

JOURNAL OF BIOCHEMISTRY, MICROBIOLOGY AND BIOTECHNOLOGY



Website: http://journal.hibiscuspublisher.com/index.php/JOBIMB/index

Antibacterial Effect of Bitter Leave (Vernonia amygdalina) on Klebsiella pneumoniae

Haruna Sa'idu¹*, Hassan Ismail Ahmad¹, Salau Abiola Olanrewaju² and Aisha Bello Mahmoud³

¹Department of Biology, Faculty of Sciences, Gombe State University, P.M.B 127, Tudun Wada, Gombe, Gombe State, Nigeria. ²Department of Natural Sciences, Gombe State College of Education,Billiri, Gombe State, Nigeria. ³National Biotechnology Development Agency, Nigeria.

> *Corresponding author: Haruna Saidu, Department of Biology, Faculty of Sciences, Gombe State University Email: <u>saiduharunn@gmail.com</u>

HISTORY	ABSTRACT
	High rate of multi drug resistance bacteria caused by <i>Klebsiella pneumoniae</i> is

Received: 12^{th} Oct 2020 Received in revised form: 13^{th} Nov 2020 Accepted: 15^{th} Dec 2020

KEYWORDS

antibacterial bitter leaf medical multi drug resistance High rate of multi drug resistance bacteria caused by *Klebsiella pneumoniae* is increasing day by day, which in turn becomes medical and economic important to humans. The present study investigates the antibacterial effect of bitter leaf on *Klebsiella pneumoniae*. Frothing test and agar well diffusion method was used in this study. The former method indicate the presence of phytochemicals such saponin, tannin, flavonoid, alkaloid and carbohydrate in the plant while the later method indicate the potential effect of the plant extract to inhibit the growth of *Klebsiella pneumoniae* under different concentration ranging 2.5, 5, 10 20 mg/mL. The various zones of inhibition obtained under these concentrations were 0.0 ± 0 , 7 ± 0.8 , 11 ± 0.6 and 14.00 ± 1.3 mm, respectively. The highest zone of inhibition recorded was 26.00 ± 2.3 mm using control experiment whereby no activity was obtained using 2.5 mg/mL plant extract. Thus, increase in the concentrations of extract was found to inhibit the growth of the bacteria under study. This research reports the potential effect of *Vernonia amygdalina* on the activity of *K. pneumoniae*.

INTRODUCTION

Bitter leaf is botanically known as *Vernonia amygdalina*. The plant has been named differently by different ethnics group around the world. For instance, In Hausa, the plant is known as *Shuwaka*, in Yoruba the plant called *Ewuro*, while in Igbo language it is called *Onugbu* [16]. The plant is a soft wooded shrub of 2 to 10 m tall with petiolate leaves of around 6 mm in diameter. In terms of classification, bitter leaf was classified under the genus *Vernonia schreb*, Family: Compositae; Order: Asterales; Classes: Dicotyledons which contains about 1000 species. More than 500 of these *Vernonia* plants species are distributed in Africa and Asia, approximately 300 in Mexico, South America and 16 species can be found in the United States.

According to World Health Organization (WHO) 80% of world population is dependent on traditional medicine. These medicines are sourced from plants extracts or from their active constituents [13]. That is why a scientific study to determine their antimicrobial active compounds is a comparatively new field of study [14]. Infectious disease, particularly skin and mucosal infections are common most complicated diseases to be treated nowadays. Typical examples of such diseases are dermal inflammation, folliculitis, skin abuses, acne and dermatitis.

Multidrug resistant bacteria have become important cause for higher skin care products. Multiple drug resistance has developed due to the indiscriminate use of commercial antimicrobial drugs commonly used in the treatment of such infectious disease. This resulted in a condition called immunocompromised leading to individuals suffering from skin infections that are difficult to cure. For instance, in 2005, 3.3% *Klebsiella pneumoniae* isolates were reported to be resistant to carbapenem in Brooklyn hospitals [4]. In 2014, two out of six WHO regions reported 50% or more resistance of *Klebsiella pneumoniae* to carbapenem [19]. Today Acinetobacter baumannii (Moraxellaceae) resists almost all known antibiotics used for the treatment of skin disorder [17].

High rate of multi drug resistance bacteria which is caused by *Klebsiella pneumoniae* is increasing day by day, which in turn becomes medical and economic important to humans. In 2016, [10] and his co-workers tested the effect of Bitter leave extract on some culturable microorganism obtained from palm wine. In the present study, the antibacterial effect of bitter leaf will be tested on *Klebsiella pneumoniae*. *Vernonia amygdalina* (Bitter leave) has been known for its antibacterial properties and effect on bacterial isolate, moreover such potentiality has not been tested on *Klebsiella pneumoniae*. Such study may contribute in resolving the novelty of finding the efficient remedy for disease caused by the bacteria.

MATERIALS AND METHODS

Study area

The research was conducted in Gombe State University, Gombe State, Nigeria. Gombe State has an area of 20.265 Km² (7.246 sqm) and a population of 2,353,000 at the 2006 census, and located at $10^{0}17^{I}N-11^{0}10^{I}E/10.283^{0}N-11.167^{0}E$. Average rainfall is 85 mm falling between April and October and dry season lasting from November to March. The temperature ranges between 29 to 39 °C [3].

Materials/equipment used

All the glass wires used for this research were thoroughly autoclave before use and for instruments were ensured to be calibrated before their employment for testing.

Collection, identification and preparation of plant material

The leaves of *Vernonia amygdalina* were collected from Gombe State University quarters. The plant was taken to the Herbarium Unit of the Department of Biological Sciences, Gombe State University for proper identification and authentication and given a voucher number of (84). After which it was shade dried, grinded to powder with mortar and pestle, extracted with methanol using cold maceration apparatus. The extract was then further evaporated to dryness using rotary evaporator to obtain the methanolic extract which was stored in an airtight container for further use. This was used for phytochemical and antimicrobial analysis [15].

Phytochemical tests

Phytochemicals such as saponins, test for steroids, test for flavonoids, test for tannins, test for alkaloids, test for cardiac glycosides, test for carbohydrates were extracted using methanol extraction method according standard method as described by [18].

Test for saponins

Frothing test was conducted for the determination. Accurately, 0.5g of the plant extracts were weighing using weighing balance and place in test tube containing water. The test tube was then place in a water bath for warming purpose. Appearance of the frothing indicated the presence of saponins.

Test for steroids

Lieberman-Buchard test was conducted for this determination. A portion of the plant extracts were place in a test tube followed by the addition of 2mL chloroform so as to dissolve the extract. To enhance the dissolving ability, 2 mL of acetic anhydride was then added plus concentrated sulphuric acid down in drop wise step. After 5 min, appearance of brown ring at the interphase of the mixture indicates the presence of steroids.

Test for flavonoids

For the determination of flavonoid, Shinoda Test was then applied. The method involves dissolving accurately 0.5g of the plant extracts in 5mL 95% methanol, followed by warming before filtration. To portion of supernatant, 3 pieces of magnesium chip was appended, followed by the addition of 3-4 drops of concentrated hydrochloric acid. After few mins, appearance of a pink, orange or red to purple color indicated the presence of flavonoids.

Test for tannins

Ferric chloride Test is a method for the determination of tannins in plant extract. The method operates by weighing 0.5 g of the sample (extracts) and placed in test tube containing 10 mL distilled water, the mixture was swirled before it was filtrate in order to separate the palate. To the portion of supernatant, 2 drops of 1% ferric chloride solution was and the formation of a blue-black (hydrolysable/gallitannins) or green or blue-green (condensed/cathehic tannins) precipitate indicated the presence of phenolic compounds.

Test for alkaloids

To test for the Alkanoid, 0.5 g of the plant extract were weight using weighing balance and placed in a test tube, 5 mL of 1% aqueous hydrochloric acid was added, the mixture was warmed in a water bath before filtration. Ammonia solution was added to the filtrate until it turns basic. 3 mL of chloroform solvent was added, swirled gently to allow separation between two layers. The chloroform layer was then collected and mixes with HCl. After a gentle shaken, one drop of Mayer's reagent was added and the appearance of white to yellowish or cream color precipitates indicated the presence of alkaloids.

Test for cardiac glycosides

Glacial acetic acid (2 mL) was added to the 5mL of the plant extract., followed by the addition of one drop of ferric chloride solution. Upon addition of 1mL of concentrated sulphuric acid, an appearance of brown ring at the interface indicated the presence of cardiac glycosides.

Test for carbohydrates

Fehling test was done for the determination of carbohydrate. 5 mL mixture of Fehling solution was added to the test tube containing plant extract. The mixture was then heated in a water bath and the appearance of brick red precipitate indicated the presence of reducing sugar.

Collection of clinical isolates

Pure culture of *Klebsiella pneumoniae* were collected from Specialist Hospital, Gombe and brought to the microbiology laboratory.

Confirmation of the collected isolate

The clinical isolate collected was sub-cultured on nutrient agar (NA) and pure isolates of the resulting growth was confirmed using gram staining and some biochemical methods.

Gram staining technique

Grams staining of the isolate was done using staining techniques whereas biochemical parameters such as Indole test, Oxidase test, Coagulase test, Urease test, were conducted using according to standard method as describe by [5].

Preparation of the McFarland's standard:

Barium Sulphate (1%w/v) standard suspension was used as turbidity standard. This was prepared using the method of [6] described below.

One percent (1% v/v) solution of sulphuric acid was prepared by adding 1mL of concentrated H₂SO₄ in to 99 mL of distilled water. One percent (1% w/v) solution barium chloride was also prepared by dissolving 0.5 g of dehydrated barium chloride in 50mL distilled water. 0.6 mL of the barium chloride solution was combined with 99.4 mL of sulphuric acid solution to yield 1% w/v barium sulphate suspension. The turbid solution formed which corresponds to the 0.5 McFarland turbidity standard was then transferred into a test tube and used as the standard for comparison.

Preparation of stock solution of extracts

A 40 mg/mL concentration of the extracts was constituted by dissolving 0.04 g in 1 mL of 20% v/v dimethyl sulfoxide (DMSO) as described previously [8].

Standardization of inoculums

Using inoculation platinum wire loop, enough material from an overnight culture of the test organism was transferred into a test tube containing normal saline until the turbidity of the suspension matched the turbidity of the 0.5 McFarland Standard [6].

Antibacterial activity tests

This assay was conducted using agar-well diffusion method as described below. A 40 mg/mL concentration of the extracts were constituted by dissolving 0.04g in 1 mL of 20% v/v dimethylsulfoxide (DMSO) and 2-fold serial dilutions was made. A single colony of the test isolate was suspended in 2mL of sterile Muller Hinton Broth. The suspension of the isolate was standardized as stated previously and used to inoculate the surface of the Muller Hinton agar and the excess fluid was drained into disinfectant jar. The inoculated agar surfaces were allowed to dry, and the plates was appropriately labeled.

Using a cork borer, four wells of 8 mm in diameter were bored in the inoculated Muller Hinton agar. Using a micropipette, 60μ l of each concentration of the test extracts were delivered into each well. The plates were left on the bench for 30 mins to allow the extracts to diffuse into the agar. Thereafter, the plates were incubated at 37 °C for 24 h. After incubation, the plates were observed for inhibition zones around the wells. The diameters of the zones were measured with meter ruler to the nearest whole millimeter. Each test was carried out thrice and the mean inhibition zone diameter was recorded to the nearest whole millimeter. The test was done separately for the plant extracts.

Minimum inhibitory concentration

This test was carried out for the extract that had shown synergistic activity in the sensitivity tests carried out. The test was carried out using agar dilution method following the procedure outlined by [6]. For the extract 40 mg (20 mg) was weighed and dissolved in 1mL of 20% v/v DMSO to get a stock solution with concentration of 20 mg/mL. Sterile test tubes were arranged in a test tube rack and 1 mL of distilled water dispensed into each of them. From the stock solution, 1 mL was transferred into the first test tube and serial dilution of the extract was carried out until the resultant concentrations in the test tubes became 20, 10, 5, and 2.5, mg/mL. 1 mL of the multer Hinton agar, mixed thoroughly and poured into separate sterile Petri dishes.

The plates were allowed to solidify and then labelled appropriately. A single colony of the test isolate was picked from the subcultured plate with wire loop and inoculated into 2 mL sterile normal saline to make a suspension of the test isolate. The suspension was standardized as stated previously. Using a micropipette, 10µl of the standardized broth culture was placed on the surface of the plates containing various concentrations of the extract. Plain Mueller Hinton agar (that is, without the extract) was also inoculated and served as negative control. Inoculated plates were incubated at 37°C for 24 h and observed for any visible bacteria growth. MIC was taken as the lowest concentration of extract that resulted in no visible growth on the surface of the agar.

RESULTS AND DISCUSSION

Phytochemical analysis of the plant extract of Vernonia amygdalina

Freshly prepared extract was subjected to preliminary phytochemical screening for various constituent and the results of the methanolic leaf extracts of Vernonia amygdalina were summarized in Table 1. After the extract of the plant were extracted, it was then characterized based on its physical properties such as colour, texture and consistency. Base on that, the plant extract was found to be dark green, smooth texture and liniments in consistency. Results of the phytochemical screening for secondary metabolite indicated that cardiac glycosides, flavonoids, alkaloid, carbohydrate, steroid, and saponins were confirmed to be present (Table 1). The presence of such listed metabolites was also confirmed to be presence in V. amygdalina by [9]. In a different research conducted [1] using same plant, they discover that the plant contains tricosane and vernolide. This prove the versatility of this plant for the composition of vast number of secondary metabolites giving it high potential for use in medicinal purpose.

 Table 1. Phytochemical Screening of Methanolic Extract of Vernonia amygdalina Leaves.

S/N	constituents	test	observation	inference
1	saponin	Frothing	frothing	+
2	flavonoid	Ferric chloride test	green precipitate	+
3	steroid	Lieberman Burchard	brown ring at the	+
			interphase	
4	alkaloid	Mayerls test	a yellow coloration	+
5	carbohydrate	Fahling solution	bright red precipitates	+
		a&b		
6	cardiac	Keller-Kiliamis test	purple-brown ring at	+
	glycosides		the interphase	

Key: + = positive, - = negative results

Antibacterial effects of methanolic extract of bitter leaf on *K. Pneumoniae*

The potential of antibacterial effect of methanolic leave extract of *Vernomia amygdalina* at different concentrations were tested against multi drug resistant bacteria (*Klebsiella pneumoniae*). The justification of varying the concentrations of the plant extract is due to the following reason; (1) to determine the optimum level of the extract to inhibit the growth of the bacteria, (2) to determine the tolerance level of the bacteria against the increase in the concentrations of the extract. The results presented in **Table 2** showed that at 20 mg/mL concentration of the plant extract was found to produce zone of inhibition of 14.00 mm. When the concentration was increase to 10, 5 and 2.5 mg/mL concentration, the zone of inhibition was found to be 11.0, 7.0 and 0.0 mm respectively. It was however noted that when compared with control, highest zone of inhibition was recorded at 26 mm.

The results further explain that 2.5 mg/mL of the plant extract does yield any activity against MDR *Klebsiella pneumoniae* isolate. This confirmed that as the concentration of the plant extract increase, the activity of the extract against the bacteria also increases and vice versa (**Table 2**). This might be due to the presence of phytochemical such as cardiac glycosides, flavonoids, alkaloid, carbohydrate, tannins, terpens,

and saponins which were all confirmed in this research and were presented in Table 1 of this study. This result was found to agree with the research of [7] which found same secondary metabolite of the plant extract when screen out the extract obtained from other plant such as A. digitata leaves, A. mannii leaves, and A. alboviolaceum fruit, A. polyanthum fruit and O. Gratissimum twigs. However, when [20] tested the effect of same Vernomia amygdalina on Klebsiella pneumoniae, he reported that no effect of the plant extract was observed on the bacteria. Perhaps the reason why this study suggested positive result was due to the differences in the measuring technique as disc diffusion method was used in the former research. Furthermore, result of research done by [1] using same plant concluded that the plant has strong activities against bacterial pathogens such S. aureus as the plant was found to contained tricosine and vernolide compounds. Pathogens like E. coli and S. typhi were also reported to loos viability in the presence of V. amygdalena extract [2]. [11] further confirm that extract of the plant under study have strong effect against food borne pathogen such as against E. coli, Staphylococcus aureus, Bacillus cereus, Shigella dysentriae and Salmonella typhimurium.

Based on the results of the minimum inhibitory concentration of *Vernomia amygdalina* leave extract, the results indicated that the plants extracts showed antibacterial activities at variable degrees against MDR bacteria, with MICs values varying from 0-14 mm (**Table 2**).

 Table 2. Inhibition zone of diameter of concentration of Vernomia amygdalina on Klebsiella pnenmoniae.

Concentration of Leave Extract (mg/mL)	Zone of Inhibition (mm)
20	14.00±1.3
10	11.00±0.6
5	$7.00{\pm}0.8$
2.5	$0.00{\pm}0$
Control (Gentamycin)	26.00±2.3

It was noted that, concentrations such as 20, 10 and 5 mg/mL inhibited the growth of bacteria, but the bacteria was able to growth 2.5 mg/mL concentration (Table 3). This further validates the results obtained in Table 2 which indicated that 2.5 mg/mL concentration produce 0.0 mm zone of inhibition. Nevertheless, [11] have reported a relatively higher zone of inhibition on food pathogen using same plant species. Furthermore, minimum inhibitory concentration of range 6.25-200 mg/mL were applied to S. aureus, P. aeruginosa, B. subtilis K. pneumoniae and E. coli using ethanolic method of extraction were reported to produce zone of inhibition at range of 0.00±0.0 to 11.5 ± 0.5 mm. The results follow the pattern of increase in concentration of extract decreases the zone of inhibition which is reverse case of our study. Perhaps it could be due to the differences in the concentrations of the extract and solvent employed (methanol/ethanol).

Phytochemical screening results of *Vernomia amygdalina* was in accordance with the results previously obtained [12]. This latter suggested that the presence of alkaloids (which interfere with cell division) in *Vernomia amygdalina* could account for its antibacterial activity. It was therefore demonstrated that methanol extract of *Vernomia amygdalina* possess inhibitory activities against *K. pneumonia*.

 Table 3. minimum inhibitory concentrations (MIC) of plant extract for bacterial growth.

Concentration of extract	Appearance of growth
(mg/mL)	
20	_
10	_
5	_
2.5	+
Control	_
Keys: += positive, - = ne	gative results

To the best of our knowledge, the effect of phytochemical composition of V. *amygdalina* on K. *pneumoniae* using methanolic extraction method was described here for the first time. The different phytochemicals found here should then explain its antibacterial activity against the bacterial strains tested. 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 biopotential chemicals and their subsequent processing into chemotherapeutic agents.

CONCLUSION

Assessment of the effect of phytochemical constituents of *Vernomia amygdalina* on *Klebsella pneumoniae* was conducted in this study. The findings indicated that methanol extract of *Vernomia amygdalina* leave contain several phytochemical constituents such as saponin, tannin, flavonoid and steroid. The constituent of the extract was found to show antibacterial activity on the growth of *Klebsella pneumoniae*. Increase in the antibacterial activity of the bacteria.

REFERENCES

- 1. Abere H, Melaku Y. Antibacterial and antioxidant compounds from the flower extracts of *Vernonia amygdalina*. Adv in Pharmacol Sci; 2018.
- 2. Alo MN, Anyim C, Igwe JC, Elom M, Uchenna DS. Antibacterial activity of water, ethanol and methanol extracts of *Ocimum gratissimum, Vernonia amygdalina* and *Aframomum melegueta*. Adv Appl Sci Res. 2012; 3(2): 844-848.
- Benjaman M. Household salid waste management in gombe metropolis, gombe state nigeria, b.sc thesis, department of geography Gombe State University, 2009.
- Bratu S, Landman D, Haag R, Recco R, Eramo A, Alam M. Quale J. Rapid spread of carbapenem- resistant *Klebsiella pneumonia* in network york city: a new threat to our antibiotic aemamentarium. Arch Intl Med. 2005; 165: 1430-1435.
- Cheesbrough, M. District laboratory practice in tropical countries part 1. 2006. Clsi. (clinical laboratory standard institutes), 2012.
- Djeussi DE, Noumeden JA, Fankam AG, Voukeng IK, Tankeo SB, Kuete V. Antibacterial activities of selected edible plants extracted against multidrug-resistant gram-negative bacteria. Bmc. Compl Alt Med. 2013; 13(1): 164.
- Egharevba HO, Ibrahim JA, Abraham CH, Kunle of. Phytochemical constituents and herbarium specimen of commonly used antidiabetics medicinal plants found in Jos North, Plateau State- a critical Review. J Folklore Med. 2015; 1(1): 12-22.
- Evbuomwan I, Chukwuka EP, Obazenu EI, Lievbare I. Antibacterial activity of *Vernonia amygdalina* leaf extracts against multidrug resistant bacterial isolates. J of Appl Sci and Environ Manage. 2018; 22(1): 17-21.
- 9. Gberikon T, Ichor Enyi TO. Effect of bitter leaf extract (*Vernonia amygdalina*) on culturable Microorganisms Isolated from palm wine in Makurdi Metripolis. Res J Microb. 2016; 11:112-118.

- Ibrahim T, Ajala I, Adetuyi F, Jude-Ojei B. Assessment of the antibacterial activity of *Vernonia amygdalina* and *Occimum gratissimum* leaves on selected food borne pathogens. Elec J of Environ Agric Food Chem. 2009; 8(11): 1213-1217.
- Lyumugabe F, Uyisenga JP, Bayingana C, Songa EB. Antimicrobial activity and phytochemicals analysis of Vernonia aemulans, Vernonia amygdalina, Lantana camara and Markhamia lutea leaves as natural beer preservatives. Am J Food Technol. 2017; 12: 35-42.
- 12. Mahato TK, Sharma K. Study of medicinal herbs and its antibacterial. A review journal of drug delivery and therapeutics. 2018; 8(5): 4754.
- Mohammed S, Modibbo A, Sa'idu H, Ibrahim SA. Preliminary Survey of Medicinal Plant Species in Gombe Metropolitan, Nigeria. Int J Herb Med. 2017: 5(3): 101-104.
- Ncube NS, Afolayan AS, Okoh AI. Assessment techniques of properties of natural compound of plants origin in current methods and trends. Afr J Biotechnol. 2009; 7(12): 1797-1806.
- 15. Oguntola S. Things of bitter leaf can do nigerian tribune. Com.ng/news. 2013/index-/15271, 2013
- Peleg AY, Seifert H, Paterson DL. Acinetobacter baumannii: emergence of successful pathogens. Clin Mirobiol Rev. 2008; 21; 583-582.
- 17. Sofowara EA. The natural guide to medicinal herbs and plant. Tiger books, 2006.
- Bhan MK, Nita Bhandari, S Sazawal, J Clemens, P Raj, Myron Max Levine and Kaper JB. Descriptive epidemiology of persistent diarrhoea among young children in rural northern India. Bull World Health Org. 67, no. 3 (1989): 281.
- Yeap SK, Ho WY, Beh BK, San Liang W, Ky H, Yousr AHN and Alitheen NB. *Vernonia amygdalina*, an ethnoveterinary and ethnomedical used green vegetable with multiple bio-activities. J Med Plant Res. 2010:4(25), 2787-2812.