^{o}C$. The least concentration, at which no growth was observed, was noted as the MBC [13].

2.25. Mode of Action of the Extracts

All plates showing no visible growth on the nutrient agar (NA) indicated bactericidal effect of the concentration of the extract used. Plates showing light growth indicated the bacteriostatic effects of

the extract concentration. Concentrations of the extracts showing moderate and heavy growth were considered to have no inhibitory effect on the organism [16].

2.26. Statistical Analysis

The data was analyzed using Statistical Package for Social Sciences (SPSS) version 20.0. The statistical tool employed was one-way Analysis of Variance (ANOVA) to determine if there was any significance among the solutions. Statistical significance tests included the use of *p-value* to assess for the role of chance. In this study, p-value = 0.05 was used to disapprove the null hypothesis

3. Results

The phytochemical analysis in table 1 shows that flavonoids, tanins, alkaloids, saponins and cardiac glycosides were present in all extracts of *V. amydalina.*

Table1. *Phytochemical Analysis of V. amydalina*

Phytochemical ethanol extract constituents	Cold H_2O Extract	Hot H_2O Extract	Cold ethanol Extract	Hot
Anthraquinones	-	-	-	-
Flavonoids	+	+	+	+
Tanins	+	+	+	+
Alkaloids	+	+	+	+
Phlobatinins	-	-	-	-
Steroids	-	-	-	-
Cardiac Glycosides	-	-	+	+
Saponins	+	+	+	+

+ = positive

- = negative

The antimicrobial properties of the aqueous extracts of *V. amydalina* against the test organisms shows that the organisms were resistant to different concentrations of both cold and hot extracts of *V. amydalina.* The result also revealed that higher concentration produced higher zones of inhibition. The univariant test showed that all the test organisms were effectively inhibited by *V. amydalina* extracts having p-values of 0.001 and 0.003 for aqueous extracts on *S . aureus* and *E. coli* respectively.

Table2. *Antimicrobial Activities of Aqueous Extracts of V. amydaline against S. aureus and E coli.*

Diameter zone of Inhibition(mm)														
	Cold aqueous extract(CAE)							Hot aqueous extract(HAE)						
Test Organismvalue	C	*12.5*	*25*	*50*	*100*	*150*	*200*	*C*	*12.5*	*25*	*50*	*100*	*150*	*200*	*P-value*
S. aureus	-	*2.0*	*3.0*	*5.0*	*8.5*	*8.5*	*10.0*	*-*	*0*	*1*	*1*	*2*	*3*	*3*	*0.001*
E coli	-	*0.2*	*1.2*	*3.0*	*7.5*	*7.5*	*9.0*	*-*	*0*	*0*	*0*	*1*	*1*	*2*	*0.003*

C = Control

The antimicrobial properties of the aqueous extracts of *V. amydalina* against the test organisms shows that the organisms were resistant to different concentrations of both cold and hot extracts of *V. amydalina.* The result also revealed that higher concentration produced higher zones of inhibition. The univariant test showed that all the test organisms were effectively inhibited by *V. amydalina* extracts having p-values of 0.011 and 0.005 for ethanolic extracts on *S. aureus* and *E. coli* respectively.

Table3. *Antimicrobial Activities of Ethanolic Extracts of V. amydalina against S. aureus &E coli*

Diameter zone of Inhibition(mm)															
	Cold ethanolic extract (CAE)							Hot ethanolic extract (HAE)							
Test organism	C	*12.5*	*25*	*50*	*100*	*150*	*200*	*C*	*12.5*	*25*	*50*	*100*	*150*	*200*	*P-value*
S. aureus	-	*1.0*	*1.5*	*3.0*	*4.0*	*5.0*	*7.0*	*-*	*8.0*	*11.0*	*14.0*	*16.0*	*18.0*	*19.0*	*0.011*
E coli	-	*1.2*	*1.8*	*2.0*	*2.5*	*3.0*	*4.0*	*-*	*7.0*	*13.0*	*16.0*	*18.0*	*19.0*	*20.0*	*0.005*

C = Control

The MIC of *S. aureus* was between 25-100mg/ml while that of *E. coli* was between 50mg/ml to 150mg/ml.

Table4. *Minimum Inhibitory Concentration (MIC) of leaf extracts of V. amydalina on S. aureus and E. coli*

Concentration of Extracts (mg/ml)								
Isolates	**200**	**150**	**100**	**50**	**25**	**12.5**	**Extracts**	**MIC**
S. aureus	-	-	-	-	-	+	CAE	25
S. aureus	-	-	-	-	+	+	HAE	50
S. aureus	-	-	-	-	+	+	CEE	50
S. aureus	-	-	-	+	+	+	HEE	100
E. coli	-	-	-	-	+	+	CAE	50
E. coli	-	-	+	+	+	+	HAE	150
E. coli	-	-	-	-	+	+	CEE	50
E. coli	-	-	+	+	+	+	HEE	150

KEY:

CAE = Cold aqueous extract

HAE = Hot aqueous extract

CEE = Cold ethanolic extract

HEE = Hot ethanolic extract

CAE = Cold aqueous extract

HAE = Hot aqueous extract

CEE = Cold ethanolic extract

HEE = Hot ethanolic extract

The MBC for *S. aureus* isolates was between the range of 25 to 50mg/ml while that of *E. coli* wasbetween the range of 25 to 100mg/ml.

Table5. *Minimum Bactericidal Concentration (MBC) of leaf extracts of V. amydalina on S. aureus and E. coli*

Concentration of Extracts(mg/ml)								
Isolates	**200**	**150**	**100**	**50**	**25**	**12.5**	**Extracts**	**MBC**
S. aureus	-	-	-	-	-	+	CAE	25
S. aureus	-	-	-	-	-	+	HAE	25
S. aureus	-	-	-	-	-	+	CEE	25
S. aureus	-	-	-	-	+	+	HEE	50
E. coli	-	-	-	-	-	+	CAE	25
E. coli	-	-	-	-	+	+	HAE	50
E. coli	-	-	-	-	-	+	CEE	25
E. coli	-	-	-	+	+	+	HEE	100

KEY:

CAE = Cold aqueous extract

HAE = Hot aqueous extract

CEE = Cold ethanolic extract

HEE = Hot ethanolic extract

CAE = Cold aqueous extract

HAE = Hot aqueous extract

CEE = Cold ethanolic extract

HEE = Hot ethanolic extract

4. Discussion

Qualitative phytochemical analysis of this study, detected the presence of alkaloids, tannins, cardiac glycosides, flavonoids and saponins in all plant extracts. Secondary metabolites of plants such as

tannins, cardiac glycosides, alkaloids and saponins and all other active principles of plants have been shown to be responsible for the antimicrobial activities shown by these extract. For instance, Glycosides serve as defense mechanisms against predation by many microorganisms, insects and herbivores [17]. Saponin has detergent properties and exhibits anti-inflammatory properties [18]. Alkaloid is a plant derived compound that is toxic or physiologically active, contains nitrogen in a heterocyclic ring with complex structure and is of limited distribution in the plant kingdom. Alkaloids are formed as metabolic by-products and have been reported to be responsible for antibacterial activity [19]. The above illustrations may therefore explain the demonstration of antimicrobial activity of the ethanolic leaf extract of *Vernonia amygdalina*

The antibacterial activity of *Vernonia amygdalina* was found to be dependent on the nature of the solvent used for extraction and the concentration of the extract. Ethanolic extract was observed to possess more antibacterial activities compared to the aqueous extract. This is attributable to the fact that ethanol due to its high volatility, extracted more of the bioactive component of the plant compared to aqueous [20]. The high activity ofthe ethanolic extracts verifies the use of the ethanolicextraction method by local herbalists [21]. Similar result was found in the works of [22].

The aqueous extracts showed no inhibition on all two isolates. [23] also reported that most active components are not water soluble. The potency of an antibacterial agent is an inverse measurement of its MIC and MBC. Plant extract or drugs that have low MIC and MBC against bacteria are said to be very potent. The reverse is also true for antimicrobial agents.

5. Conclusion

This research work has shown that *Vernonia amygdalina* has potential bioactive phytochemicals that are responsible for its antibacterial activities. It has also proven that bitter leaf extract is a more antibacterial substance than conventionally used antibiotics. Therefore, more research should be carried out to enable the purification of the specific bio-potential chemicals and their subsequent processing into chemotherapeutic agents.

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