

THE ANTIBACTERIAL ACTIVITY OF LEAVES OF VERNONIA AMYGDALINA ON SOME ENTERIC BACTERIA

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Abstract

Serial exhaustive extraction was carried out using n-hexane, ethyl acetate, methanol and water to obtain extracts from leaves of *Vernonia amygdalina*. Phytoconstituents present in the extracts were evaluated qualitatively and quantitatively. Activity of resulting extracts was investigated against *Klebsiella pneumoniae* strain Pmk1, *Escherichia coli* ST2747 and *Salmonella enterica* sub specie entericaserovar Typhimurium strain 08-1736 using agar well diffusion technique. Minimum inhibitory and minimum bactericidal concentrations (MIC and MBC) were determined by agar plate method. Effect of standard antibiotics (Gentamycin and Ofloxacin) against test strains were compared with those of crude extracts. The yields obtained from extraction were 4.52g, 4.77g, 4.91g and 5.21g for hexane, ethyl acetate, methanol and aqueous extracts respectively. Phytochemical screening revealed presence of flavonoids, alkaloids, phenols, glycoside, saponins and tannins in all extract types with tannins being highest in composition (9.62mg/ml) in methanolic extract. Extract from polar solvents demonstrated higher activities with methanolic extract showing the highest zones of inhibition (6.2mm) followed by the aqueous extract. However, all the extract types exhibited MIC at same concentration of 125mg/ml. For all extracts, no bactericidal actions were observed at all tested concentrations. The activity of reference antibiotics was higher than crude extracts, however, gentamycin had no effect on *Klebsiella pneumoniae* strain Pmk1 and *Salmonella enterica* sub specie entericaserovar typhimurium strain 08-1736 while three of the extracts produced zones of inhibition against the same test strains. It is evident from this study that extraction technology utilizing serial exhaustive extraction posed an influence on the degree of activity of the various extracts toward the tested bacterial strains.

Keywords: Successive extraction, agar diffusion, antibiotics, phytochemicals, inhibition.

Introduction

More than half of all modern clinical drugs are plant-derived showing that plant products play a significant role in the development of drugs by the pharmaceutical industry. Also, the consumption of plant materials by man is believed to contribute immensely to the improvement of human health and nutrition (Oseni, 2013). Over 80% of the populations of Africa depend on medicinal plants for medical care. Therefore, information on the nutritional and medicinal potentials of plants found in Africa is of immense importance (WHO, 2008).

The indiscriminate use of synthetic anti-microbial drugs commonly used in the treatment of infectious diseases has led to the development of multiple drug-resistant strains of bacteria over the years (Samrot et al., 2010). In addition to this problem, adverse effects on the host including hypersensitivity, immunosuppressant and 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 possess less undesirable side effects (Samrot et al., 2010).

Extraction, as the term is used pharmaceutically, involves the separation of medicinally active portions of plant or animal tissues from the inactive or inert components by using selective solvents in standard extraction procedures. The products so obtained from plants are relatively impure liquids, semisolids or powders intended only for oral or external use (Sukhdev et al, 2008). Successful determination of biologically active compound from plant material is largely dependent on the type of solvent as well as the extraction technology employed. Several extraction techniques abound of which the serial extraction is one. Serial exhaustive extraction which involves successive extraction with solvents of increasing polarity from a non polar to a more polar solvent in order to ensure that a wide polarity range of compounds could be extracted.

Vernonia amygdalina is a member of the Asteraceae family it is a small shrub that grows in the tropical Africa to a height of 2-5m. The leaves are elliptical and up to 20cm long, it has a rough bark (Ijeh et al., 2011). *Vernonia amygdalina* is commonly called bitter leaf in English because of its bitter taste. Some of its common African names include: grawa (Amharic), ewuro (Yoruba), etidot (Ibibio), onugbu (Igbo), ityuna (Tiv), oriwo (Edo), chusar-doki (Hausa), mululuza (Luganda), labwori (Acholi), olusia (Luo) and ndoleh (Cameroon) (Egedigwe, 2010).

Bitter leaf has a grey or brown coloured bark, which has a flaked rough texture. The herb is an indigenous African plant which grows in most parts of sub-Saharan Africa. It is a medicinal plant and of great importance in human diet most especially in its fresh state because of the presence of vitamins and mineral salts (Sobukola et al. 2007). It is a very important protective food as a result of its medicinal value and can be used as an anticancer agent (Clement et al. 2013) antibacterial agent, anti-malaria and anti-parasite agent (Izevbigie et al. 2003). Some principal chemical constituents found in bitter leaf herb are a class of compounds called steroid glycosides- type vernonioside B₁. These chemical substances possess potent anti-parasitic, anti-tumour, and antibacterial effect. The bitter leaf is mainly employed as an agent in treating schistosomiasis, which is a disease caused by parasitic worms. It is also useful in the treatment of diarrhea and general physical malaise. Bitter leaf also helps to cleanse vital organs of the body like the liver and the kidney as well as in the treatment of skin infections such as ringworm, rashes and eczema. However, bitter leaf and other vegetables contain both essential and toxic metals over a wide range of concentrations (Radwan & Salama, 2006).

Vernonia amygdalina has been used traditionally in blood clotting and has elicited a substantial reduction in the level of glucose in the blood at post-prandial time point (Udochukwu et al, 2015). The hypoglycemic activity of *V. amygdalina* has been demonstrated by the reduction in fasting blood sugar in alloxan-induced diabetic rat observed after treatment with different concentrations of aqueous extract of its leaf extract (Fasola et al. 2011).

This work aims at investigating the influence of serial exhaustive extraction on the activity of the extracts against selected bacterial strains and possibly determine their mechanism of action.

Materials and Methods

Collection and Identification of Plant Materials

Fresh leaves of *Vernonia amygdalina* were obtained from University of Ilorin GRA quarters, Ilorin, Kwara state, Nigeria in the month of December 2015. This plant was authenticated and deposited at the herbarium unit of the Department of Plant Biology, University of Ilorin, with voucher specimen number UILH/ 001/302.

Collection and maintenance of test strains

Test bacterial strains; *Klebsiella pneumoniae* strain Pmk1, *Escherichia coli* ST2747 and *Salmonella enterica* sub specie *entericaserovar typhimurium* strain 08-1736 were obtained from the culture collection unit of the Department of Microbiology, University of Ilorin and maintained on appropriate agar slants after which they were routinely sub cultured for the purpose of purity and refrigerated at 4°C (Adetunji et al. 2013).

Plant Preparation and Extraction

The fresh plant materials collected were air-dried for a period of two weeks, pre-crushed in a mortar and subsequently pulverized into fine powder using electric blender. A serial exhaustive extraction was carried out as described by Adetunji et al (2013) with slight modifications. Hexane, ethyl acetate, methanol and water were the solvents of choice used according to their order of increasing polarity. One hundred grams (100 g) of the powdered leaves were soaked into 1000 ml of the first solvent (hexane), left on a rotary shaker for 24hr and filtered. The residue was air dried for a few hours and subsequently soaked in the second solvent (ethyl acetate) and the procedure was observed up till the last solvent (water) to obtain the respective filtrates.

Qualitative and Quantitative determination of phytochemicals

Qualitative phytochemical screening

Test for flavonoids was carried out as described by Ladipo et al (2010). The method of Obdoni and Ochuko (2001) was used to determine the presence of cardiac glycosides. Alkaloids and tannins were determined by the method of Vijay and Rajendra (2014). Saponins and phenols and terpenoids were determined using the method of Ifemeje et al (2014).

Quantitative Determination of Phytochemicals

Total flavonoid content was determined by aluminium chloride colorimetric assay adopted by Vijay and Rajendra (2014). The percentage cardiac glycoside was calculated following Ifemeje et al. (2014). The percentage alkaloid content was calculated as following the methods of Harborne, (1973), Obdoni and Ochuko (2001) and Ifemeje et al. (2014). The percentage of saponins present after dissolution in 20% acetic acid and ethanol and filtration upon concentration in water bath was carried out as described by Obdoni and Ochuko (2001) and Ifemeje et al. (2014). Total tannin content was determined by Folin - Ciocalteu method (Vijay and Rajendra, 2014). The concentration of phenolics in plant extracts was determined using spectrophotometric method following Vijay and Rajendra (2014). The dried ether extract was treated as total terpenoid (Ladan et al., 2014).

Antibacterial Susceptibility Testing

Agar Well Diffusion Technique

Agar well-diffusion technique described by Adetunji et al. (2013) was adopted in determining the activity of extracts against test organisms. A day old inoculums (0.1ml) of each test organism was separately streaked onto the surface of sterile Mueller-Hinton agar plates. A known volume of each extract at the prepared concentrations (300 and 150mg/ml) was introduced separately into wells at the centre of the agar plates and incubated at 37°C for 24 hours. Diameter of zones of inhibition was measured to the nearest milliliters around the well of the agar plates and recorded.

Minimum Inhibitory Concentration

Extract solutions at concentrations of 125, 100, 75, 50 and 25mg/ml were prepared. A day old inoculums (0.1ml) of each test strain was separately streaked onto the surface of sterile Mueller-Hinton agar plates. Extract solution at the different concentrations were separately

introduced into wells at the centre of each agar plate and incubated at 37°C for 24 hours. Diameters of zones of inhibition were measured to the nearest milliliters around the well of the agar plates and recorded (Doughari, 2006).

Determination of Minimum Bactericidal Concentration (MBC)

The method of Gumgumjee et al.(2012) was adopted. Sterile peptone water (9ml) was measured into 3 test tubes, 0.8ml each extract concentrations and 0.2ml of each test strain were added. This was incubated at 37°C for 24hours. Following incubation, 0.1ml of broth culture was taken from each of the tubes and inoculated into sterile petri dishes using pour plate method. Plates were incubated at 37°C for 24hours and observed for possible growth. Concentrations of extracts that showed no growth following incubation were regarded as the MBC.

Antibiotic Sensitivity Assay

This was determined using modified Kirby-Bauer method of 1966. Antibiotic discs gentamicin and ofloxacin (oxid) were placed on agar plates streaked with a day old inoculums of each test organisms and incubated at 37°C for 24hours. The diameter of zones of inhibition were measured and recorded after 24 hours (Ladipo et al, 2010).

Results

Recovery of crude extracts from leaves of *Vernonia amygdalina*

Following extraction and concentration of crude extracts using different solvents of increasing polarity the yield obtained for each extract were weighed and recorded as follows: Aqueous (5.21g), Methanol (4.91g), Ethyl acetate (4.77g) and n-hexane (4.52g).

Qualitative and quantitative evaluation of phytochemicals from crude extracts

The qualitative and quantitative evaluations of phytochemical present in the various extracts are as shown in Table 1. and Figure 1. respectively. From the qualitative screening, it was observed that methanolic and ethyl acetate extracts had the presence of all tested (7) phytochemicals. In the quantitative assay, flavonoids was highest in the aqueous extract while phenols was least in composition in the same extract (Figure 1).

Antibacterial activity of extracts of *V. amygdalina* against test bacterial strain

The effect of extracts of *V. amygdalina* against test strains revealed varying degree of activity as shown in Figure 2. The highest inhibition (6.2mm) was observed with methanolic extract in the assay against *Salmonella enterica* sub specie *enterica* serovar *typhimurium* strain 08-1736 at a concentration of 300mg/ml. Generally all the test strains demonstrated considerable zones of inhibitions. However, all three test strains resisted all concentrations of n- hexane extract.

Table 1: Qualitative phytochemical screening of four extracts of *V. amygdalina*

Phytochemicals	Extracts			
	N-hexane	Ethyl acetate	Methanol	Aqueous
Flavonoids	+	+	++	++
Glycosides	++	+	++	+
Saponins	+	+	++	++
Tannins	+	++	+	+
Phenols	+	+	+	+
Terpenoids	—	++	+	—
Alkaloids	++	+	++	+

Keys: + + = Abundantly present
+ = present
- = Absent

Keys:

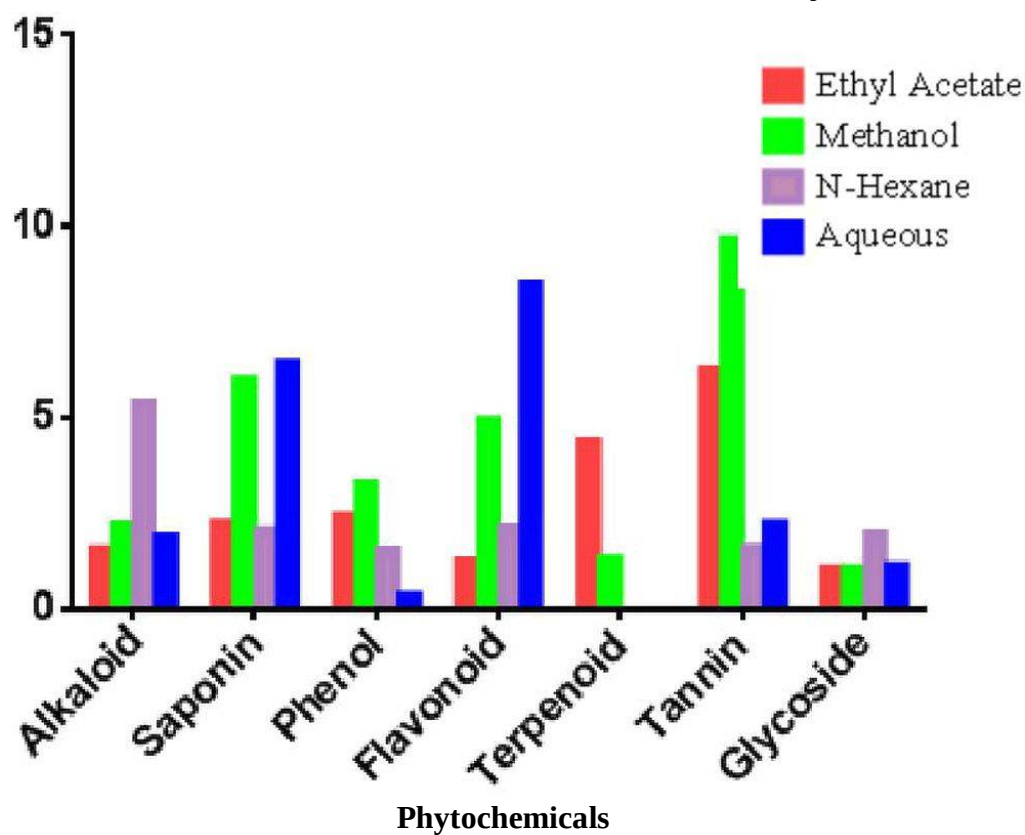


Fig 1: Quantitative phytochemical profile of extracts of *V. amygdalina*

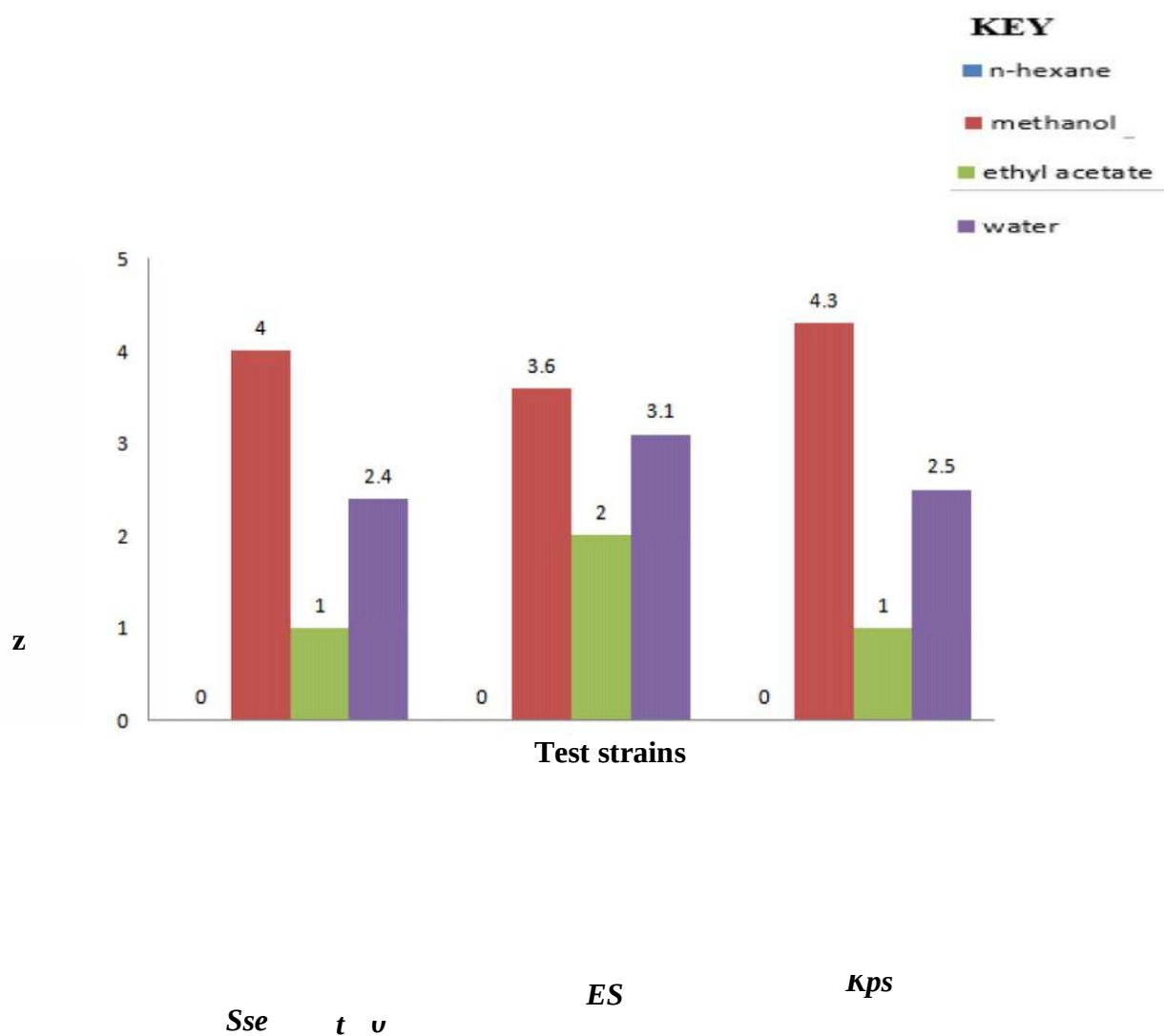


Fig 2: Susceptibility pattern of test strains to extracts of *V. amygdalina* at 150mg/ ml

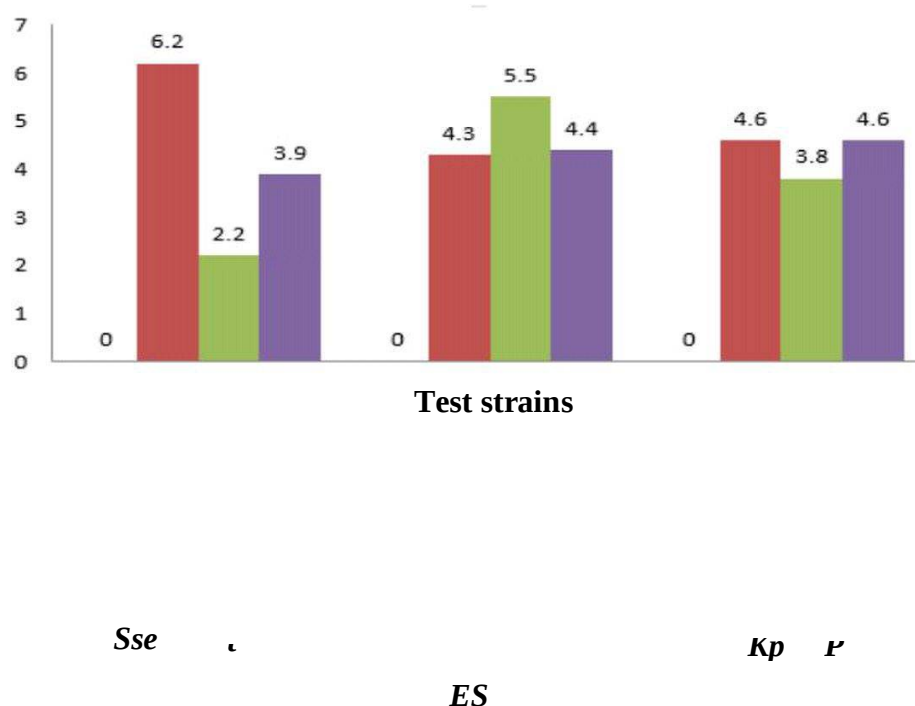


Fig 3: Susceptibility pattern of selected bacterial strains to extracts of *V. amygdalina* at 300mg/ ml

Evaluation of Minimum Inhibitory and minimum bactericidal concentrations of extracts of leaves of *Vernonia amygdalina*

The lowest MIC value (125mg/ml) was observed in the assay of all extract types against the test strains with the exception of ethyl acetate extract as shown in Table 2. All extracts exhibited bacteristatic activity, hence no MBC values were obtained.

Comparison of antibacterial activity of crude extracts with reference antibiotics

The activities of reference antibiotics were generally higher than those of the crude extracts. However, gentamycin had no effect on *Klebsiella pneumoniae* strain Pmk1 and *Salmonella enterica* sub specie *enterica* serovar typhimurium strain 08-1736 while three of the extracts produced zones of inhibition against the same test strain.

Table 2: Minimum inhibitory and minimum bactericidal concentrations of extracts of *V. amygdalina*

Test strains	MIC of extracts(mg/ ml)			MBC of extracts(mg/ ml)
	Ethyl acetate	Methanol	Aqueous	
08-1736	-	125	125	-
ST2747	125	125	125	-
Pmk1	125	125	125	-

KEY:

- = No MBC

08-1736= *Salmonella enterica* sub specie entericaserovar typhimurium strain 08-1736ST2747= *Escherichia coli* ST2747Pmk1= *Klebsiella pneumoniae* strain Pmk1**Table 3: Comparison of antibacterial activity of extracts of *V. amygdalina* with reference antibiotics**

Test strains	Diameter of zone of inhibition (mm)					
	n-hexane	Ethyl acetate	Methanol	Aqueous	Gentamycin	Oflaxacin
ST2747	0.0	5.5	4.3	4.4	6.0	8.0
Pmk1	0.0	3.8	4.6	4.6	-	16.0
08-1736	0.0	2.2	6.2	3.9	0.0	<u>15.0</u>

KEY:

- = No activity

08-1736= *Salmonella enterica* sub specie entericaserovar typhimurium strain 08-1736ST2747= *Escherichia coli* ST2747Pmk1= *Klebsiella pneumoniae* strain Pmk1**Discussion**

This work has demonstrated the possible effects of various extracts of *V. amygdalina* against some selected test strains with considerable efficacies. The yield obtained from the study imply that the solvent system and technology of serial extraction used were adequate for the release of active plant components into the crude extracts. According to the study, the highest yield obtained from the aqueous and methanolic extracts is an indication that polar solvents are better for dissolution and extraction of phytochemicals compared to non polar ones for the leaves of *V. amygdalina*. This is in line with the work of Uzoigwe and Agwa (2011) indicating that polar solvents like ethanol is suitable for extraction of phytochemicals from leaves and roots of *V. amygdalina*. The screening for the presence and quantity of active phytoconstituents present in the crude extracts is in line with some past literatures, however, very few literatures exists on the use of successive extraction for the test plant used in this study. Similarly, Adetunji et al (2013) and Yasmazura et al. (2016) have reported the presence of same phytochemicals in *V. amygdalina*. In terms of composition, several factors are responsible for the distribution of phytoconstituents in plant tissues and one of which is environmental factors. Thus the difference existing in the types and composition of these active compounds among different authors may be explained for by environmental influences such as soil nature, topography, climatic conditions among others (Prashant et al., 2011).

Methanolic extract exhibited the highest activity against the test stains when compared to other extracts derived from other three solvents. Similar incidence has been reported by Oseni et al. (2014). This can be attributed to its polar nature due to the presence of several hydroxyl groups which enhances its formation of hydrogen bonds with other molecules. Subsequently, interaction of these hydroxyl groups are more likely to result in binding to microbial proteins since proteins comprise of several arrangements of various functional groups of which hydroxyl atoms are one. Although, the antibacterial assay revealed some form of reduced susceptibility by the test organisms towards all the extracts as reflected by

reduced zones of inhibition contrary to some past literatures. This could be as a result of the strain types used in the assay. According to literature it has been established that the genetic traits of microbial cells are resident in the different strains of such organisms. Hence strains have often become more important in determining the specific behavior of microorganisms. This implies that the types of strains used in this study may possess some resistance plasmids which contributed to reduced susceptibility. Similarly, Gram negative organisms have been known to possess virulent characteristics such as the presence of efflux pump system known to pump out unwanted chemical substances. For instance, Min-Jung et al. (2016) reported *Escherichia coli* ST2747 which was one of the test strains used in this study, to be pathogenic causing both urinary tract infections and neonatal meningitis. The strain has a genome analysis of NCCP15653 which makes it belong to Group-D *E. coli* strain. Also, *Klebsiella pneumoniae* strain Pmk1, possess a circular chromosome, plasmid (Ppmk1-NDM; circular), nucleotides (5990738), protein genes (5594) and RNA genes (113) which differ from other species of *K. pneumoniae* (Stoesser et al., 2014).

The assay of the minimum inhibitory and minimum bactericidal concentrations also buttress the fact stated above on the virulence of the test strains used. Hence MIC was obtained at a much higher concentration when compared with some other existing literatures such as Adetunji et al. (2013). This could have accounted for reasons why extracts showed no bactericidal activity.

In the comparative analysis between the reference antibiotics and *Vernonia amygdalina* crude extracts, it was observed that extract activity were better than gentamycin against *Klebsiella pneumoniae* strain Pmk1 and *Salmonella enterica* sub specie *enterica* serovar typhimurium strain 08-1736. This agrees with the observation of Mukhar and Huda (2005) that, some plant extracts are more potent than source antibiotics. According to Sharma and Sharma (2010), the acetonic, methanolic and ethanolic extracts of *V. amygdalina* showed a higher antifungal activity against *S. cerevisiae* than the control ciprofloxacin and gentamycin. However, due to the broad spectrum nature of Ofloxacin, it could have been able to attack various strain types. Generally however, the enhanced activity of the antibiotics may be associated with the fact that they are purified synthetic chemicals which are likely to be more definite in action unlike crude extracts which represent a combination of several compounds both inert and active, some of which could militate against antimicrobial action. This is in line with the work of Muhammad et al. (2014).

From the results emanating from this study, it is evident extracts of *Vernonia amygdalina* possess activity against the selected test strains despite their established virulence. With the increasing interest in the search for alternative remedies for the treatment of microbial infections threatening human existence, it is evident that secondary plant metabolites have become very promising in proffering solutions to this effect and *Vernonia amygdalina* may be just one of such promising agents. Also, its ready availability and affordability as well as its non-toxicity at required concentration makes it a good candidate for exploitation and possibly incorporation into orthodox health delivery.

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