Antibacterial and effect of dosage of Methanol and Omidun Extract of Root of *V. paradoxa* on some diarrheagenic bacteria

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Abstract: Root of *V. paradoxa* extracted using methanol and omidun was tested for efficacy by *in vitro* assay (Minimum Inhibitory Concentration (MIC) and disc diffusion assay) and effect of dosage on albino rats was determined. The test organisms used include *Salmonella typhi*, *Shigella flexneri,* Enteropathogenic *Escherichia coli* (EPEC ATCC 43887), Enterohaemorrhagic *Escherichia coli* (EHEC ATCC 43889) and *Escherichia coli* ATCC 25922. Four groups (I-IV) of five rats per group was treated thus: Group I were infected with EPEC and given ciprofloxacin; group II rats were only infected, group III were infected and treated with omidun extract and group IV rats were not infected nor treated rats. The procedure was replicated for each organism, methanol extract and 50mg/mL /100mg/mL of the extracts respectively. Liver and kidney samples of the treated were collected after six hours of observation for diarrhea symptom. Methanol extract gave higher percentage yield (20.80%) more than omidun (19.2%) extract. MIC of methanol extract (3.15 µg/mL -50.0µg/mL) was significantly different ($P<0.05$) from that of omidun extract (6.25 µg/mL -50.0 µg/mL) on all the tested microorganisms. Higher concentration tested and methanol extract (17.00mm) gave wider inhibition zones than omidun extract (12.67mm). Amounts of active constituents were slightly higher in methanol extracts than in omidun extracts. Livers and kidneys of rats in groups I and IV showed no abnormality. Mild inflammation to focal lymphocytic aggregate was observed in the portal area of liver and kidneys of rats treated with 50mg/mL. Intense infiltration, marked distortion of architecture and vacoular degeneration was seen in group II rats and those of rats treated with 100mg/ml concentration. Hence, omidun is a good extraction solvent and the root extract of *V. paradoxa* can be toxic at high concentration.
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1. Introduction

Medicinal plant is defined as any substance with one or more of its organs containing substances that can be used for therapeutic purposes or which can be used as precursors for the synthesis of antimicrobial drugs (Sofowora, 1984; Tapsell *et al.,* 2006). Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been generated from natural products (Lahlou, 2007).

In developing countries, it is estimated that about 80% of the population rely on herbal medicine for their primary health care (Matu and Van-Staden, 2003), because of better cultural acceptability, belief of better compatibility with the human body and fewer side effects (Mohammed *et al.,* 2010). It has been used in many parts of the world as a rich tradition for the treatment of many infectious diseases, including diarrhea and abdominal pain (Brantner and Grein, 1994).

The major cause of diarrhea among children in developing countries is malnutrition. To nullify the problem of diarrhea which is a leading cause of mortality in developing countries, the World Health Organization has constituted a diarrheal disease control programe (CDD) which includes studies of traditional medicinal practices, together with the evaluation of health education and prevention approaches (Sheba *et al*., 1999). Search for potent herbal drugs require the bringing together of ethnobotanical, ethnopharmacological, chemical, biological, pharmacological and toxicological studies (Gurib-Fakim, 2006).

Considering the importance of plants as a source of medicine, in thepresent study a plant *Vitellaria paradoxa*, often known as Shea butter tree, family-Sapotaceae, which is in use traditionally in the treatment of many diseases was selected. Hence, this plant has been extracted traditionally using omidun along with conventional methanol as extraction solvents of extraction and the different solvent extracts comparatively investigated for potential use in treating diarrhea.

2. Materials and Methods

Collection of Diarrheaogenic bacteria

Clinical diarrheagenic isolates (*Salmonella typhi* and *Shigella flexneri*) and typed cultures Enteropathogenic *Escherichia coli* (EPEC ATCC 43887), Enterohaemorrhagic *Escherichia coli* (EHEC ATCC 43889) *Escherichia coli* ATCC 25922 used in this study were obtained from Sacred Heart Hospital Lantoro, Abeokuta Nigeria and National institute of medical research, Yaba Lagos Nigeria respectively. Pure cultures of the isolates were promoted by further subjecting them to cultural and morphological identification and finally biochemical characterization using protocols described by Cheesbrough (2002). The pure cultured isolates were then maintained on appropriate media for further use.

Collection and preparation of roots of *V. paradoxa*

Roots of *Vitellaria paradoxa* (Shea butter tree) were collected in Ilorin, Kwara State of Nigeria, and confirmed by local farmers before further authentication and identification in the Herbarium Laboratory of the Department of Forestry and Wildlife Management, Federal University of Agriculture, Abeokuta where it was assigned identification Number UAHA NO. 015/001. The root was washed with sterile water and dried under shade; it was reduced into small pieces with a surface-sterilized scalpel before milling with electric blender (Model Marlex BL 238). A quantity of 150 g each of the fine powder were weighed into two seperate 1000ml-capacity conical flasks and 500 ml methanol and omidun were added to each powder in the conical flasks respectively. Each was allowed to stand for 48hours with constant shaking at regular intervals to facilitate extraction (Asuzu and Onu, 1994). The percolates were then filtered and the resulting volume on filtration was reduced to dryness with a Rotary evaporator (RE 300) at 45 ± 10ºC. The extracts were then collected, weighed, packed in sterile air tight containers and labeled. They were kept in the refrigerator at 4ºC until needed for analysis.

Quantitative phytochemical screening

The phytochemicals which are present in the methanol and omidun extracts of root of V. paradoxa were determined and quantified by standard procedures as described by these authors: Harborne, 1973; Hagerman et al., 2000; Obadoni and Ochuko, 2001; Kumaran and Karunakaran, 2006 and AOAC, 2010):

Preparation of Dilute Stock of Extracts

Stock solution of root extract was prepared in three test tubes labeled 1 to 3. A stock concentration of 100mg/mL of the extract was prepared in the first test tube, subsequently; 5ml of distilled water was then introduced into the remaining two test tubes. 5mL of the stock was withdrawn from the first tube and added to the second test tube which was mixed thoroughly to obtain a concentration of 50mg/mL. Another 5ml was withdrawn from the second tube and then transferred to the third tube which was also thoroughly mixed to give a concentration of 25mg/mL.

Experimental Animal

Albino rats made up of either sex (weighing between 180g – 220g) but from the same genetic lineage were procured from the University of Ibadan, Animal House. They were kept in Laboratory cages and maintained according to the NIH guidelines of care and use of laboratory Animals published by Saha *et al*. (2001). They were acclimatized to standard laboratory conditions (temperature 24 ± 1ºC and a 12 hours photoperiod), fed twice daily with standard commercial feeds (Vital Feeds, Nigeria) and distilled water adlibitum for one week before the commencement of the experiment.

Ethical Considerations

Albino rats used for this research were appropriately selected. Rats were kept in Laboratory cages and maintained according to the NIH guidelines of care and use of laboratory Animals published by Saha *et al*. (2001) while standards of the animal care and administration met those required by applicable international laws and regulations. Painful procedures were performed under anesthesia to avoid distress and pain to the albino rats.

Experimental Design

A total number of 155 male (75) and female (80) experimental rats (weighing between 180-220g) were randomly assigned into 4 groups (I- IV) of 5 rats each. They were fasted overnight prior to the experiment but allowed free access to water ad libitum. The various groups were treated as follows:

Group I: Received organisms and Ciprofloxacin (positive control).

Group II: Received organisms only (negative control).

Group III: Received organisms and methanol as well as omidun extract of root of *V. paradoxa* respectively.

Group IV: Received nothing.

Rats in all groups were weighed prior to and after the experiment to determine the growth index, each rat in groups I-III were orally given 0.5mL of 18- hr broth culture of *S. typhi* to induce diarrhea. One hour later, all groups I-III were treated by intraperitoneal route as follows: Group I received 2mLs of commercially prepared Ciprofloxacin, two subgroups of III received 2mLs of 50mg/kg and 100mg/kg of methanol extracts of the root of *V. paradoxa*, group IV received nothing. The procedure was replicated for *E. coli* ATCC 25922, omidun extracts and two dosages of the extract (50mg/kg and 100mg/kg) respectively. The treated rats were then placed in separate cages over clean white paper and observed for the presence of diarrhea every

one hour for six hours for the onset of diarrhea and number of diarrhea episodes.

Histology

At the end of the *in vivo* assay, laparotomy was carried out by placing experimented rats in a jar containing cotton wool soaked with chloroform followed by jugular puncture with a sharp sterile blade. Thin sections of each of Liver and kidney samples (3µm) were quickly excised and fixed in 10% formalin for histopathological studies. The organ sections were histologically processed, stained with Haematoxylin and Eosin (H & E) and observed with light microscope for histopathological changes.

Data Analysis

The experiments were done in triplicate and data were expressed as mean ± Standard deviation (S.D). Statistical comparison was performed between the groups by one-way Analysis of Variance (ANOVA) and Duncan Multiple Range Test used to separate the means which was determined at the 5% probability level using SPSS 16.0 for Windows (SPSS, 2007).

3. Results

Methanol extraction from root of *V. paradoxa* gave the higher percentage yield (20.80%) than omidun (19.2%).

Table 1. Percentage Yield after Extraction of root of *V. paradoxa* with methanol and omidun

Extraction solvent	Dry Raw powder (g)	Yield from dry raw powder (g)	Percentage yield%
Methanol	150	31.2	20.8
Omidun	150	28.8	19.2

Table 2 shows the result obtained for Minimum inhibitory concentration (MIC) of methanol and omidun extracts of root of *V. paradoxa*. There was significant difference (P<0.05) in the MIC of methanol extract (3.15 µg/mL -50.0µg/mL) and omidun extract (6.25 µg/mL -50.0 µg/mL) of root of *V.paradoxa* on all the tested microorganisms.

Table 2. Minimum Inhibitory Concentration (MIC) of Methanol and Omidun Extracts (µg/mL) of root *V. paradoxa* on Diarrhoea Inducing Bacteria tested

Extract	EHEC ATCC 43889	EPEC ATCC 43887	*S. typhi*	*Sh. Flexneri*	*E. coli* ATCC 25922
Omidun root	12.50 [a]	50.00 [a]	6.25 [a]	12.50 [a]	6.25 [b]
Methanol root	6.25 [b]	50.00 [a]	6.25 [a]	6.25 [b]	3.15 [b]

Values with different superscript on the same column are significantly different (p< 0.05)

The antibacterial assay result of omidun and methanol extracts of root of *V. paradoxa* revealed a concentration dependent zone of inhibitions (Table 3). But at all concentrations tested, methanol extract gave higher zones of inhibitions (ranging from 2.67mm to 17.00mm) than omidun extract (ranging from 2.33mm to 12.67mm)

Table 31. *In vitro* Inhibition zone (mm) of Different Concentrations of Omidun and Methanol extracts of Root of *V. paradoxa* on tested Microorganisms

Concentration of Extract	Extraction Solvent	EHEC ATCC 43889	EPEC ATCC 43887	*S.typhi*	*Sh.flexneri*	*E.coli* ATCC 25922
100 (mg/mL)	Methanol	12.67±1.16	6.67±2.08	17.00±1.00	14.67±1.16	15.00±1.00
	Omidun	11.00±1.00	4.67±1.16	12.33±1.53	10.33±0.58	12.67±1.53
50(mg/mL)	Methanol	8.00±1.00	4.67±1.16	13.33±4.93	13.33±4.93	9.33±0.58
	Omidun	7.00±1.00	4.67±0.58	9.33±0.58	9.33±0.58	11.00±1.00
25(mg/mL)	Methanol	6.33±1.16	3.00±1.00	2.67±1.16	3.00±1.00	4.67±0.58
	Omidun	4.67±0.58	2.67±0.58	2.67±0.58	2.33±0.58	3.67±0.58

Result expressed as mean ± SD of triplicate measurement

Qualitative phytochemical analysis of the extracts revealed the presence of all the tested active ingredients (tannin, saponin, flavonoids, alkaloids, oxalate, steroid, terpene, Phlobatannin, cardiac glycoside and anthraquinone) in methanol, omidun, sterile-omidun and sterile distilled water extracts of leaf, bark and root of *V. paradoxa* (Table 4).

Table 42. Preliminary Phytochemical Constituents of Methanol and Omidun extracts of Root of *V. paradoxa*

Phytochemical Constituents	Methanol	Omidun
Tannin	+ve	+ve
Saponin	+ve	+ve
Alkaloid	+ve	+ve
Oxalate	+ve	+ve
Flavonoid	+ve	+ve
Steroid	+ve	+ve
Terpene	+ve	+ve
Phlobatannin	+ve	+ve
Cardiac glycoside	+ve	+ve
Anthraquinone	+ve	+ve

+ve Present

The active ingredients present methanol and omidun extracts of root of *V. paradoxa* were found present in varying proportion (Figures 1). Although, these constituents were slightly higher in methanol extracts of than omidun extracts. Oxalate was found present in larger amount than other active ingredients, some of which were found present in moderate quantities (tannin, saponin, flavonoid and anthraquinine) while others (alkaloid, steroid, terpene, phlobatannin and cardiac glycoside) were present in small quantities.

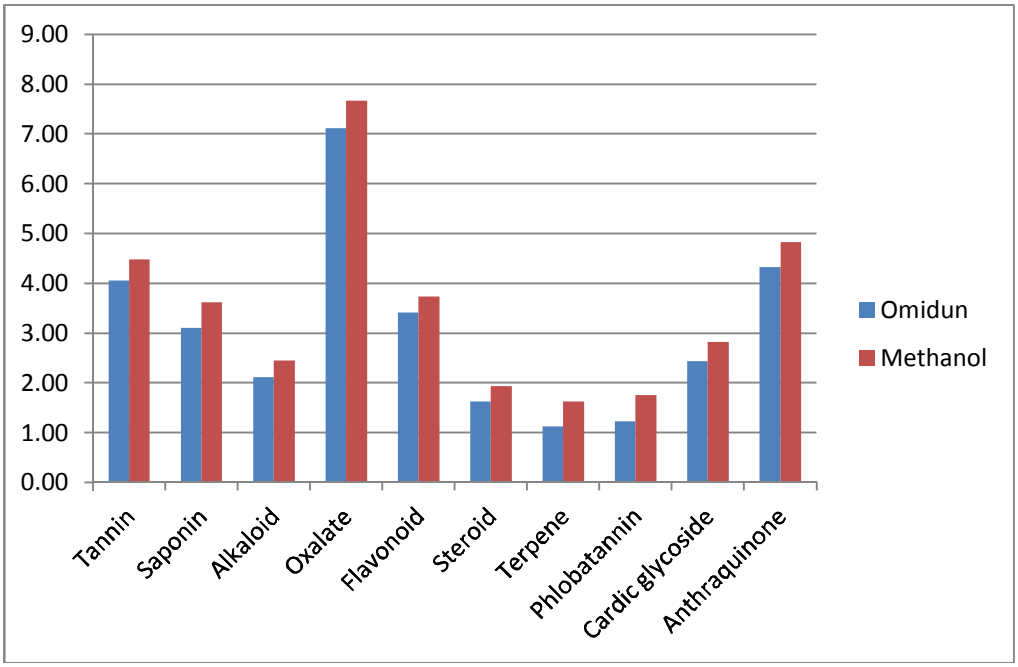

Figure 1. Comparative Phytochemical result of omidun and methanol extracts of root extracts of *V. paradoxa*

Similar presentations with no histopathological changes (plate 1 (A) and E) was observed in Livers and kidneys of rats in the non-challenged (with the tested microorganisms) nor treated groups and those of rats in the grouptreated with commercial antibiotics. However, moderate inflammation was observed in liver of rats in the group treated with low concentration (50mg/mL) of solvents extracts (methanol and omidun) of the root as in plate 1(B) while livers of rats in the group treated with high concentration (100mg/mL) of root extract showed moderate vacoular degeneration of centrilobular hepatocytes as shown in plate 1 (C). Kidneys of rats treated with 50mg/mL concentration of solvent extracts of the root revealed mild to moderate mononuclear cellular infiltrate (Plate 2 (F)) and at high concentration (100mg/mL), inflammation and moderate degeneration was observed in the kidneys of rats in this group as shown in plate 2 (G).

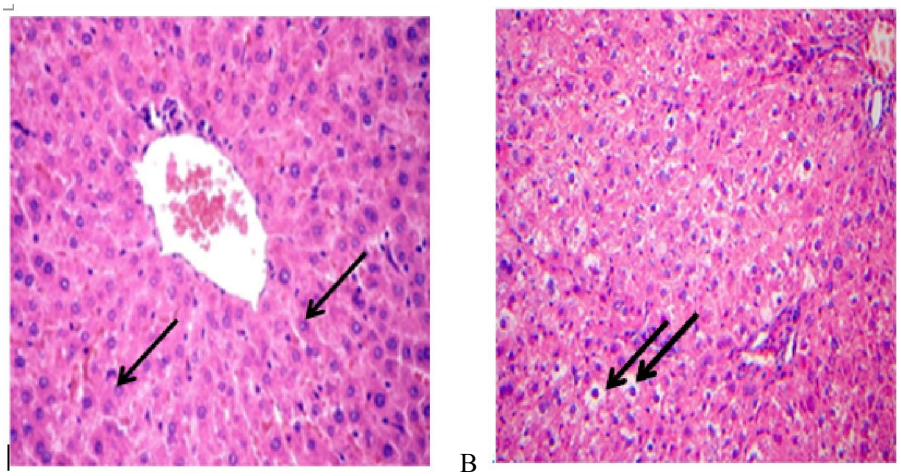

Plate 1: Microscopic Presentation of liver of Diarrhoea Induced Albino Rats Treated with 50mg/mL and 100mg/mL root extracts of *V. paradoxa*

A. Normal hepatic presentation. (H & E. X400). B: moderate mononuclear cellular infiltrate in the portal area (H & E. X400).

B. moderate mononuclear cellular infiltrate in the portal area (H & E. X400).

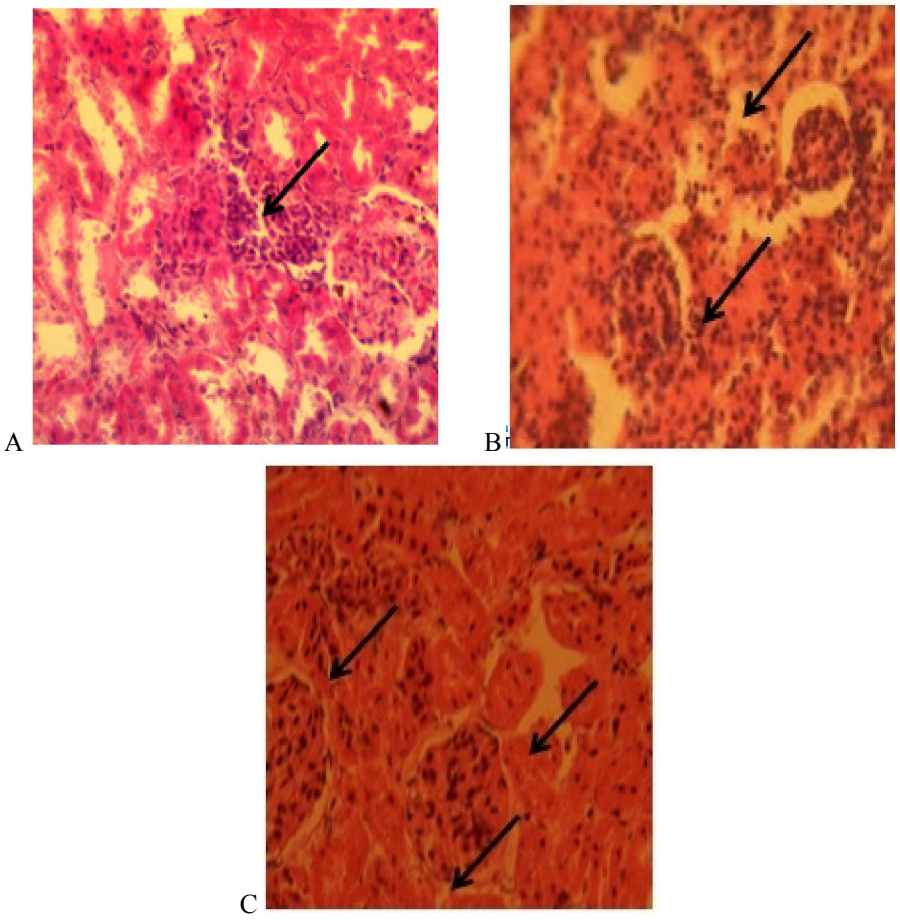

Plate 2: Microscopic Presentation of Kidney of Diarrhoea Induced Albino Rats Treated with 50mg/mL and 100mg/mL of root extract of *V. paradoxa*

A: Focal interstitital lymphocytic aggregate in the kidney. (H & E. X400).

B: marked distortion of the architecture and vacuolar Degeneration. (H & E. X400).

C: Normal Kidney presentation with prominent structural. (H & E. X400).

4. Discussions

The present study was designed to evaluate the effects of conventional (using methanol) and traditional (using omidun) extraction of root of *V. paradoxa* on some diarrhoea causing organisms using the *In vitro* and *In vivo* assay methods. Methanol as solvent for extraction of the root of *V. paradoxa* gave higher percentage yield (20.8%) more than omidun (19.2%). This slight disparity may be as a result of variance in polarity of these two solvents. Reports by Ncube *et al*. (2008) and De Boer *et al*., (2005) state that that choice of solvent used in extraction of plants may have effect on the yield after extraction and active components of plants are more soluble in organic solvent. Similar report was given by Sun *et al.* (2005) who stated that methanol was most effective in extracting active components from oat bran in contrary to 52% yield of water extracts of *Senna obtusifolia* obtained by Doughari *et al*. (2008). Owolabi *et al.,* (2007) also reported a yield of 10.74% for water extract and 3.78% of their ethanolic extracts.

Minimum inhibitory concentrations (MIC) of the extracts on the tested organisms were 50mg/mL, 25mg/mL, 12.5mg/mL, 6.25mg/mL and 3.25mg/mL. Crude plant extracts are generally a mixture of active and non-active compounds and MICs of less than 100mg/mL suggest good antimicrobial activity (Webster *et al.,* 2008). Thus, the MICs observed in this work were lower than 100mg/mL and this implies high antimicrobial activity of root of *V. paradoxa*. The low MIC is of great significance as this plant could be used as an alternative to orthodox medicine in the treatment of microbial infections, with rapid increase in microbial resistance to known antibiotics (Singleton, 1999).

Methanol extract gave lower MIC (3.15 µg/mL - 50.0 µg/mL) than omidun extract (6.25µg/mL -50.0 µg/mL). This could be an indication that there is a variation in the levels of compounds in two solvent extracts (methanol and omidun) of root of *V. paradoxa* (Anjorin *et al*., 2010) and may also mean the presence of the more dissolved phytochemicals in methanol than omidun extract of the plant part. This is in agreement with the report of De Boer *et al*. (2005) who reported that active components of plants are more soluble in organic solvent. This finding is in conformity with the work of Yahya *et al* (2012) in which methanolic extract of the stem bark of *Combretum glutinosum* showed the highest level of activity on *Salmonella typhi* and *E. coli* while the aqueous extract showed less response.

The antibacterial activities by disc diffusion method revealed that omidun and methanol extracts of root of *V. paradoxa* inhibited growth of the tested microorganisms (EPEC ATCC 43887, EHEC ATCC 43889, *S.typhi*, *Sh. flexneri* and *E. coli* ATCC 25922). Even though all the tested microorganisms were susceptible to methanol and omidun extracts at all concentrations tested, the zones of inhibitions obtained were concentration dependent. Concentration dependent zones of inhibitions have also been reported in antimicrobial activities of roots of *R. sativus* (El-Tohamy *et al*., 2010). Akinpelu *et al.* (2011) also reported the bioactivity of the methanolic crude leaf extract of D. guineense on some bacteria.

Better potency of omidun extracts may probably have resulted from the synergistic effects of microorganisms (such as lactic acid bacteria and *Aspergillus fumigatus*) found in it and the active compounds present in root of *V. paradoxa*. These microorganisms have been shown to control some diarrhoea organisms in *in vitro* study by Falana *et al*. (2012). Lactobacilli are important lactic acid bacteria in foods, which contribute not only to acidification of foods, but are also important in the preservation of food, prevention of pathogens and improve the palatability of foods (Hounhouigan *et al.,* 1993). Lactobacilli exert strong antagonistic activity against many microorganisms including food spoilage organisms and pathogens (Obadina *et al*., 2006; Valenzuela *et al*., 2008; Ma *et al*., 2009). Furtado *et al.* (2005) has also reported the production of some antimicrobial metabolites from *A. fumigatus*.

Although, previous work on *V. paradoxa* by Ayankunle *et al.* (2012) suggests that Gram- positive bacteria are more susceptible to the antimicrobial effect of ethanol extract of *V. paradoxa* than Gram-negative bacteria, this study however showed that the two tested solvent extracts of root of *V. paradoxa* were found inhibitory against the tested Gram-negative organisms. This agrees with the report of Aibinu *et al*. (2007) that omidun extracts of *Bryophyllum pinnatum* and *Kalanchoe crenata* were found inhibitory against some Gram-negative organisms. This result further support the report of Bibitha *et al*. (2002) that variations exist in the antibacterial activities of different plant extracts.

The effect produced by the positive control agent (Ciprofloxacin) on the tested microorganisms was higher than the tested concentrations of methanol and omidun extracts of root of *V. paradoxa*. This indicates that the commercial antibiotic (orthodox medicine) was more effective against the tested microorganisms than herbal medicine. This disparity may be due to the mixtures of bioactive compounds present in the extract compared to the pure compound contained in the standard antibiotics (Gatsing *et al.,* 2010). The solvent extracts contain many active compounds that are in minor which are working either in synergy (Davicino *et al.,* 2007) some of which might have been degraded

by the tested microorganisms and some might be having antagonistic effects with others, hence reducing their antimicrobial activities. This study reveals that methanol and omidun extracts of root *V. paradoxa* showed appreciable antimicrobial effects against all the tested diarrhoea causing microorganisms. Also, methanol and omidun extracts conferred similar patterns of protection at both concentrations of treatments from diarrhoea. The root extracts was safe on livers and kidneys at low concentration but toxic at high concentration and should therefore be carefully consumed and should be avoided at higher dosage.

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