Journal of Agriculture, Environmental Resources and Management
ISSN2245-1800(paper) ISSN 2245-2943(online)
6(4)1-800:**July**.2024; pp31-40
www.saerem.com

Actions of *Vernonia amygdalina* (Bitter Leaf) Extracts on a Gastroenteritis Bacteria in a Coastal Agricultural Environment in Southwestern Nigeria

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ABSTRACT

The main target of this research was to investigate the activities of extracts of Vernonia amygdalina on Salmonella enteritidis causing gastroenteritis, isolated from a coastal agricultural environment where livestock manure mainly cow dung is used for soil improvement in crop production. The plant materials were obtained by using extraction medium of ethanol, water and chloroform. The susceptibility of the test bacteria was determined using the agar-well technique where the diameter of the zones of inhibition was measured in millimeters (mm). The minimum inhibitory concentration was determined using the agar-dilution method for each of the extracts. Microsoft office excel 2010 version was used to compute the charts for the zones of inhibition (mm) and Statistical Package for Social Science (SPSS) version 25 was used to determine range mean and $p<0.05$ based on a one-way ANOVA test was used to determine statistically significant difference between the results obtained from the three extracts. The three extracts of Vernonia amygdalina all showed antimicrobial properties inhibiting the growth of the test bacteria. Ethanolic extract was observed to be more potent and show larger inhibition when compared to the other two extracts. The ethanolic extract showed the largest zone of inhibition among the three extracts with the value of 21±1.00 mm at 125 mg/ml. The potency of the aqueous extract was lower than that of the ethanolic extract with values for zones of inhibition ranging from 11±0.56 mm to 17±1.00 at 25 mg/ml and 125mg/ml respectively. The least potent of the three extracts was the chloroform extract that inhibited the growth of the test organism at higher concentrations of 100 mg/ml and 125 mg/ml with zones of inhibition of 9±2.0 mm and 10±1.5 mm respectively. The minimum inhibitory concentration was higher for the chloroform extract due to its low potency, it was observed to be 100 mg/ml which is higher than the two other extracts that were found to be 25 mg/ml. phytochemical analysis was carried out on the extracts and bioactive components found present are flavonoids, alkaloids, reducing sugars, saponins, tannins, anthraquinones, steroids, terpernoids, cardiac glycosides and these were contributory factors to the antimicrobial properties of the extracts The extracts from Vernonia amygdalina was generally observed to inhibit the growth of Salmonella enteritidis, this implies that the extracts of the plant have antibacterial properties. Further research is recommended to understand more on the structure of the components of the bioactive substances in Vernonia amygdalina and their principles of action as a lead way to the formulation of potent antibacterial agents and drugs.

Keywords: Gastroenteritis, Bacteria, Vernonia amygdalina, inhibition, Bitter leaf extract, Salmonella enteritidis, susceptibility

Introduction: Herbs and plants are abundant in nature which form the main source of traditional medicines used for relief in some infectious diseases and are still widely used all over the world (Ajose, 2017). Medicinal plants have a long history of use and their use is spread across both developing and developed countries kavitha (2016). According to the report of the World Health Organization (WHO), 80 per cent of the world's populations rely mainly on traditional therapies which involve the use of plant extracts or their active substances (WHO, 1993). Plants generally contain certain bioactive substances that make them relevant over the years in traditional medical practices and these extracts are modified in drug industries for pharmaceutical use (Adebanjo AO, Adewumi CO and Essien EE. 1983). From the advent of drug development, it is evident that many drugs have been derived from medicinal plants (Yuan, H., Q. Ma, L. Ye and G. Piao, 2016). Plant-derived compounds have been precursors to drug formulation and many are used as drugs, either in their original or semi-synthetic forms and have been proven to be effective. In recent times, Antimicrobial drug resistance has received increased attention from several international bodies and is more generally recognized as a threat to global health (Davies and Davies, 2010, Fanta and Gemechu, 2017). Overtime, much concern in the treatment of infectious diseases caused by many of the bacterial pathogens associated with epidemics of human disease is their evolution into multidrug-resistant (MDR) forms subsequent to antibiotic use, which render therapy more precarious, costly and sometimes unsuccessful causing fatality in many cases (Ventola, 2015). These increasing concerns of the MDR bacteria pathogens are now a great concern to both the clinicians and pharmaceutical industries and this has made it significant to search for newer drugs that are highly effective, affordable, acceptable and available (Martino, PD; Gagniere, H; Berry, H; Bret, L 2002; Akinjogunla, OJ; Ekoi, OH; Odeyemi, AT; Etok, CA; Oshoma, CE. 2011). This have also increased the demand for drugs from plants in recent times, as many plants or herbs are scientifically proven to contain bioactive compounds that can be precursor to novel drugs coupled with the global campaign for consumption of plant based substances as food supplement compared to synthetic substance usage (Vadhana, 2015). It is stated by Ashraf, A., R.A. Sarfraz, A. Mahmood and Moin ud Din. (2015) that some medicinal plants in which their activities are either not yet confirmed scientifically or need further exploitation after confirmation even though they are traditionally used by the local communities. Some plants known to be used primitively to alleviate symptoms of illnesses have been screened to have medicinal importance, some of which include Azadirachta indica (Dogonyaro), V. amygdalina (Bitter leaf), Allium sativum (Garlic), O. gratissimum (Scent leaf), and Zingiber officinale (Ginger). These plants have been reportedly used in the treatment of ailments such as stomach disorder, fever symptoms and cough traditionally (Evbuomwan L, Chukwuka EP, Obazenu EI, Ilevbare L, 2017). This study will conduct its research using Vernonia amygdalina. According to study of Anibijuwon et al. (2012), Vernonia amygdalina is considered to be readily availabile and useful to homes, but it is still categorized as an underutilized crop. The main bioactive constitutions in bitter leaf according to Abere et al. (2018) was identified as Sesquiterpene Lactones in which its subunits are made up of Vernoniosides A1, Vernoniosides 2, Vernoniosides B1, Vernoniosides B2, Vernodalin, Vernolepin, Vernomygdin, Vernodalol and Vernodalinol. Constituents of Sesquiterpene Lactones are said to be responsible for the bitter taste of *Vernonia amygdalina* and observed to possess antimicrobial and antitumor activities. *Vernonia amygdalina* (Bitter Leaf) is one of the medicinal plants used in the treatment of many diseases (Ogundare, 2011 and Abere, 2018). The plant is a shrub usually about five-

meter high, the leaves are simple and entire (5x15 cm) finely glandular below and displaying few lateral nerves. Anibijuwon II, Oladejo BO, Adetitun DO, O.M. Kolawole (2012), explained that the plant leaves are used in the preparation of soup in food in many homes, especially in the southern part of Nigeria and east African countries like Ethiopia and Kenya. The plant is also used in the treatment malaria, helminth infection, gastrointestinal disorder and fever.

The presence of microorganisms like *Escherichia coli*, *Shigella* spp, *Salmonella* and other enteric bacteria in agricultural environment are boosted by the use of manure from animal dung (Black Z, Balta I, Black L, Naughton P, Dooley JSG, Corcionivoschi N, 2021). Manure is used to improve soil conditions in order to make plant nutrients readily available, this became necessary to support intensification of agriculture to meet the rising global population (Chaudhari, 2021). "The use of animal manure for soil amendment intensifies the spread of Antimicrobial Resistance Genes (ARGs) as they are prominent in the animal gut due to the overuse of antibiotics in farming or intensive use of in-feed antibiotics" [Zhao Q, Xiong W, Sun Lin YX, Dong Y. 2018 and Zeng M, de Vries W, Bonten IT, Zhu Q, Liu T. 2018]. "When Crops are produced under these conditions they have potentials to contain organisms found within the soil micro-biome, creating a potentials route for pathogens finding their way into the food chain causing various illnesses" (Grarchi-Sanchez L, Melero B, Rovira J. 2018). "Manure application can inadvertently spread zoonotic diseases to humans" (Tran DT, Bradbury MI, Ogtrop FFV, Bozkurt H, Jones BJ, McCONCHIE, R. 2020). Bacteria pathogen found within manure associated with bacterial outbreaks include, but are not limited to *Campylobacter*, *Salmonella* and strains of pathogenic *E.coli* such as O157:H7" (Swanenburg M, Urlings HAP, Snijders JMA, Keuzenkamp DA, Van Knapen F. 2001 and Zhong et al. 2020). "*Salmonella* is a facultative anaerobe with many serotypes capable of causing gastroenteritis in humans" [Black et al. 2021]. "The infective dose of Salmonella is debated in many literatures, but generally it is agreed that the dose required is higher than that of *Campylobacter and E.coli"* (Hara-Kudo and Takatori 2011). "An increase in the occurrences of antimicrobial resistant strains of *Salmonella"* has been reported by (Williamson DA, Lane CR, Easton M, Valcanis M, Strachan J, Vaitch MG. 2018). "Crops exposed to *salmonella* contaminated soils may be enablers of bacterial population growth and maintenance as well as a vehicle for transmission to human populations" [Black et al. 2021]. Guo X, Chen J, Brackett RE, Beuchat LR. (2002) Reported that "*Salmonella* levels remained constant within the soil over an initial 14-day period with little decline over a 45-day period". Ge C, Lee C, Lee J. (2012) Reported that normal techniques of washing with water may not remove or reduce the risk of infection in humans of salmonella on some food crops. The prevalence, antimicrobial resistance and infectious nature reported to have been exhibited by *salmonella spp*. (Black et al. 2021) forms the thrust of this research work. This work investigates the activities of *Vernonia amygdalina* on *Salmonella enteritidis* that have been reported to be a cause of infectious gastroenteritis in humans.

Materials and Methods: Collection and Identification of Plant Materials

Leaves of *Vernonia amygdalina* were collected in an agrarian community of Itebu-Manuwa, Ilushin area of Ogun waterside LG in Ogun state. Leaves were also collected in Igbotako in Okitipupa LG and Igbokoda in Ilaje LG areas of Ondo state, all within the coastal region of the southwestern part of Nigeria in the month of February 2024. The cow dung samples from which the bacteria were isolated were collected during the period between the month of January and Feburary 2024. The plant is cultivated on farms, gardens and backyards in the areas and it is used in preparation of soup for food and also used as medicine. The

leaves were identified by Olusegun Koko an Agricultural officer at the Planning Research and Statistics Department of the Ogun state Ministry of Agriculture. Five (5) kg of *Vernonia amygdalina* leaves were collected from each of the four locations making a total of twenty (20) kg and air dried for one week and grinded into powder using a blender and stored in a bottle container till when needed.

Preparation of Crude Extract and Plant Extract: The method adopted by Idris, S; Ndukwe, GI; Gimba, CE (2009) was used to prepare crude extract of *Vernonia amygdalina*. Fifty grams of powdered plant material was soaked in 250 ml of each of distilled water, Chloroform and ethanol for 24 hours. A sieve was used to filter the extract to remove debris and passed through filter paper. The filtrate obtained was evaporated in a water bath at 40°C to get the crude extract. The extracts for ethanol and aqueous was stored at 4°C until required for phytochemical and antimicrobial test. The plant extract was later prepared by putting one gm each of the crude extracts of ethanol, aqueous and chloroform extract into 5ml of ethanol, distilled water and chloroform respectively and diluted to give a concentrations of 125 mg/ml, 100 mg/ml, 75 mg/ml, 50 mg/ml and 25 mg/ml.

Sterilization Techniques: Glassware used was sterilized in a hot air oven at 170°C for 2 hours after proper washing and drying. Aluminum foil was used before sterilization to wrap each of the material. Distilled water was autoclaved at 121°C for 15 minutes, Cork borer and glass rods were flamed using Bunsen burner after been dipped into 70% alcohol. Constant swabbing of the work bench during the experiment was carried out.

Phytochemical Analysis of Plant Extracts: Phytochemical parameters tested in Vernonia amygdalina using standard methods were glycosides, steroids, flavonoids, tannins, alkaloids, proteins, saponins, quinines and sugars.Test for glycosides: 25 ml of dilute sulfuric acid (H_2SO_4) was added to 5ml of plant extract in a 100 ml volume flask, boiled for 15 minutes cooled and further neutralized with sodium hydroxide (NaOH). Fehling solution A and B (5 ml) was added to the neutralized solution where a brick red precipitate of reducing sugars indicates the presence of glycosides.Test for steroids: 1 g of the plant extract was dissolved in a few drops of acetic acid, warmed and cooled under the tap water. Sulfuric acid (H_2SO_4) was added along the sides of the test tube. Appearance of green coloration indicates the presence of steroids. Test for tannins: The plant extracts were mixed with basic lead acetate solution. The absence of Formation of white precipitate indicated the absence of tannins. Test for alkaloids: Plant extracts were shaken with few drops of 2N HCL. An aqueous layer was formed which was decanted, one or two drops of Mayer's reagent was added and the observation of the formation of white precipitate indicated the presence of alkaloids. Test for flavonoids: Few magnesium turnings and concentrated hydrochloric were added to the plant extract in alcohol and then boiled for 15 minutes. Appearance of red coloration indicates the presence of flavonoid. Test for saponin: The plant extract was shaken vigorously with water and formation of foamy leather indicates the presence of saponin. Test for quinines: Indication of blue green or red color on addition of sodium hydroxide to the extract indicates the presence of quinine.

Test Microorganism: The test microorganism used was *Salmonella enteritidis* which was isolated from the manure applied for soil improvement on the farms in the study area. The availability of large numbers of cattle herds in the area makes farmers have access to enough cow dungs that is used on farmlands. The bacteria strains were grown in nutrient agar plates at 37°C.

Antibacterial Susceptible Testing of the Extracts with Organism: The test organisms were inoculated in nutrient broth and incubated for 24 hours at 37°C and the cultures were diluted to 0.5 McFarland

turbidity standards after the incubation. 0.2milliliters of the culture was diluted using normal saline. Inoculation of culture was done using glass rod into solidified nutrient agar by using the spreading technique. The agar well technique was used to determine the ability of the extract to inhibit the growth of the test organisms.The inoculated nutrient agar plates were allowed to dry and wells are bored on the surface of the inoculated agar plates using sterilized 6 mm cork borer 0.3 ml of each of the extracts at different concentrations were filled into the well using a sterilized Pasteur pipette. To prevent overlapping of the zones of inhibition, the wells were adequately spaced. Incubation of the plates was done at 37°C for 24 hours. The experiment was performed in triplicate and the zones of inhibitions obtained were recorded as mean ± standard error.

Determination of Minimum Inhibitory Concentration: Minimum inhibitory concentration of the extracts was determined by agar dilution method, the dilutions of the extracts were prepared in 1% dimethyl sulfoxide, which is clear of antimicrobial activity against the test organism. Two portions of extracts were prepared and 2 ml of aliquots of the various concentrations of the different extracts were added to 18ml pre-sterilized Mueller Hinton agar at 50°C to produce a final 20 mg/ml which was then poured into pre-labeled sterile Petri dishes on a level surface. Petri dishes containing only the growth media prepared in the same way were also made available so as to compare the growth of the test organism with that including the extract. The lowest concentration of each of the extracts which inhibited the growth of the test organism is taken as its minimum inhibitory concentration.

Data Presentation and Analysis: The data were analyzed using statistical Package for Social Science (SPSS) version 25. Mean values of the results were obtained and data were interpreted based on the standard interpretive results of zones of inhibition diameter in millimeters (mm) of extracts at the different concentrations on the test organisms. $P<0.05$ based on one-way ANOVA was used to indicate statistically significant differences in the results obtained for the three different extracts and results were presented in tables and charts.

Results: Phytochemical analysis of the different extract shows the presence of flavonoids, tannins, glycosides, reducing sugars, terpenoids, saponins, anthraquinones, alkaloids and Steriods. The results are presented in Table 1. These phytochemicals were present in all three extracts, except for the absence of tannins in both ethanolic and aqueous extracts and the absence of anthraquinones and alkaloids in the chloroform extract.The results for Zones of inhibition obtained for each extracts are presented in Fig. 1 Results showed that the ethanolic extract (BLE) showed zones of inhibitions at all concentrations.

Discussion

The activities of *Vernonia amygdalina* on *Salmonella enteritidis* was observed to be dependent on the medium used for extraction and the concentration of the extract. Ethanolic abstract was observed to show zones of inhibitions at all concentrations and therefore exhibit more antibacterial activities when compared to aqueous and chloroform extract this could be as a result of the fact that ethanol extracted more bioactive substances present in the plant a similar result was obtained in the work of [Evbuomwan et al. 2017]. This may also be due to the higher volatility of the ethanol which tends to extracts more bioactive compounds from the samples than water this was also reported by [Anibijuwon, 2012].

The results showed the highest zone of inhibition at the highest concentration of 125mg/ml. The values of these zones of inhibition ranged from 15±1.0mm at 25mg/ml and 21±1.0mm at 125mg/ml. the minimum inhibitory concentration obtained from the result for the ethanolic extract was found to be 25mg/ml. when the results obtained were subjected to ANOVA test it was found that there was significant difference

between the results when compared to the other two extracts with $p<0.05$. This finding was similar to the work of [Ogundare, 2011] who recorded antimicrobial susceptibility of some microorganisms to ethanolic extracts of *Vernonia amygdalina*. The antibacterial property exhibited by the ethanolic extract can be linked to the different secondary metabolites or phytochemicals present and they have been described by [Fanta et al. 2017] to have antimicrobial properties.

Fig 1. Map showing the study area

Table 1. Phytochemical analysis of ethanol, aqueous and chloroform extract of *Vernonia amygdalina*

Phytochemicals	Solvents		
	Ethanol	Aqueous	Chloroform
Flavonoids	+	+	+
Tannins	-	-	+
Glycosides	+	+	+
Reducing Sugars	+	+	+
Terpenoids	+	+	+
Saponins	+	+	-
Anthraquinones	+	+	-
Alkaloids	+	+	-
Steroids	+	+	+

Key: + = present; - = absent

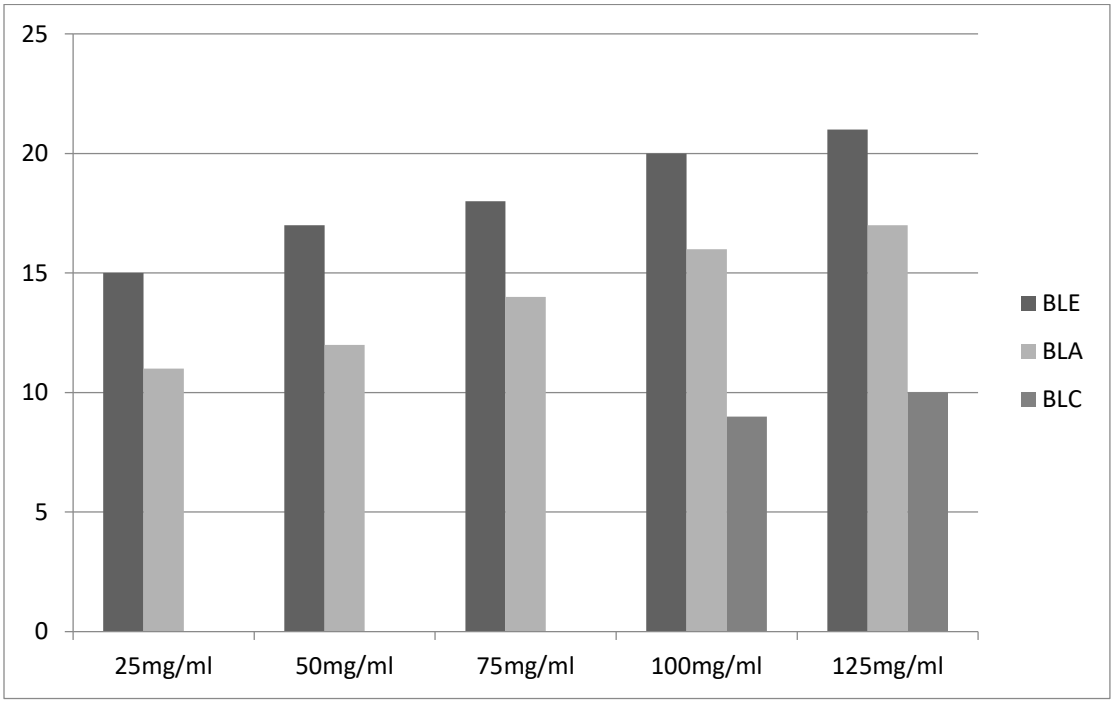

Fig. 2. Antibacterial activity of the three extracts (zones of inhibition are measured in millimeters)

The aqueous extract (BLA) was found to also exhibit antibacterial properties but the zones of inhibitions observed was less in diameter when compared to that of the ethanolic extract. The zones of inhibitions increases as concentration increases, the lowest zone of inhibition was recorded at 25 mg/ml concentration with the value of 11±0.56 mm and the highest zone of inhibition was recorded at the highest test concentration of 125 mg/ml with the value of 17±1.00 mm in diameter. The minimum inhibitory concentration found for the aqueous extract is 25 mg/ml similar to the concentration of the ethanolic extract. Using ANOVA test at $p<0.05$ the result showed significant

difference in the zones of inhibition obtained when compared to the other two extracts. The chloroform extract (BLC) is the less effective of the three tested extracts this may be connected to low availability of bioactive substance in the extract when compared to the other two extracts. The result showed that the test bacteria were not susceptible to the extract at 25, 50 and 75 mg/ml but was susceptible at higher concentrations of 100 and 125 mg/ml, the zones of inhibition ranged between 9±2.0 mm and 10±1.5 mm at these concentrations respectively. The results obtained showed significant difference when compared with the results of the two other extracts at $p<0.05$. The minimum inhibitory concentration of the chloroform extract was found to be 100mg/ml higher than that of the two other extracts. generally the result showed that the values obtained for each of the three extracts of Ethanol, water and chloroform using ANOVA test, were statistically significantly different at $p<0.05$.

Conclusion: The results obtained in this research work indicated that the leaves of *Vernonia amygdalina* contain antimicrobial properties that can inhibit the growth of *Salmonella enteritidis*. The three extracts showed varying activities on the test bacteria with the ethanolic extracts being more potent than the other two therefore, the plant extracts should be further explored to develop antibacterial drugs. The extracts from the leaves of *Vernonia amygdalina* should be tested in-vivo after the bioactive components in the plant have been isolated.

Recommendation: Further study of the structural components and principle of action of these bioactive should be explored for subsequent processing into chemotherapeutic agents.

Competing Interests: Authors have declared that no competing interests exist.

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