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Research Paper

Antibacterial activity of *Vernonia amygdalina* aqueous leaf extracts on bacteria associated with fresh beef meat sold in different areas of Sokoto metropolis

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This study was carried out to determine the antibacterial activity of *Vernonia amygdalina* extracts at concentrations of 30 mg/ml, 60 mg/ml and 90mg/ml respectively. The bacteria were isolated from meat samples obtained from five (5) designated areas in Sokoto State. The bacteria isolated were *Escherichia coli, Salmonella typhi, Pseudomonas aeruginosa, Staphylococcus aureus, Shigella* spp and *Bacillus subtilis.* Agar well diffusion method was used to test the antibacterial activity of the extract at diffusion concentration on the bacteria and the following results were obtained: At 30 mg/ml, *Escherichia coli* (9 mm), *Staphylococcus aureus* (7 mm), *Pseudomonas aeruginosa* (9mm), were susceptible but *Bacillus subtilis, Shigella* spp and *Salmonella typhi* were resistant. At 60 mg/ml, *Escherichia coli* (12 mm), *Staphylococcus aureus* (10mm) *Pseudomonas aeruginosa* (11 mm) were susceptible but *Bacillus subtilis, Shigella* spp and *Salmonella typhi* were still resistant. However, at 90 mg/ml, activity was only seen in *Salmonella typhi* (6 mm) among the resistant bacteria, while the rest were still susceptible. However, the activity of the extract increases as the concentration increases. This might be due to the presence of secondary metabolite like Tannins, Saponins, Flavonoids, Steroids and saponin glycosides.

Key word: Antibacterial, *Vernonia amygdalina,* beef meat, Sokoto, metropolis

INTRODUCTION

Food-borne pathogens are the leading cause of illness and death in developing countries costing billions of dollars in medical care and social costs (Fratamico *et al.,* 2005). Changes in eating habits, mass catering complex and lengthy food supply procedures with increased international movement and poor hygienic practices are major contributing factors (Hedberg *et al.,* 1992). Contaminated raw meat is one of the main sources of food-borne illness (Bhandare *et al.,* 2007; Podpecan *et al.,* 2007). Meat is the main edible part of domestic mammals; however, recent definition includes species, as well as fish, shellfish, poultry and exotic species such as frogs and allegation (Nakai and Moddler, 2000). Similarly, meat refers to animal tissue used as food, mostly skeletal muscles and associated fat but it may also refer to organs including lungs, livers, skin, brains, bone marrow, kidney and a variety of other internal organs as well as blood (Hammer, 1997).

Meat, like any food, can also transmit certain diseases, but complete cooking and avoiding recontamination reduces this possibility (Ikeme, 1990). Minced beef can be contaminated during slaughter with disease-causing *Escherichia coli* O157:H7 originating from the intestinal tract or hide if proper precautions (such as steam pasteurization or organic acid treatment) are not taken (Ikeme, 1990). It has been pointed out that during slaughter, dressing, and cutting, microorganisms come chiefly from the exterior of the animal and its intestinal tract but more are added from knives, clothe, air, workers, carts, boxes, and equipment in general (Bhandare *et al.,* 2007). A great variety of kinds of organisms are added, and so it can be assumed that under ordinary conditions most kinds of potential spoilage organisms are present and will be able to grow if favourable conditions present themselves (Bhandare *et al.,* 2007).

Herbal medicine is the oldest form of healthcare known to mankind and over 50% of all modern clinical drugs are of natural products origin and natural products play important roles in drug development in the pharmaceutical industry (Preethi *et al.,* 2010). The use of plant continues to play essential roles in traditional medicine for the treatment or management of various human diseases, especially in rural Africa where infectious diseases are endemic due to poverty and poor sanitations (Preethi *et al.,* 2010).

Vernonia amygdalina commonly called bitter leaf is a perennial shrub of 2-5 m in height that grows throughout tropical Africa. It belongs to the family Asteraceae and it's a highly appreciated vegetable in west and central Africa where it's commonly used in traditional medicine. Leaf decoctions are used to treat fever, malaria, diarrhoea, dysentery, hepatitis and cough as a laxative and as fertility inducer (Ijeh *et al.,* 1996). The plant has acquired relevance recently, having been proven in human medicine to possess potent antimalarial and antihelminthic properties (Abosi and Raseroka, 2003) as well as antitumorigenic properties (Tula *et al.,* 2012). Various workers had reported the phytochemical and antibacterial activity of the plant parts against food borne pathogen (Ibrahim *et al.,* 2009), urinary tract pathogens (Uzoigwe and Agwa, 2011) and other clinical isolates (Oboh and Masodje, 2009; Ibrahim *et al.,* 2009: Tula *et al.,* 2012).

Bacteria are on the increase and imposes severe threat to mankind, existing procedure to control bacterial infections rely almost exclusively on curative or preventive treatment with synthetic antibiotic. However, widespread and indiscriminate use of these potent drugs has resulted in the antimicrobial resistance strains of bacteria. The urgent and rational solution to the problem is to develop an affordable, acceptable and effective drug from reasonable inexpensive source for use as supplement to commercial drugs.

Plant extracts are continuously being sort for as effective and cheaper alternative sources of medication due to the development of multiple drug resistant strains of bacteria and adverse effects on the host which are sometimes attributed to the use of anti-microbial drugs. *Vernonia amygdalina* plant is known to exhibit various medicinal properties and is widely recommended for the treatment of varieties of ailments. It is therefore necessary to confirm its acclaimed anti-bacteria properties on bacteria associated with fresh beef meat sold within the metropolis base on its ethno medicinal use in Nigeria in order to provide an alternative to microbial resistant antibiotics that has less adverse effect on the host as well as the phytoconstituents of the aqueous plant extracts. The research is aimed at determine the antibacterial activity of *Vernonia amygdalina* extracts at concentrations of 30 mg/ml, 60 mg/ml and 90 mg/ml.

MATERIALS AND METHODS

Sample collection

Beef meat samples were purchased from different locations within Sokoto metropolis. The designation sites of which includes; Sokoto Central Market, Runjin Sambo, Bado Quarters, Mabera Area and Usmanu Danfodiyo University Sokoto School Mini-Mart. The meat samples were collected in a sterile polythene bags and transported to Microbiology Laboratory, Usmanu Danfodiyo University, Sokoto for further analysis.

Sample preparation

Ten (10) gram of each fresh meat sample was weighed using a weighing balance and placed into a sterile blender, 90 ml of distilled water was added and the mixture was homogenized to obtain a thoroughly blended meat. The homogenized meat was transferred into a sterile beaker.1 drop of the homogenized meat was transferred into a test tube containing 9ml of sterile distilled water and 6fold serial dilution was carried out (Mboto *et al.,* 2012).

Media used

The media used in this research work includes Nutrient agar and Mueller Hinton agar and were prepared according to manufacturer's instructions (Atlas, 1993).

Sample inoculation

A small quantity (0.1 ml) of the dilution factor of 10-6 was dispensed into the petri dish and a sterile glass rod was used to spread the sample on the media. It was then

incubated at 37°C for 24 h. After 24 h incubation, there was presence of colonies which were counted using colony counting machine. The colonies were sub cultured into a fresh nutrient media plate and was incubated for 24 h.

Gram staining technique

A smear of colonies isolated after incubation was made on a clean glass slide using sterile wire loop which was dried and heat fixed. The smear was flooded with crystal violet for about 60 sec and then washed with tap water. It was tipped off with Lugols iodine for 30 sec and then washed with tap water, then counter stained with safranin after decolorizing with acetone followed by rinsing with tap water. It was allowed to air dry, oil immersion was added to the stained slide and it was viewed under a microscope using X100 objective lens for the morphological characteristics of the isolates (Oyeleke and Manga, 2008). The test organisms were identified on the basis of microscopic examination and relevant biochemical test were carried out.

Biochemical test for identification of bacteria

The following biochemical test was carried out on the bacteria isolates Indole test, coagulase test, Methyl Red test, Triple Sugar Iron Agar test (T.S.I), Vogues-Proskauer test, Catalase test.

Indole test

A speck of each isolates was inoculated into 5ml of sterile peptone water, which was enriched with 1% tryptophan in the test tube and incubated at 37°C for 48 h, 0.5 ml of kovac's indole reagent was added and shaken gently. In a positive test, indole dissolves in the reagent which becomes pink or red and forms a layer at the surface of the medium. A yellow layer at the surface of the medium denotes a negative result (Oyeleke and Manga, 2008).

Catalase test

The container containing hydrogen peroxide solution was mixed vigorously to expel the dissolved oxygen. One drop of the solution was placed on a clean glass slide, followed by the addition of a loopful of 24 h' inoculum on the slide. Presence of gas bubbles indicates a positive test while absence of gas bubbles indicates negative reaction (Cheesbrough, 2000).

Methyl red test (M.R test)

A speck of each isolate was inoculated into the medium, which was incubated at 37°C for 48 h. Few drops of methyl red were added to the culture. M.R positive test indicated red colour while no changes denotes negative (Oyeleke and Manga, 2008).

Triple sugar iron agar test (T.S.I test)

A speck of each isolate was inoculated by streaking and stabbing into the medium and incubated at 37°C for 24 h. Fermentation of any of the sugar was indicated by a change in colour, from red to yellow and crack or raised in the medium indicates gas production (Oyeleke and Manga, 2008).

Coagulase test

About 2 or 3 colonies were emulsified in 0.05 ml of saline contained in a serological tube. 1ml of plasma was added and incubated at 35-37°C and checked after (1, 2, 3 and 4) h of incubation for signs of clotting of the plasma. Increase in viscosity or complete clotting indicates a positive coagulase test, while absence of viscosity or clotting indicates a negative coagulase test (Oyeleke and Manga, 2008).

Voges-Proskauer test (V.P test)

A speck of each isolate was inoculated into glucose phosphate water medium and incubated at 37°C for 2 days. Ethanoic solution of 5% α-naphthol 1.2 ml and 0.4 ml potassium hydroxide solution were added to 2 ml of culture and was shaken vigorously. It was placed in a sloping position (for maximum exposure of the culture to air) and was examined after 30 to 60 min. The evolution of red colour indicates a positive test for voges-proskauer (Singleton, 1999; Oyeleke and Manga, 2008).

Motility test

A small quantity of each isolate was stabbed into triple sugar iron agar and incubated at 37°C for 24 h. Motility was observed by spread of the organism outwards from the stabbed area (Singleton, 1999)

Urease test

A speck of each isolate was inoculated into Christensen's urea agar and incubated at 37°C for 24 h. Liberation of red color indicates urease positive test while initial yellow color indicated negative test (Singleton, 1999)

Aqueous extraction

Fifty grams of the powdered *Vernonia amygdalina* was dispensed into 500 ml of distilled water in a 1 L capacity

conical flask. The mixture was stirred vigorously and then allowed to stand for 48 h. It was stirred again and then filtered through a whatman No.1 filter paper line funnel into a conical flask. The filtrate was evaporated at 40°C in a hot air oven to obtain the solid crude extract (Bukar *et al.,* 2013).The extracts were then made into concentrations of 30 mg/ml, 60 mg/ml and 90 mg/ml.

Antibacterial Activity of the Crude Extract of the Plant

Fifteen milliliter (15 ml) of sterile Mueller Hinton agar was poured into each sterile Petri dish of equal sizes and allowed to solidify. 0.1 ml aliquot of each of the standardized bacterial cell suspension was transferred onto the surface of the dried agar plate and spread evenly using a sterile bent glass. A cork borer (8 mm in diameter) was sterilized by flaming and used to create four wells on each Petri dish. Two drops of plain agar were used to seal the bottom of each well created to prevent cross diffusion and then 0.2 ml of different concentration of leave extract which includes 30 mg/ml, 60 mg/ml and 90 mg/ml was poured into the designated wells which carry the concentration of the extract. The plates were allowed to stand for 30 min before sealing the top with plain agar. The plates were inverted back and incubated at 37°C for 24 h. At the end of the incubation period, the diameter of the zone of inhibition was measured in millimeter using a meter rule. Ciprofloxacin was set as control.

Phytochemical screening of the aqueous extract

Test for flavonoid

Three (3) ml aliquot of the filtrated was mixed with 1ml of 10% NaOH sodium hydroxide, a yellow colour was developed, and this indicates the possible presence of flavonoid compounds (Harborne, 1998).

Test for tannins

Ferric Chloride solution 5% was added drop by drop to 2-3 ml of the extract and the colour produced was noted. Condensed tannins usually give a dark green colour; hydrolysable tannins give blue-black colour (Harborne, 1998; Trease and Evans, 1989).

Test for saponin

Five (5) ml of the extract was placed in a test tube + 5ml of water and shaken strongly. The whole tube was filled froth that lasts for several minutes (Harborne, 1998).

Test for glycosides

Two and half (2.5) ml of 50% H_2SO_4 was added to 5cm^3 of the extracts in a test tube. The mixture was heated in boiling water for 15 min. Cool and neutralize with 10% NaOH, and 5 ml of fehlin's solution was added and the mixture is boiled. A brick-red precipitate is observed which indicate the presence of glycosides (Harborne, 1998).

Test for alkaloids

About 2 ml of each extract was stirred with 2 ml of 10% aqueous hydrochloric acid. 1 ml was treated with a few drops of Wagner's reagent and second 1 ml portion was treated similarly with Mayer's reagent. Turbidity or precipitation with either of these reagents was taken as preliminary evidence for the presence of alkaloids (Harborne, 1998).

Test for cardiac glycosides (Keller-killiani's test)

To one of the extract, 2 ml of 3.5% ferric chloride solution was added and allowed to stand for 1 min. 2 ml of H_2SO_4 was carefully poured down the wall of the tube so as to form a lower layer. A reddish brown ring in the interface indicates the presence of cardiac glycoside (Evans, 2000).

Test for steroids

This was carried out according to the method of Harborne (1998). 5ml of the extract was dissolved in 2 ml of chloroform. 2 ml of sulphuric acid was carefully added to form lower layer. A reddish-brown colour at the interface indicates the presence of a steroidal ring.

Test for saponin glycosides

Two and half (2.5) ml of the extract was added to 2.5ml of Fehling's solution A and B. A bluish green precipitate showed the presence of saponin glycosides (Trease and Evans, 1989).
The extract was mixed with equal volume of 90% ethanol. 2 drops of alcoholic ferric chloride solution were added to the mixture. A dark green colour indicates the presence of balsams (Evans, 2000).

Test for anthraquines

Five (5) ml of each plant extract was shaken with 10ml benzene, and 5ml of 10% ammonia solution was added. The mixture was shaken and the presence of a pink, red, or violet colour in the ammonia Cal (lower) phase indicated

Table 1. Mean bacteria load of meat samples.

Designation	Mean Bacteria load (cfu/ml)
Mabera	7.6×10^7
Runjin Sambo	4.6×10^7
UDUS School Mini Market	8.1×10^7
Bado Mini Market	6.8×10^7
Central Market	7.4×10^7

Table 2. Bacteria isolates and their percentage occurrences.

Bacteria Isolates	No. of occurrences	% frequency of occurrences
Escherichia coli	5	31.3
Salmonella typhi	2	12.5
Staphylococcus aureus	4	25
Pseudomonas aeruginosa	3	18.8
Shigella spp	1	6.3
Bacillus subtilis	1	6.3
Total	16	100

Table 3. Antibacterial activity of aqueous leaf extract of *Vernonia amygdalina.*

Bacteria Isolates	Concentrations			
	30 mg/ml	60 mg/ml	90 mg/ml	Ciprofloxacin 100 mg/ml
Salmonella typhi	0	0	6	20
Escherichia coli	9	12	15	23
Staphylococcus aureus	7	10	11	22
Pseudomonas aeruginosa	9	11	13	22
Bacillus subtilis	0	0	0	15
Shigella spp	0	0	0	19

Keys:0 = Resistance, C = Ciprofloxacin (control).

the presence of anthraquines (Evans, 2000).

Test for volatile oils

One (1) ml of the fraction was mixed with dil. HCl. A white precipitate was formed which indicates the presence of volatile oils (Harborne, 1998).

RESULTS AND DISCUSSION

The result of mean bacterial load of meat samples obtained is presented in (Table 1), with the beef meat obtained from UDUS Minimarket having the highest bacterial load of 8.1×10^7 cfu/ml while the meat sample obtained from Runjin Sambo had the least mean bacterial load of 4.6×10^7 cfu/ml. This is however in agreement with the results of Gayathri and Anu-Swedha, (2015) as well as Igile *et al*. (2011) whom all determine high mean bacterial load for meat samples exposed in the atmosphere. However, this high mean bacterial load may be attributed to the human and environmental conditions the meats are being placed before being sold. The bacteria load obtained is not within the tolerable limit as set by WHO (2007).

The bacteria isolated from this research based on their percentage frequency of occurrences are; *Escherichia coli* (31.3%), *Staphylococcus aureus* (25.0%), *Pseudomonas aeruginosa* (18.8%), *Salmonella typhi* (12.5%), *Shigella* spp (6.3%) and *Bacillus subtilis* (6.3%) as presented in (Table 2).The occurrence of these organisms might be due to the hygienic practices by the meat vendors and the exposure to the atmosphere. This result is in agreement with Gayathri and Anu-Swedha (2015) who isolated *Escherichia coli* in high percentage compared to other organisms. The crude antibacterial activity of the aqueous leaf extract of *Vernonia amygdalina* at different concentrations of 30 mg/ml, 60 mg/ml and 90 mg/ml is shown in (Table 3).The crude antibacterial activity of the aqueous extract of *V. amygdalina* leaf on *Escherichia coli, Salmonella typhi, Pseudomonas aeruginosa, Staphylococcus aureus, Shigella* spp. and *Bacillus subtilis* at concentrations of 30 mg/ml, 60 mg/ml and 90 mg/ml recorded zones of inhibition with *Escherichia coli* having the highest zone of

Table 4. Phytochemical analysis of *Vernonia amygdalina* leaves.

Constituents	Aqueous Extract
Flavonoid	++
Tannins	+
Saponin	++
Alkaloid	–
Glucoside	–
Cardiac glycoside	–
Steroid	++
Saponin glycoside	++
Balsalms	–
Anthraquines	–
Volatile oil	–

Keys: **++=** Abundantly present, **+=** Present in low concentration, –= Absent (not detected)

inhibition in all concentrations and *Salmonella typhi* having the least zone of inhibition. While *Bacillus subtilis* and *Shigella* spp. showed no zone of inhibition. Ciprofloxacin being the control was sensitive to all the test bacteria at concentration of 100 mg/ml with *Escherichia coli* showing the highest zone of inhibition of 23 mm in diameter and *Bacillus subtilis* with the lowest zone of inhibition of 15 mm in diameter. This research is in conformity to the findings of Adetunji *et al*. (2013) whom stated that cold aqueous extract of *V. amygdalina* leaf had activity on *Escherichia coli*, indicating that the plant possess antibacterial activity on the organism. The resistance of *Bacillus subtilis* and *Shigella* spp. could be as a result of acquired resistance, mutation or environmental factors.

The phytochemical screening revealed the presence of some secondary metabolite like tannins, Saponins, Flavonoid, Steroids and Saponin glycoside in the aqueous extract (Table 4). Flavonoids have been reported to be synthesized by plants in response to microbial infections and are good antibacterial agents and tannins have been demonstrated to have antibacterial activities (Akiyama *et al.,* 2001). This study indicates that the aqueous plant extract possess antibacterial activities. Also it has been reported to contain antiparasitic activity (Odeh and Usman, 2014) thus Awareness should be created on the benefit of bitter leaf to wellbeing of humans. Thus giving more room for studies to be carried out on other parts of the plants such as the roots and stem.

Conclusion and Recommendation

The extracts *Vernonia amygdalina* is widely consumed and has a great activity on the bacteria isolated from fresh beef meat at varying concentrations (30 mg/ml, 60 mg/ml and 90 mg/ml). Though, at 30 mg/ml and 60 mg/ml Salmonella typhi isolated from the meat was totally resistant except at an increase concentration, i.e. extraction of 90mg/ml with little activity on S. typhi. Thus, at an increase concentration of V*. amygdalina* leaves, it has a greater antibacterial activity. Base on the above reason, it is recommended that, beef meat should be boiled or fried properly before consumption.

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