



Leaf Extracts of *Vernonia amygdalina* Del. from Northern Ghana Contain Bioactive Agents that Inhibit the Growth of Some Beta-lactamase Producing Bacteria *in vitro*

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Authors' contributions

This work was carried out in collaboration between all authors. Author OLA designed the project, managed the protocol for qualitative screening, antimicrobial analysis, performed the statistical analysis and wrote the first draft of manuscript. Author AJ designed and wrote protocol for quantitative analysis of phytochemicals and managed the literature searches. Authors SBK and OPJ searched for samples, prepared samples for analysis and managed parts of laboratory works. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: The current study was aimed at evaluating the phytochemical profile and *in vitro* bacteria growth inhibitory potential of different solvent leaf extracts of *V. amygdalina* from northern Ghana.

Study Design: Different solvent extracts of the plant were quantitatively and qualitatively evaluated for phytochemicals. *In vitro* bacteria sensitivity assay of the extracts was evaluated using some beta-lactamase producing bacteria as test microbes.

Methodology: Ethanolic, methanolic, petroleum ether and aqueous leaf extracts of *Vernonia amygdalina* were studied *in vitro* for growth inhibition against beta-lactamase producing bacteria such as *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* using agar well diffusion method. Saponins, flavonoids, glycosides, alkaloids, tannins, phenolics, reducing sugars, anthracenosides, terpenes and phytosteroids were determined qualitatively and quantified.

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Results: All the phytochemicals tested were found to be present in both the methanolic and ethanolic leaf extracts. The aqueous extract showed the presence of saponins, reducing sugars and anthracenosides. Glycosides, flavonoids, saponins and alkaloids were the only groups of phytochemicals found in the petroleum ether extract. The methanolic extract showed the greatest amount of saponins (14.23%), flavonoids (2.15%), alkaloids (7.49%), tannins (5.4%), terpenes (10.20%) and phenolics (8.24%). The methanolic extract at concentration of 4 mg/ml showed growth inhibitory activities against all the test organisms with zone of inhibitions ranging from 16.00±0.50 (against *E. coli*) to 20.50±0.03 mm (against *S. aureus*). The ethanolic extract showed activity against only two of the test organisms viz. 23.00±0.33 mm against *P. aeruginosa* and 12.00±0.00 mm against *S. aureus* at similar concentration. All test organisms were resistant to both aqueous and pet ether extracts.

Conclusion: The antibacterial activities of the methanolic and ethanolic extracts were significant ($P < 0.05$) and may be mediated by the presence of saponins, flavonoids, tannins, terpenes and alkaloids. Results from present study corroborate previous findings and also presents methanolic leaf extract of the plant as a credible candidate for the discovery of new phytotherapeutic agents against the beta-lactamase producing bacteria tested.

Keywords: Phytochemical evaluation; leaf vegetable; Vernonia amygdalina; bacteria growth inhibition; agar well diffusion; beta-lactamase.

1. INTRODUCTION

Beta-lactam antibiotics structurally consist of a thiazolidine ring connected to a beta-lactam ring, which is attached to a side chain. These drugs include the penicillins, cephalosporins, carbapenems and monobactams. They have been reported to be the most widely used antibiotics due to their high efficacies [1]. However, there have been reports on increase resistance among most common pathogens in recent times. Most notable microbes that have shown resistance to these drugs are the beta-lactamase-producing bacteria (BLPB). BLPB can have a direct pathogenic impact in causing an infection as well as an indirect effect through their ability to produce the enzyme beta-lactamase (BL) [2]. The enzyme hydrolyzes the beta-lactam rings causing their inactivation. As BLPB releases the enzyme into the environment, they may not only resist beta-lactam antibiotic therapy but can also as in a recent study [2], protect other bacteria sensitive to these antibiotics. In this regard several bacteria long known to be resistant to these antibiotics, and previously sensitive organisms have increasingly shown resistance to these antibiotics.

The indiscriminate use of synthetic anti-microbial drugs commonly used in the treatment of infectious diseases has also led to the development of multiple drug-resistant strains of bacteria over the years. In addition to this problem, adverse effects on the host including hypersensitivity, immunosuppression, gastrointestinal upset and allergic reactions are sometimes attributed to the use of these anti-microbial drugs. This has drawn the attention of the scientific community to biologically active compounds derived from medicinal plants since they present less desirable side effects [3].

More than half of all modern clinical drugs are plant-derived [4]. This shows that plant products play a significant role in the development of drugs by the pharmaceutical industry [5,6]. The consumption of plant materials by man is also believed to contribute immensely to

the improvement of human health and nutrition. Over 80% of the population of Africa depends on medicinal plants for medical care [7]. Therefore, information on the nutritional and medicinal potentials of plants found in Africa is of immense importance.

Vernonia amygdalina is a tropical plant that grows up to 3 meters in height. The shape of the leaf is elliptic [8]. It has a short life cycle and can be harvested twice per month for up to seven years. The plant is a leaf vegetable that is indigenous to sub-saharan Africa [9]. It commonly called bitter leaf (English), Ndole (French), ewuro (Yoruba), shiwaka (Hausa), awonwon (Twi) and onugbu (Igbo). The leaves are dark green coloured with a characteristic odour and bitter taste. The plant grows well at almost every part of Ghana. *V. amygdalina* can be found along drainage lines, in natural forests or at homes and commercial plantations [10].

V. amygdalina Del. is probably the most used medicinal plant in the genus *Vernonia* [11]. Huffman & Seifu [12] and Ohigashi et al. [13] reported on the medicinal effects of the plant on apparently sick wild chimpanzee. This report attracted the attention of the phytomedicine community and this has led to numerous scientific studies on the medicinal values of different extracts of the plant [14].

Both the leaves and the roots of *V. amygdalina* plant are used traditionally in phytomedicine to treat fever, hiccups, kidney disease and stomach discomfort. Antihelmintic, antitumorigenic, hypoglycaemic and hypolipidaemic properties of this plant has also been reported [15,16,17]. The plant has also been reported in traditional medicine as anti-malaria and a laxative [18]. Other traditional use of this plant includes digestive tonic, appetizer, febrifuge and for the topical treatment of wounds [19]. *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* were found to be sensitive to the sap of the leaves of *V. amygdalina* [20]. Methanolic leaf extract of the plant was found to show inhibitory activity against *K. pneumonia*, *P. aeruginosa*, *S. aureus*, *Bacillus subtilis*, *Proteus vulgaris* and *Shigella dysenteriae* [21].

Different solvent extracts of medicinal plants contain various phytochemicals and their medicinal values vary accordingly. Furthermore, the phytochemical composition of a medicinal plant may vary according to geographical location, time of harvest and to some extent the prevailing weather condition.

For the first time, the phytochemical profile and the antibacterial potential of different solvent extracts of *V. amygdalina* leaves harvested in the northern part of Ghana were evaluated in current study. Ethanolic, methanolic, petroleum ether and aqueous leaf extracts of *V. amygdalina* were screened qualitatively and quantitatively for some phytochemicals. Bacteria growth inhibition potentials of these extracts against beta-lactamase producing bacteria such as *K. pneumonia*, *P. aeruginosa*, *S. aureus* and *E. coli* were evaluated *in vitro*.

2. MATERIALS AND METHODS

2.1 Materials and Equipment

2.1.1 Chemicals and reagents

All chemicals used in this study were of analytical grade and were used without further purification. All solvents and other consumables were obtained from British Drug House (BDH).

2.1.2 Equipment and apparatus

Equipment such as UV visible spectrophotometer, electronic balance, freeze dryer and other basic laboratory apparatus were used.

2.1.3 Plant material

Fresh leaves of *Vernonia amygdalina* were collected from a piece of land in Navrongo in the Upper East Region of Ghana in November 2011. The leaves were later taken to the Herbarium Department of the Centre for Scientific Research into Plant Medicine, Mampong-Akuapim, for botanical identification.

2.1.4 Microorganisms

Four different microbes of standard strains were purchased at the Komfo Anokye Teaching Hospital (KATH) Kumasi, Ghana with standard codes. The microbes were: *E. coli* (ATCC 25922), *K. pneumonia* (ATCC 33495), *P. aeruginosa* (ATCC 27853), *S. aureus* (ATCC 25923).

2.2 Methodologies

2.2.1 Extraction of plant material

Fresh leaves of *V. amygdalina* were dried in shade and milled. The various extracts were prepared from the powdered leaves. Five hundred grams of the powdered material were soaked in 5 litres of the respective solvents to obtain the 70% ethanolic, 70% methanolic, pet-ether and aqueous extracts. The suspension was then filtered through Whatman No. 1 filter paper. The filtrate was concentrated using rotary evaporator and then freeze dried.

2.2.2 Phytochemical screening

Qualitative tests were carried out to detect the presence of phytochemicals present in all the extracts of *V. amygdalina* using standard procedure reported by Trease and Evans [22], Sofowora [23] and Harborne [24].

Quantitative phytochemical analysis was carried out using the methods reported by Edeoga et al. [25] with little or no modification. Extracts were defatted with 100 ml of diethyl ether using a soxhlet apparatus for 1hr. Determination of terpenes (essential oils) was carried out using steam distillation on the extract.

2.2.2.1 Alkaloid content determination

To about 1 g of the defatted sample, 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 dropwise to the extract until the precipitation was complete. The precipitate was collected and washed with dilute ammonium hydroxide and then filtered to obtain the alkaloid residue. This was dried and weighed.

2.2.2.2 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 No 42 (125 mm). The filtrate was evaporated to dryness in a crucible over a water bath and weighed to a constant weight.

2.2.2.3 Saponin content determination

About 50 ml of 20% aqueous ethanol was added to 2 g of grinded samples in a conical flask. The samples were heated over a hot water bath for 4 hours with continuous stirring at about 55°C. The mixture was filtered and the residue re-extracted with another 100 ml 20% ethanol. The combined extracts were evaporated to about 20 ml over water bath at about 90°C. The concentrate was transferred into a 250 ml separating funnel. About 10 ml of diethyl ether was then added and shaken vigorously. The aqueous layer was recovered and the ether layer discarded. The purification process was repeated; 30 ml of n-butanol was added. The combined n-butanol extracts were washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated in a water bath to evaporate and the samples dried to a constant weight. The saponin content was then calculated as a percentage.

2.2.2.4 Determination of total phenols by spectrophotometric method

About 0.5 g of sample was boiled with 50 ml of ether for the extraction of the phenolic component for 15 minutes. Ten millilitres of distilled water was added to 5 ml of the extract in a 50 ml flask. Two millilitres of ammonium hydroxide solution and 5 ml of concentrated amyl alcohol were also added. The samples were made up to the mark and left to react for 30 min for colour development. The absorbance was measured at 505 nm.

2.2.2.5 Tannin content determination by spectrophotometric method

To about 500 mg of the sample in a 100 ml plastic bottle, 50 ml of distilled water was added and shaken for 1 hour in a mechanical shaker. This was filtered and the filtrate transferred into a 50 ml volumetric flask and made up to the mark. Then 5 ml of the filtrate was pipetted out into a test tube and mixed with 2 ml of 0.1 M FeCl_3 in 0.1 N HCl and 0.008 M potassium ferrocyanide. The absorbance was measured at 505 nm within 10 min.

2.2.2.6 Determination of terpenes (as essential oils) by steam distillation

Briefly, about 300 ml of distilled water was added to 10 g of the ground plant material in a 500 ml round-bottom flask fitted with a condenser and a collecting flask. The system was then heated gently to generate drops of oil-water mixture in the collecting flask. Small

volumes of water were added periodically to the boiling mixture in the round-bottom flask via a separating funnel. The condensate was transferred to a 250 ml separating funnel and about 40 ml of diethyl ether added. This was shaken for about a minute and the water layer drained off. The ether fraction was drained into a collection flask. The extraction of oil from the oil-water mixture was repeated two more times and the ether fractions combined in the same flask. Traces of water were removed by the addition of small amount anhydrous Na_2SO_4 to the ether fraction. The liquid was then decanted into a beaker and the beaker placed on a water bath to boil off the ether. The oil was then transferred to a weighed vial and its mass determined.

2.2.3 Antibacterial assay

About 1 ml of the test culture (previously diluted to 10^7 CFU/ml) was inoculated into 20 ml of molten nutrient agar in a petri plate and then spread uniformly using a sterile glass spreader. This was allowed to solidify and a sterile cork borer (5 mm in diameter) was used to make wells on the media in the plates for introduction of extracts.

One hundred microliters of 4 mg/ml of each extract prepared in 20% dimethyl sulphoxide (DMSO) were introduced into the wells. Each extract was introduced into each hole in triplicate. The plates were kept at room temperature for the extract to diffuse into the media before it was placed in the incubator at 37°C for 24 hours. The relative susceptibility of the organism to the extract is indicated by clear zone of inhibition produced after incubation. Diameters of inhibition zones were measured by calculating the difference between cork borer (5 mm) and the diameters of inhibition [26,27,28]. Chloramphenicol at 5 μml and DMSO were used as positive control and negative controls respectively.

Data collected in the study are expressed as the mean \pm standard error of mean (S.E.M.) and statistical analysis was carried out using unpaired t-test. $P < 0.05$ is considered significant.

3. RESULTS AND DISCUSSION

Qualitative phytochemical screening revealed the presence of various phytochemicals in the extracts (Table 1). Both ethanolic and methanolic leaf extracts of *V. amygdalina* were found to contain all the phytochemicals tested. Of all the extracts, the aqueous extract showed the least number of extracts viz. saponins, reducing sugars and anthracenosides. Saponins, flavonoids, glycosides and alkaloids were the only phytochemicals found in the pet ether extract.

Even though the classes of phytochemicals found to be present in both ethanol and methanol extracts were identical, the quantities varied as presented in Table 2. The methanolic extract showed the greatest of amount of all the phytochemicals tested viz. saponins (14.23%), flavonoids (2.15%), alkaloids (7.49%), tannins (5.40%), phenolics (8.24%) and terpenes (10.2%). Antimicrobial activities in plants have been attributed to the presence of alkaloids, saponins, tannins, flavonoids and terpenes [29,30]. These phytochemicals were all found to be present in both methanolic and ethanolic leaf extracts of *V. amygdalina*. This may be attributed to similarity in their polarity indexes.

Table 1. Results of qualitative phytochemical screening of solvent extracts of leaf of *V. amygdalina*

Phytochemical constituents	Leaf extracts of <i>V. amygdalina</i>			
	Ethanol	Methanol	Pet ether	Aqueous
Saponins	+	+	+	+
Flavonoids	+	+	+	-
Glycosides	+	+	+	-
Alkaloids	+	+	+	-
Tannins	+	+	-	-
Phenolics	+	+	-	-
Reducing sugars	+	+	-	+
Anthracenosides	+	+	-	+
Terpenes	+	+	-	-
Phytosteroids	+	+	-	-

+ = phytochemical present; - = phytochemical not detected

Table 2. Results of quantitative phytochemical screening of leaf extracts of *V. amygdalina*

Phytochemical constituents	Amount of phytochemical in leaf extracts of <i>V. amygdalina</i>			
	Ethanol	Methanol	Pet ether	Aqueous
Saponins	10.40%	14.23%	8.60%	2.00%
Flavonoids	1.80%	2.15%	0.60%	nd
Alkaloids	2.50%	7.49%	1.20%	nd
Tannins	3.25%	5.40%	nd	nd
Phenolics	5.36%	8.24%	nd	nd
Terpenes	6.00%	10.20%	nd	nd

nd: Phytochemical not detected.

The results of antibacterial activities of the extracts of *V. amygdalina* have been presented in Table 3. From the data, it is evident that only the methanolic and ethanolic leaf extracts showed activities. The aqueous and pet ether extracts did not show inhibitory activity against the growth of any of the test organisms. The presence of flavonoids, tannins, alkaloids, saponins and terpenes in the methanol and ethanol extracts may be responsible for the growth inhibitory activities shown by these extracts as in Table 3. Of the bacteria tested *E. coli* and *K. pneumonia* were resistant to the ethanolic extract. The mean diameters of zones of inhibition for the ethanolic extract were 12.00 ± 0.00 mm against *S. aureus* and 23.00 ± 0.33 mm against *P. aeruginosa*. The methanolic extract however, showed activities against all the test organisms.

Table 3. Antibacterial activities of crude solvent extracts of *V. amygdalina* against test organisms

Test organism	Mean diameter of zone of inhibition of extract against organism (mm)					
	Ethanol	Methanol	Pet ether	Aqueous	DMSO	Control (positive)
<i>S. aureus</i>	12.00±0.00	20.50±0.03	0.00	0.00	0.00	20.00±0.00
<i>K. pneumonia</i>	0.00	18.00±0.50	0.00	0.00	0.00	20.00±0.00
<i>P. aeruginosa</i>	23.00±0.33	18.00±0.80	0.00	0.00	0.00	18.00±0.00
<i>E. coli</i>	0.00	16.00±0.50	0.00	0.00	0.00	12.00±0.00

Values are expressed as mean±SEM (n=3); Control (positive) = 5 µg/ml chloramphenicol

Both methanolic and ethanolic extracts were found to contain similar classes of phytochemicals. But while all the bacteria showed sensitivity to the methanolic extract, *E. coli* and *K. pneumonia* showed resistance to the ethanolic extract. This could probably be as a result of the presence high amounts of the phytochemicals in the methanolic extract. In addition, differences in the actual compounds found in the various groups of phytochemicals may also explain this observation. Of all the test drugs, the ethanolic extract showed the greatest activity against *P. aeruginosa* (23±0.33 mm). The activity of the methanolic extract was greatest against *S. aureus* (20.50±0.03 mm) at 4 mg/ml while *E. coli* was the least susceptible to the extract (16.00±0.50 mm). The activity of the methanolic extract was significant ($P < 0.05$) against *S. aureus*, *K. pneumonia* and *P. aeruginosa*. This was comparable to the positive control (Chloramphenicol at 5 µg/ml). However, the methanolic extract showed a better activity than the positive control against *E. coli*. Our results are consistent with the works of Akinpelu [21], Kambizi & Afolayan [31], Ogbulie et al. [32] and Kola [33]. The results further suggest that the antibacterial activity of *V. amygdalina* may not be attributed to saponins since this was present in all the extracts.

The broad-spectrum activity shown by the methanolic extract suggests that the methanolic leaf extract of *V. amygdalina* is a very good potential source of antibacterial agents that could be used to treat infections caused by *S. aureus*, *K. pneumonia*, *P. aeruginosa* and *E. coli*. Prescott et al. [34], reported that *S. aureus* and *E. coli* are the bacteria responsible for nosocomial infection hence the growth inhibitory effects of the methanolic and ethanolic extracts against such organisms proves crucial in the clinical management of nosocomial infections. Moreover, studies have implicated *S. aureus*, *E. coli* and *P. aeruginosa* as the leading causative agents of community infections [35,36]. Therefore, it appears that the methanolic leaf extract of *V. amygdalina* could present an important treatment option of infections caused by these organisms.

Among the several modes of action of resistance to beta-lactam antibiotics is the production of beta-lactamase enzymes which either hydrolysis the beta-lactam ring rendering it inactive or by binding tightly to the beta-lactam keeping it outside the bacteria cell. The good activity demonstrated by the methanolic and ethanolic extracts seem to suggest that the active agents in these extracts are not beta-lactam based and the mechanism of action may allow binding on to the bacteria cell wall and preventing cell wall biosynthesis.

4. CONCLUSION

Medicinal plants are constantly screened for bioactivity in our quest for the discovery of new and effective therapeutic agents. Leaf extracts of *V. amygdalina* from northern Ghana have been screen for phytochemicals and bacterial growth inhibition potentials. The methanolic

and ethanolic leaf extracts were found to contain significant quantities of bioactive agents. The methanolic leaf extract of *V. amygdalina* was found to possess broad-spectrum growth inhibitory activity against beta-lactamase producing bacteria such as *S. aureus*, *K. pneumonia*, *P. aeruginosa* and *E. coli in vitro*. This justifies the use of the plant in folkloric medical practices in the treatment of a range of infections caused by these bacteria. We conclude that methanolic leaf extract of *V. amygdalina* is a potential candidate for subsequent isolation of effective antibacterial agent for the treatment of infections caused by beta-lactamase producing bacteria. However, a wider study with an increase in the number of clinical isolates and the identification of the active compounds in the crude extract is desired. The presence of numerous compounds in a crude extract may interfere with the activity of the active compounds hence work on separation of compounds in the crude extracts is underway. Further work on *in vivo* antibacterial potentials of the extracts will also be undertaken.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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