Original Research Article

Phytochemical, Antibacterial and Toxicity Study of Leaf Extracts of *Vernonia Amygdalina,* Delile on *Salmonella* Species

ABSTRACT

Aim: The aim of the study is to evaluate the bioactive compounds, toxicity and anti-bacterial activities of leaf extract of *V. amygdalina*.

Methodology: The Phytochemical analysis of *Vernonia amygdalina* to detect the presence of bioactive compounds (oxalate, tannins, saponins, flavonoid, cardiac glycoside, alkaloids, steroid, balsams, essential oil and saponin glycoside) was detected using standard methods. The antibacterial activity of the leaf extracts was determined using agar well diffusion method against clinical isolates of *Salmonella typhi* and *Salmonella paratyphi*. The toxicity testing was carried out with albino rats using standard method.

Results: Different secondary metabolites were found to be present in the leaf extracts after the phytochemical screening. They include tannins, saponins, flavonoids, cardiac glycosides, alkaloids, glycosides, steroid, saponin glycoside, volatile oil and belsalms. Highest zone of inhibition of 10.00mm and 20.0mm was recorded against *S. typhi* and *S. paratyphi* aqeous and ethanolic extracts of the leaves respectively. Minimum Inhibitory Concentration (MIC) of 100mg/ml of the aqueous extract was recorded against *S. typhi* and *S. paratyphi*. A MIC of 25mg/ml of the ethanolic extract was recorded against *S. typhi* and *S. paratyphi*. The acute toxicity tests reveal no occurrence of death after 2 weeks of administering 5000 mg/kg body weight of the extracts to the albino rats.

Conclusion: The results revealed promising potentials of the leaves of *V. amygdalina* in the treatment of infectious diseases, due to its low toxicity. However, further studies need to be conducted to isolate and characterize the active metabolites present in the leaves.

Keywords: *Vernonia amygdalina, S. typhi, S. paratyphi, Antibacterial activity, Acute toxicity.*

INTRODUCTION

Bitter leaf, scientifically known as *Vernonia amygdalina*, belongs to the family Astercieace. It originated from Tropical Africa [1]. It is mainly cultivated in the Southern part of Nigeria as a vegetable and for medicinal purpose. In Nigeria, it is called 'Ewuro jije' by the Yoruba's, 'Olugbo' by the Igbo's and 'Shiwaka' by the Hausa's [2]. It is used for curing diseases such as diarrhea, as well as management of fever [3]. The medicinal value of some plants lies in some chemical substances that produce definite physiological actions in the human body; examples of these most important bioactive constituents are alkaloids, tannis, flavonoids

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essential oil and phenolic compound [3]. The leaves of *Vernonia amygdalina* are green with a characteristic odour and bitter taste. *Vernonia amygdalina* is a valuable medicinal plant that is widespread in West Africa, it is known as bitter leaf due to its characteristic bitter taste and flavour, and can be used as an active anticancer, antibacterial, antimalarial and antiparastic agent [3]. This plant contains complex active components that are useful pharmacologically.

Many of these indigenous medicinal plants are used as spices and food plants [4, 5]. 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. They do so without knowing that these agents were used against some pathogenic microorganisms [6]. 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 [7]. Secondary metabolites which include tannins, cardiac glycosides, alkaloids and saponins were reported to be present in higher plants [8], with *V. amygdalin* reported having contained many secondary metabolites [2].

Fasola *et al.* [9] reported *V. amygdalina* to possess hypoglycaemic activity. They observed a close-dependent reduction in fasting blood sugar level in alloxan-induced diabetic rats after treatment with different concentrations of the aqueous leaf extracts. Yedjou *et al.* [10] also demonstrated *V. amygdalina* leaf extracts as a DNA-damaging of the anticancer agent in the management of breast cancer. The aim of this research is to carry out phytochemical analysis of the leaf of *Vernonia amygdalina*, study the antibacterial effects of the leaf extracts of *V. amygdalina* on selected Enterobacteriaceae and to estimate the toxic effects of aqueous and ethanolic extracts from *V. amygdalina* in albino Rats.

2.0 MATERIALS AND METHODS

2.1 Collection and Identification of Leaf Materials

Vernonia amygdalina (Bitter leaf) was obtained from Meat Market, Sokoto, Nigeria. The collected leaf was identified and authenticated at the Herbarium Section of the Department of Biological Sciences, Botany Unit of Usmanu Danfodiyo University Sokoto, Sokoto State, Nigeria. Voucher specimen number UDUH/ANS/0100 was obtained.

2.2 Preparation and Extraction of Leaf Extracts

The fresh leaves were allowed to dry completely at room temperature under shade before using them for this study. The leaf material was pulverized using mortar and pestle into a fine powder. Two different solvents were used for the extraction namely: water and ethanol. A 100g of the powdered leaf was soaked in 1000ml of each solvent in accordance with Ugochukwu *et al.* [3]. Each solution was stirred intermittently and allowed to stand for 48h, and then filtered by first, using a clean muslin cloth and then, No. 1 Whatman filter paper. The filtrate was concentrated using rotary evaporator. This stock solution of the extract was sterilized by filtration through Millipore membrane filter [11]. The sterile extract obtained was stored in sterile capped bottles and refrigerated until when required for further analysis.

2.3 Characterization and Identification of *Salmonella* Species

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2.3.1 Source of Test Organism

The test organism for this study (*Salmonella* species) is member of the family Enterobacteriaceae. The pure clinical isolates of *Samonella typhi* and *Samonella paratyphi* were obtained from the Department of Medical Microbiology and Parasitology, Specialist Hospital Sokoto, Nigeria. All the clinical isolates were checked for purity by sub-culturing the isolates onto Salmonella-Shigella Agar medium. After 24hrs of incubation, there were growths of the isolates and they were maintained on nutrient agar slants at 4^0C in the refrigerator until required for further use.

2.3.2 Biochemical Characterization and Serotyping of *Salmonella*

The ISO-6579 [12], standard recommendation was used for biochemical confirmation of *Salmonella*. The subculture of the characteristic colonies from each Petri dish of Salmonella-Shigella agar medium was made. The triple sugar iron agar (TSI agar), Urea agar/broth, L-lysine decarboxylase, β-galactosidase (ONPG), Voges Proskauer and Indole tests were followed in this order.

In serotyping, a subculture of characteristic colonies from each Petri dish of Salmonella-Shigella agar was transferred onto nutrient agar slopes and incubated overnight at 37^0C. Using a wire loop, 3 separate drops (each 0.02 ml) of saline solution were placed onto a clean microscope slide. Growth from the agar slope was added and emulsified to produce a homogeneous suspension. A loopful of *Salmonella* polyvalent 'O' (PSO) anti-serum was mixed with the first drop of suspension and a loopful of *Salmonella* polyvalent 'H' (PSH) anti-serum with the second drop. It was rocked gently back and forth and examined for agglutination against a black background. Positive results were recorded if agglutination occurred within 20 min after shaking against dark background. In order to exclude any spontaneous agglutination (auto-agglutination), a negative control (using physiological saline solution and bacterial colony to be tested) was included in the test.

2.3.3 Standardization of Bacteria Cell Suspension

The nutrient broth cultures of the organisms for this study were taken and inoculated at 37^0C on a fresh agar plate of nutrient agar for 24 hours. Sterile distilled water (2ml) was poured on it and then mixed with the inoculums, 1ml of each was taken and transferred into 9ml of sterile distilled water and diluted to 0.5 Macfarland Standard giving a load of 10^5- 10^6 organisms/ml. One hundred microlitres of these were taken and poured onto the surface of the agar and then spread evenly with the use of a spreader on the plate to be used for the study.

2.3.4 Preparation of Extracts Concentration

The different extracts of the sample were reconstituted with sterile distilled water. The initial concentration of each leaf extracts (1g) was diluted using 10ml of sterile water to obtain the stock extract. From this stock extract, different concentrations were obtained 100mg/ml, 50mg/ml, 25mg/ml, 12.5mg/ml, 6.25mg/ml, and 3.125mg/ml for each of the extracts (water and ethanol).

2.4 Determination of Antibacterial Activities of Leaf Extracts

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Agar-well diffusion Method was employed for the antibacterial testing [13]. The antibacterial screening of the extracts was done as described by [13]. One (1) gram of each crude extract (aqueous and ethanolic) was poured into 10ml distilled water. Prepared and sterilized nutrient agar was poured in sterile Petri dishes and was allowed to solidify. A loopful of the test culture of MacFarland standard was dropped on the solidified agar and the organism was spread all over the surface of the agar using a spreader (wire loop). The inoculated plates were allowed to dry after which wells of approximately 5mm in diameter were made on the surface of the agar medium using a sterile cork borer. Then, 0.2ml of different concentrations of the extract was separately introduced into the different wells that have been labelled accordingly. This procedure was repeated in triplicate and allowed to stay for 30mins on the bench after which they were incubated for 24h at 37^0C. At the end of incubation, observed zones of inhibition were measured and recorded to the nearest millimeter.

2.5 Determination of Minimum Inhibitory Concentration of the Extracts

This was carried out using the agar diffusion method following the recommendations of the Clinical and Laboratory Standard Institute [14]. Different concentrations 100, 50, 25, 12.5, 6.25 and 3.125 mg/ml of the extracts were prepared and 1ml from each of the concentrations of the extracts was added onto molten nutrient agar and was mixed thoroughly. Then, 1µml of an overnight nutrient broth culture of the test isolates were added to each plate of the Mueller-Hinton agar containing the extracts and incubated at 37^0C for 24 h. The experiment was conducted in triplicate for all the test isolate. Plates without visible growth of the organisms in each concentration were taken as the MIC [11].

2.6 Phytochemical Screening of Leaf

The pulverized leaf obtained was subjected to phytochemical screening to determine the presence of bioactive compounds.

2.6.1 Test for Tannins

Five percents (5%) ferric chloride were added drop by drop to 3ml of each extract and observed for brownish green or a blue black colouration [15].

2.6.2 Test for Saponins

Two grams (2g) of the powdered sample of each extract was boiled in 20ml of distilled water in a water bath and filtered. Then, 10ml of the filtrate was mixed with 5ml of distilled water and shaken vigorously for a stable persistent froth. The frothing was mixed with 3 drops of olive oil, shaken vigorously and then observed for the formation of emulsion [16].

2.6.3 Test for Flavonoids

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One milliliter (1ml) of 10% NaOH solution was added to a portion of the aqueous filtrate of the each plant extract, followed by the addition of concentrated H_2SO_4. A yellow colouration observed in the extract indicated the presence of flavonoids [15].

2.6.4 Test for Cardiac Glycosides

Five millilitres (5ml) of each extract was treated with 2ml of glacial acetic acid containing I drop of ferric chloride solution (3.5%). The content was allowed to stand for one minute. One milliliter (1ml) of concentrated H_2SO_4 was carefully poured down the wall of the tube. A reddish brown ring of the interface indicated a deoxysugar characteristic of cardenolides [17].

2.6.5 Test for Alkaloid

Two milliliter (2ml) of each extract was stirred with 2ml of 10% dilute hydrochloric acid. Then, 1ml was treated with a few drops of Wagner's reagent and second 1ml portion treated with Mayer's reagent. Deep brown precipitation indicated a positive test [17].

2.6.6 Test for Glycosides

The 2.5ml of 50% H_2SO_4 was added to 5ml of each of the extracts in test tubes. The mixture was heated in boiling water for 15minutes. Cooled and neutralized with 10% NaOH, 5ml of Fehling's solution was added and the mixture was boiled again. A brick-red precipitate was observed, which indicated the presence of glycosides [17].

2.6.7 Test for Balsams

Each of the extracts was mixed with an equal volume of 90% ethanol. Two drops of alcoholic ferric chloride solution were added to the mixture. A dark-green colour indicated the presence of balsams [18].

2.6.8 Test for Steroids

This was carried out according to the method of [17]. One (1) ml of each leaf extract was added in 2ml of chloroform, and 2ml of sulphuric acid (H_2SO_4) was added thereafter. A red colouration confirmed the presence of steroids.

2.6.9 Test for Saponin Glycosides

Two point five (2.5ml) of each of the extracts was added to 2.5ml of Fehling's solutions A and B. A bluish-green precipitate showed the presence of saponin glycosides [18].

2.6.10 Test for Volatile Oils

One milliliter (1ml) of each of the extract fractions was mixed with 5ml of dilute HCL. A white precipitate was formed, which indicated the presence of volatile oils [16].

2.7 Toxicity Study of the Leaf Extracts of *Vernonia amygdalina*

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Acute oral toxicity test was carried out using the procedure of the Organization for Economic Cooperation and Development [19]. Twenty (20) randomly selected Albino rats were used. The rats of both sexes weighing 160-200g were used for the study. The animals were obtained from the Faculty of Veterinary Medicine, Usmanu Danfodiyo University, Sokoto. The animals were acclimatized for a period of seven days. All animals were housed, caged and allowed free access to food and water before they were used for the experiment. The animals' weights were taken and starved of food. Then 5000mg/kg body weight of the extract was administered in a single concentration. Concentrations were calculated according to the body weight of the animals. Oral administration of extracts was done using a graduated syringe and cannula. They were placed under observation for 48 hours for behavioural changes and daily for 14days for mortality [19], upon which the number of deaths and LD_{50} were determined.

3.0 RESULTS AND DISCUSSION

The phytochemical screening of the leaf revealed the presence of bioactive compounds including alkaloids, tannins, glycoside, saponin, cardiac glycoside, steroid and flavonoids, etc (Table 1). These phytochemicals have been proved to possess biocidal and inhibitory activities against a wide range of microorganisms [20, 21, 22]. The presence of these phytochemicals in the extracts could, therefore, explain their antibacterial activities as observed in this study. From this study, it was observed that bioactive components are abundant in these leaves and that the ethanol extracts exhibited higher inhibitory activity on the test organisms. This can be deduced to the ability of ethanol to extract more of the essential oil and secondary plant metabolites which are believed to exert antibacterial activity on test organisms [23]. The leaf appeared to have the best activity at the extracts concentration of 100 mg/ml in the agar well diffusion experiments. The aqueous extract of *V. amygdalina* had a decrease in the level of inhibition against isolates at the highest concentration compared to the positive control, inhibition zones ranging from 10.0 – 8.0mm. This analysis shows that ethanol extract of *V. amygdalina* showed an increase in the level of inhibition against *Salmonella typhi* to *Salmonella paratyphi*. Ogundare [23] reports have shown results similar to this.

Table 4 shows the minimum inhibitory concentrations (MICs) of both aqueous and ethanolic extracts on test organisms using agar dilution method. Low MIC is an indication of high efficacy of the leaf extracts while high MIC indicates low efficacy or possible development of resistance by the microorganisms to the antimicrobial [24]. The aqueous extract showed its MIC at high concentration of 100mg/ml while ethanolic extract. The minimum inhibitory concentrations (MIC) of aqueous and ethanolic leaf extracts on the test organisms ranged between 25mg/ml –100mg/ml. The minimum inhibitory concentrations of ethanolic extracts of *V. amygdalina* as 25mg/ml while aqueous leaf extracts of *V. amygdalina* had their MIC as 100mg/ml as shown in (Tables 4).

Oral administration of a single dose of ethanol and aqueous extracts of *V. amygdalina* of 5000mg/kg body weight of the test animals produced no mortality in them. The general signs and symptoms of toxicity were observed for a period of 14 days after administration of the extracts (Table 5). However, the following observations were made during the exposure period; slow movement, scratching of hair and mouth, tremor, raised hair coat and weakness. Thus, the median dose (LD_{50}) of the leaf extracts was estimated to be greater than 5000mg/kg because 5000mg/kg is the highest dose according to Guideline for testing chemicals [19]. The oral administration of 5000 mg/kg body weight of the ethanolic and aqueous extracts of *V. amygdalina* resulted in no mortality. This suggests that the LD_{50} is greater than 5000 mg/kg and can be classified as

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practically non-toxic using the Organization for Economic and Cooperation Development (OECD) guideline classification of a range of LD_{50}. This suggests the possibility of using the ethanol extracts of *V. amygdalina* in treating the diseases caused by the test organisms.

The results of phytochemical screening of *V. amygdalina* leaves revealed the presence of the following secondary metabolites tannins, saponins, flavonoid, steroid, cardiac glycoside, glycosides, alkaloid, saponin glycoside, volatile oil and balsams (Table 1).

The results revealed that the aqueous extract of *V. amygdalina* has less inhibitory activity on the test organisms (Table 2), while the ethanolic extracts of *V. amygdalina* (Table 3) had antibacterial activity against the isolates tested. At 100mg/ml concentration, the ethanolic extracts showed greater antibacterial activity than the aqueous extracts as indicated by zones of inhibition. At 12.5mg/ml – 3.125mg/ml, the ethanolic extracts of *V. amygdalina* (Table 3) was not effective on the isolates. While at 50mg/ml – 3.125mg/ml the aqueous extracts of *V. amygdalina* (Table 2) was not effective on the isolates. This indicates that the antibacterial activity of this leaf extracts is concentration dependent. Ethanolic extract showed high inhibitory zones than aqueous extract and when compared to a standard antibiotic such as Pemaclav drug had an appreciable zone of inhibition on the test organisms.

Table 1: Phytochemical properties of the leaf of *Vernonia amygdalina* Leaf Extract

Phytochemical	**Inference**
Oxalate	+
Tannins	+
Saponins	+
Flavonoid	+
Cardiac glycoside	+
Alkaloid	+
Glycosides	+
Steroid	+
Saponin glycoside	+
Volatile oil	+
Balsams	+

KEY
- = Not detected.
+ = Detected

Table 2: The Antibacterial Activities of Aqueous Leaf Extracts of *V. amygdalina*

Test Organism	Zone of inhibition(mm)						
Concentration (mg/ml)	100	50	25	12.5	6.25	3.125	+ve ctrl
Salmonella typhi	10.0	×	×	×	×	×	20.0
Salmonella paratyphi	08.0	×	×	×	×	×	20.0

Key:
Values are mean of three replicates (n=3)
× = No zone of inhibition
+ve ctrl = Pemaclav drug

Table 3: The Antibacterial Activities of Ethanolic Leaf Extracts of *V. amygdalina*

Test Organism	Zone of inhibition(mm)						
Concentration (mg/ml)	100	50	25	12.5	6.25	3.125	+ve ctrl
Salmonella typhi	20.0	14.0	10.0	×	×	×	21.0
Salmonella paratyphi	5.0	12.0	11.0	×	×	×	20.0

Key:
Values are mean of three replicates (n=3)
× = No zone of inhibition
+ve control = Pemaclav drug

Table 4: Minimum Inhibitory Concentration of the Aqueous and Ethanolic Leaf Extracts of *V. amygdalina* against Salmonella spp

Bacterial Isolates	Aqueous extract MIC (mg/ml)	Ethanol extract MIC (mg/ml)	Pemaclav drug (Amoxicillin combination) MIC (mg/ml)
Salmonella typhi	100	25	12
Salmonella paratyphi	100	25	12

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Table 5: Acute Toxicity Results on Ten Randomly Selected Albino Rats.

Dose (mg/kg)	Time Duration	No. of Animals	No of Deaths	Observation Signs
5000	0-30 minutes	5(a)	0	Weakness, slow movement immediately after administration.
	1 hour			Continuously scratching of mouth part, fur and body, tremor.
	24 hours			Ruffled fur, scratching of their nostril.
	48 hours			Normal movement and less scratching of body part.
	2 weeks			No death rate recorded.
5000	0-30 minutes	5(b)	0	Increased breathing
	1 hour			Scratching of mouth and body parts
	24 hours			Ruffled fur
	48 hours			No scratching of body part
	2 weeks			No death rate recorded

Key: a = the first 5 rats were given aqueous leaf extracts of *O. gratissimum*, b = the last two 5 rats were given ethanolic leaf extracts of *O. gratissimum*

5.2 CONCLUSION

This study, however, can justify the use of the leaves of *V. amygdalina* in traditional medicine practice as a therapeutic agent and can explain the traditional use of these plants. It further suggests their possible exploitation as a source of natural products for future use in the management of multi-drug resistant bacterial infections in Nigeria. The results provide justification for the use of these plants in the treatment of various infections in herbal medicine. The result also revealed promising potentials of the leaves of V. amygdalina in the treatment of infectious diseases, due to its low toxicity. However, further studies need to be conducted to isolate and characterize the active metabolites present in the leaves.

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