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Phytochemical analysis and Antimicrobial Activity of Ethanolic Stem Extracts of *Cnestis ferruginea* on Multidrug Resistant Bacteria Isolated from Raw Retail Meat Sold in Awka, Nigeria.

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Abstract

Objectives:

To demonstrate the phytochemical properties and evaluate the antimicrobial activity of the ethanolic extract of the stem of *Cnestis ferruginea* on multidrug resistant bacteria isolated from retail raw meats.

Methods:

The antimicrobial effects of ethanolic extracts of the stem of *Cnestis ferruginea* were evaluated by the agar well diffusion method and microbroth dilution methods on multidrug resistant *Escherichia coli, Staphylococcus aureus* and Salmonella spp isolated from raw retail meat sold in Awka, Nigeria. Phytochemical screenings were also conducted on the *Cnestis ferruginea* extracts.

Results:

Saponin, flavonoid, cardiac glycoside, and anthroquinone were moderately present while tannin and reducing sugar were mildly present in the plant. The observed inhibition zone diameters (IZDs) produced by the extract ranged from 3-18 mm; and these were concentration dependent. The MIC and MBC of *Cnestis ferruginea* extracts against the bacterial isolates were in the range of 3.2 – 6.3 mg/ml.

Conclusion:

This study have presumptively reported putative antimicrobial potentials of stem extract of *Cnestis ferruginea* on some multiple antibiotic resistant bacteria; and thus the plant should be validated for future medicinal purposes. Further molecular studies are also required to characterize the bioactive components responsible for the antibacterial activity of *Cnestis ferruginea* extracts in order to develop novel antibacterial drugs from them.

Keywords: *Cnestis ferruginea*, Antimicrobial activity, pathogenic bacteria, Raw retail meat, Nigeria.

INTRODUCTION

In recent years, medicinal plants have attracted the attention of the pharmaceutical and scientific communities as evidence has demonstrated the promising potentials of antimicrobial plant-derived substances. The antimicrobial effects of plant extracts have formed the basis of many medical applications such as pharmaceuticals, alternative medicine and natural therapies since antiquity (1-2). And in traditional medicine, plant materials are of wide use especially in developing countries, where they are the major resources readily available for the treatment of various infections/ailments in the rural commuities(1-3). Plants are rich in several secondary metabolites and are a major source of chemical diversity; thus, they are potential sources of new drugs for man's use (4). The foodborne pathogens impose a significant burden of infections in the developing countries. Salmonella, *Staphylococcus aureus* and *E. coli* are amongst the most common pathogens responsible for food poisoning and food related infections in parts of the globe (5-6). The prevalence of antimicrobial drug resistant strains among foodborne pathogens including *E. coli, S. aureus* and Salmonella is on the increase due to the irrational use of antibiotics especially those meant for human use in animal husbandry and for other veterinary purposes. Such practices allow resistant microbes to emerge via selective pressure; and thus spread in the community. Multidrug resistant Salmonella are frequently isolated from food sources (7). The increasing incidence of antibiotic-resistant pathogens has drawn the attention of the pharmaceutical scientists towards studies on the potential antimicrobial activity of plant-derived substances with putative antimicrobial activity (1). Herbal remedies used in traditional medicine provide an interesting but largely undiscovered source to develop potentially new chemotherapeutic drugs that might help to overcome the growing problem of resistance and toxicity of some currently available antibiotics(8). *Cnestis ferruginea* is a shrub which belongs to the family *Connaraceae* (9). It is locally known as "amunkita" in Southeastern part of Nigeria. The plant has been found useful in treating conjunctivitis, syphilis, gum pain, wounds, dysentery and gonorrhea (9). Its fruit is widely used in oral hygiene in Nigeria against gingivitis and Streptococci infections of the mouth. Its root is used as laxative and the stem is rubbed on the skin and as a medicine for throat infections. The plant has been reported to contain bioactive compounds inhibitory to bacterial growth (10). In a study carried out in south-western Nigeria, Ndukwe *et al* (11) reported that its

fruit has appreciable activity against some bacterial isolates. Similarly, Studies carried out by Akharaiji *et al* (10) showed that the root, stem and leaf of *Cnestis ferruginea* have an antimicrobial activity against some species of microorganisms. Therefore, this study was carried out to demonstrate presumptively the phytochemical properties and the antimicrobial activity of the ethanolic extract of the stem of *Cnestis ferruginea* on multidrug resistant *S. aureus*, *E. coli* and Salmonella spp isolated from retail raw meats sold in an abattoir in Awka metropolis, Anambra State of Nigeria.

MATERIALS AND METHODS

Plant materials: The plant samples of *Cnestis ferruginea* (stem) were collected at Nsukka; Enugu State of Nigeria between April to May, 2014 and it was identified and authenticated at the Botany Department, University of Nigeria Nsukka by a taxonomist Mr. Alfred Ozioko. Samples of the plant were kept in the herbarium of the Department where the identification was conducted for record keeping purposes.

Preparation of the plant extract: Four hundred grams (400 g) of the stem of the plant was cold macerated with two litres of ethanol in two different maceration containers for 48 hours. At the end of the extraction, the suspension was filtered with Whatman filter paper No. 1 (Whatman, Maidstone, England) and the filtrates were dried in a hot air oven regulated at 50^{o}C (AMPUL dryer) and the dried filtrates were stored at 4^{o}C for further use.

Microorganisms: Multidrug resistant bacteria isolates of *S. aureus* (n=8), *E. coli* (n=8) and Salmonella spp (n=8) were obtained from raw meat from an abattoir in Awka metropolis, Anambra State, Nigeria. Their morphological, cultural and biochemical characteristics were confirmed by standard microbiological methods (12). A total of 24 multidrug resistant bacteria isolates were used for this study.

Antimicrobial agents: The antibiotic discs used were: cotrimoxazole (COT) 250 µg, Cloxacillin (CXC) 5 µg, Erythromycin (ERY) 5 µg, Gentamycin (GEN) 10 µg, Augmentin (AUG) 30 µg, Streptomycin (STR) 10 µg, Tetracycline (TET) 10 µg, Chloramphenicol (CHL) 10 µg; and these were procured from ABTEK (ABTEK, India).

Phytochemical analysis: The phytochemical screenings were conducted according to description the description of Trease and Evans (13) and Akinjogunla *et al*. (14).

Sensitivity test: The assay was conducted using the agar well diffusion method as described by Esimone *et al* (15). A 150 mg/mL concentration of the ethanolic extracts was prepared by dissolving 0.3 g of the extract in 2 mL of 10 % DMSO and then a 2-fold serial dilution was done to obtain graded concentrations for further use. With the aid of a sterile swab stick, the standardized suspension of the isolates was inoculated on the surface of the sterile agar plates. The inoculated agar plates were allowed to dry and the plates were properly labeled. A sterile cork borer was used to bore six holes in the agar with a diameter of 6 mm. Using a micropipette, 50 µL of the reconstituted plant extracts was delivered into four of the labeled wells; 10 % DMSO was introduced into one of the wells as negative control and 50 µL of a 0.2 mg/mL erythromycin (DHE, India) was used as a positive control in the sixth hole. The plates were left for 30 minutes at room temperature to allow the extracts and the drug to diffuse into the agar. This was done in duplicates for both the root and stem extract against the three microorganisms used. The plates were incubated at 37^{o}C for 24 hrs. After incubation, the plates were observed for inhibition zones around the wells. The diameters were measured and the mean inhibition zone diameter (IZD) was recorded to the nearest whole millimeter.

Determination of the minimum inhibitory concentration (MIC): Microbroth dilution method was used for the determination of MIC as described by the European Committee for Antimicrobial Susceptibility Testing (EUCAST). Briefly, the plant extracts were reconstituted in 10 % DMSO in a concentration of 150 mg/mL. A twofold serial dilution was made to obtain concentrations of 75 mg/mL, 37.5 mg/mL and 18.75 mg/mL. A 19 ml of the molten Muller-Hinton agar was mixed with 1 mL of the plant extracts dilutions in sterile Petri dishes. It was allowed to solidify and a sterile wire loop was used to streak a loopful of the standardized inoculum on the surface of the dried agar. The plates were then incubated at 37^{o}C for 24 hrs, after which the plates were observed for the presence of turbidity. The lowest dilution giving no turbidity was recorded as the MIC (16). A control experiment was run in parallel to study the impact of the solvent (i.e. DMSO) on the growth of the tested organism. The tests were done in duplicate against *E. coli*, Salmonella spp and *S. aureus* and the mean MIC was recorded to the nearest whole concentration.

Determination of Minimum Bactericidal Concentration (MBC): The agar plates showing no growth of visible colonies in the MIC evaluation were used to determine the MBC. Disc were cut out from each plate with no visible growth and transferred into a test tube containing fresh medium of double strength nutrient broth. The test tubes were incubated at 37^{o}C for 72 hours after which the test tubes were observed for the presence of turbidity.

RESULTS

The result of the phytochemical screening of the stem extracts are shown in the Table 1. The extracts were found to be abundantly rich in alkaloid. Saponin, flavonoid, cardiac glycoside, and anthroquinone were moderately present while tannin and reducing sugar are mildly present in the plant. Tables 2, 3, and 4 show the results of the antibiotic resistance profile of the bacterial isolates. From table 2 the resistance profile of *S. aureus* isolates are in the order of Augmentin erythromycin > tetracycline > cloxacillin > streptomycin > chloramphenicol > cotrimoxazole > gentamicin. The isolates of *E. coli* (Table 3) were all resistant to most of the antibiotics evaluated except isolates 2 and 3 that showed some levels of intermediate susceptibility. Almost all the isolates of Salmonella were resistant to the antibiotics tested (Table 4). Four (4) isolates each of *S. aureus*, *E. coli* and Salmonella spp were selected to evaluate the activity of the plant extracts. Antimicrobial activity of ethanolic stem extracts

of *Cnestis ferruginea* on these multidrug resistant strains was evaluated in terms of IZD, MIC and MBC. It was observed that the IZDs ranged from 3-18 mm; and 10 % DMSO had no activity on any of the isolates (Tables 5-7). It was also observed that the sensitivity decreased with decrease in concentration of the extract. This could possibly be due to the fact that the inhibitory effect of a test substance on a particular organism is proportional to the concentration of the test substance in the medium (17). Figures 1, 2, and 3 show the MIC and MBC results of the extract against some selected strains of *S. aureus*, Salmonella species and E. *coli* respectively. The recorded MIC and MBC values against the isolates of *S. aureus* were in the range of 3.2-6.3 mg/ml. The MIC and MBC of the stem extracts against Salmonella species was observed to be in the range of 6.2 mg/mL (except for strain 1 with MIC of 3.2 mg/mL). The MBC for the Salmonella strains with positive MIC was observed to be 150 mg/mL. The MIC result against the strains of *E. coli* was observed to be 3.2 mg/mL while the MBC was mostly at 6.3 mg/mL for the four selected isolates.

Table 1 : Phytochemical screening of the plant extracts

S/no	Phytochemical test	Root extract	Stem extract
1	ALKALOID	+++	++
2	SAPONIN	++	++
3	FLAVONOID	++	++
4	TANNIN	+	+
5	CARDIAC GLYCOSIDE	++	++
6	ANTHROQUINONE	++	+
7	REDUCING SUGAR	+	+

KEY: + = Mildly Present, ++ = Moderately Present, +++ = Abundantly Present

Table 2: :Antibiogram of *S. aureus* showing the susceptibility/resistance patterns

	Antibiotics							
***S.aureus* strains**	**COT (25µg)**	**TET (10µg)**	**AUG (30µg)**	**ERY (5µg)**	**GEN (10µg)**	**CXC (5µg)**	**CHL (10µg)**	**STR (10µg)**
1	S	R	R	R	S	R	S	S
2	S	R	R	R	S	R	S	R
3	S	R	R	R	S	R	S	S
4	S	R	R	R	S	R	S	R
5	R	R	R	R	S	R	R	S
6	S	R	R	R	R	R	R	R
7	S	S	R	R	S	S	S	S
8	S	S	R	R	S	R	R	R

Key: S= sensitive , R= Resistance

Table 3: Antibiogram of E. coli showing resistance/sensitivity pattern

	Antibiotics							
***E.coli* strains**	**COT (25µg)**	**TET (10µg)**	**AUG (30µg)**	**ERY (5µg)**	**GEN (10µg)**	**CXC (5µg)**	**CHL (10µg)**	**STR (10µg)**
1	R	R	R	R	I	R	R	R
2	R	I	R	I	S	R	R	R
3	R	R	R	R	S	R	R	R
4	R	R	R	R	R	R	R	R
5	R	R	R	R	R	R	R	R
6	I	R	R	R	R	R	R	R
7	R	R	R	R	S	R	R	R
8	R	R	R	R	R	R	R	R

Key :R= Resistant, S= sensitive, I = Intermediate susceptible.

Table 4: Antibiogram of *Salmonella spp* showing the susceptibility pattern

	Antibiotics							
Salmonella strains	**COT (25µg)**	**TET (10µg)**	**AUG (30µg)**	**ERY (5µg)**	**GEN (10µg)**	**CXC (5µg)**	**CHL (10µg)**	**STR (10µg)**
1	R	R	R	I	R	R	R	R
2	S	R	R	R	I	R	R	R
3	R	R	R	R	R	R	R	R
4	R	R	R	R	R	R	R	R
5	R	R	R	R	R	R	R	R
6	S	R	R	R	R	R	R	R
7	S	R	R	R	R	R	R	R
8	R	R	R	R	R	R	R	R

Key R= Resistant, S= sensitive, I = Intermediate susceptible.

Table 5: Inhibitory zone diameter of stem extract against *S. aureus*

	IZD(mm)			
Concentration (mg/ml)	**Isolate 1**	**Isolate 2**	**Isolate 3**	**Isolate 4**
7.5	12	13	9	10
3.75	8	7	4	6
1.87	4	5	0	3
0.0004	0	0	0	0
10%DMSO	0	0	0	0
Erythromycin 10µg	16	17	13	17

Table 6 : IZD of stem extract against *E. coli*

IZD (mm)				
Concentration (mg/ml)	**Isolate 1**	**Isolate 2**	**Isolate 3**	**Isolate 4**
7.5	12	9	12	13
3.75	7	5	8	7
1.87	3	0	3	4
0.0004	0	0	0	0
10%DMSO	0	0	0	0
Erythromycin 10µg	14	18	15	18

Table 7: IZD of stem extract against *Salmonella spp.*

Concentration (Mg/Ml)	IZD (mm)			
	Isolate 1	**Isolate 2**	**Isolate 3**	**Isolate 4**
7.5	10	11	8	7
3.75	9	7	3	5
1.87	6	4	0	0
0.0004	0	0	0	0
10%DMSO	0	0	0	0
Erythromycin 10µg	15	18	13	15

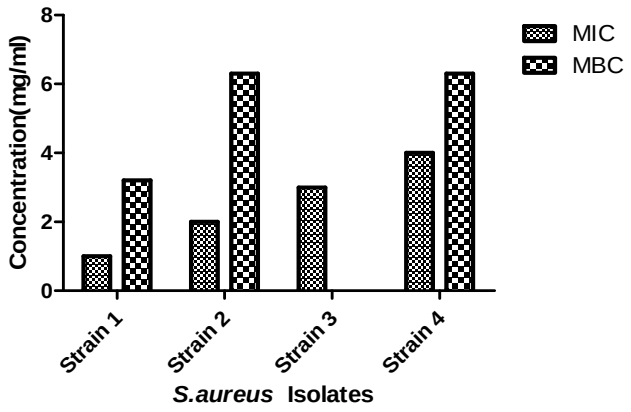

Figure 1: MIC and MBC of the stem extract against *S. aureus*

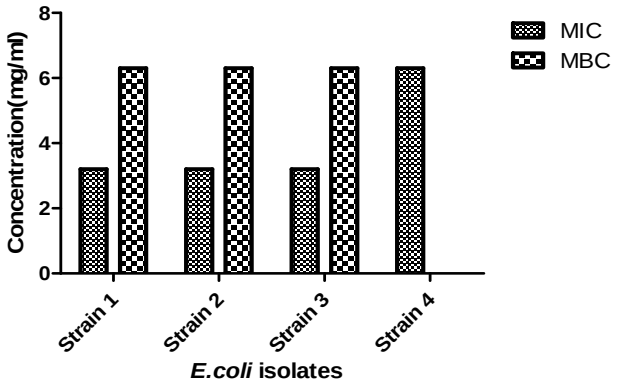

Figure 2: MIC and MBC of the stem extract against *E. coli*

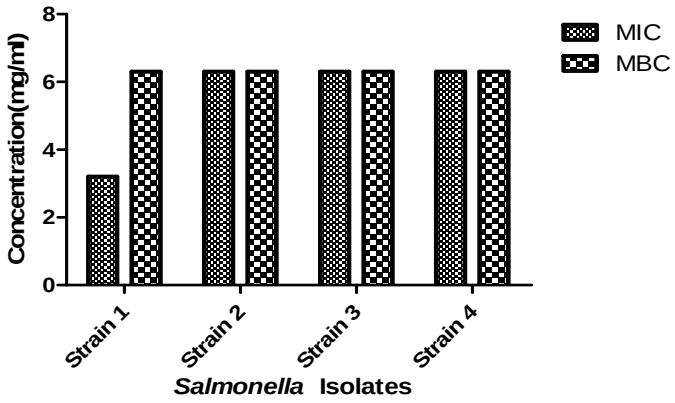

Figure 3: MIC and MBC of the stem extract against Salmonella spp

DISCUSSION

The development and spread of antibiotic resistant strains of pathogenic bacteria and the occurrence of undesirable side effects of some antibiotics due to this phenomenon have heightened the interest on exploiting plant materials as alternatives to some conventional antibiotics that are less efficacious in the treatment and management of some infectious diseases (2,18-19). Medicinal plants constitute a reservoir of new antimicrobial substances yet to be discovered. Their traditional use as herbal medicines provides an interesting potential to overcoming the growing challenges of resistance and toxicity of some currently used antibiotics especially in this era of antibiotic resistance (14,20). One significant way to contain the menace of antibiotic resistance is to use new compounds that are not based on the existing antimicrobials (14). Such novel compounds are frequently found in herbal plants. The stem bark of *Cnestis ferruginea* has been reported to be of highest inhibitory potency than the leaf and root extracts (10). Most of the phytochemical compounds identified in the ethanol extract have been reported to be of high therapeutic importance (21-22). Thus, the presence of these numerous phytochemicals in the stem extracts is strong indications that the plant has both pharmacological and medicinal values (19, 23-24). We observed in this study that the stem extract of *Cnestis ferruginea* have some antimicrobial effects on the multi-antibiotic resistant bacteria and the level of the sensitivity of the test microorganisms varies as well. The Gram negative organisms (*E. coli* and Salmonella spp) were less susceptible to the plant extracts than the Gram positive organism (*S. aureus*). Similar low sensitivity of Gram negative bacteria was reported of aqueous stem extract of *Cnestis ferruginea* by Ndukwu *et al* (11). We observed that the sensitivities of test organisms increased with increase in concentration of the extract. This shows that the inhibitory effect of the test extract on a particular organism is proportional to the concentration of the test substance in the medium. Thus the activity of the extract was a function of the concentration of the bioactive substance contained in the extract that reaches the organism (17). We equally observed that the MIC and MBC values were almost the same for Salmonella spp. This could be interpreted to mean that the concentration needed to inhibit the growth of the bacteria is equally bactericidal in effect.

CONCLUSION

The stem extract of *Cnestis ferruginea* contain alkaloid, saponin, flavonoid, cardiac glycoside, and anthroquinone. And the tested ethanolic stem extracts of *Cnestis ferruginea* were found to possess antimicrobial activity against MDR bacterial isolates. Thus *Cnestis ferruginea* could be an effective alternative to the conventional antibiotics in the treatment of infections caused by these multi-resistant bacterial strains. We recommend that the plant *Cnestis ferruginea* should be validated and be used to treat infections caused by *Escherichia coli*, *Staphylococcus aureus* and Salmonella spp.

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