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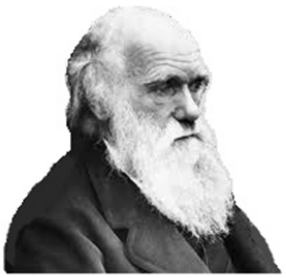

World News of Natural Sciences

An International Scientific Journal

WNOFNS 25 (2019) 188-198 EISSN 2543-5426

Antimicrobial studies and phytochemical analysis of the fruits and leaves of *Cnestis ferruginea*

R. U. B. Ebana[1], U. O. Edet[1,*], D. E. Atang[2] & L. A. Iyere[1]

[1]Obong University, Faculty of Natural and Applied Sciences, Department of Microbiology, Obong Ntak, Etim Ekpo, Akwa Ibom State, Nigeria

[2]Department of Physiology, University of Uyo, Uyo, Akwa Ibom State, Nigeria

*E-mail address: uwemedet27@gmail.com

ABSTRACT

Few studies have shown that *Cnestis ferruginea* possess some therapeutic properties. The present study was aimed screening the extracts of the fruits and leaves of *Cnestis ferruginea for* phytochemicals using crude screening and gas chromatography-mass spectrophotometer (GC-MS) methods, and also antimicrobial activity against clinical isolates. Preparation of the extracts (aqueous and ethanolic), identification of isolates, and antimicrobial sensitivity were done using previously reported standard methodologies. Replicate readings for the antimicrobial sensitivity were analysed using analysis of variance at 95% level of significance. The result of the crude qualitative screening revealed the presence of phenol, terpenes, alkaloids, saponins, tannin and polyphenol in various amounts in both the leaves and fruits. Quantitative analysis using GC-MS revealed a total of 14 similar phytochemicals each in the leaves and fruits of the study plant in varying amounts. In the fruits, phenol had the highest concentration of 15.01%, followed by terpenes (10.64%), alkaloid (5.43%), and tannins (5.16%). Others were anthocyanins, phytate, phytosterol, steroids, saponins, cardiac glycosides, oxalate, flavonoids, cyanogenic glycosides and coumarin with concentrations that ranged from to 0.01- 4.21%. On the other hand, in the leaves, alkaloids had the highest concentration of 31.62%, followed by phenol (20.59%), phytate (15.18%), and tannin (12.34%). Others include were flavonoid, terpenes, phytosterol, cardiac glycosides, saponins, cyanogenic glycosides, oxalate, anthocyanins, steroids and coumarin with concentrations that ranged from 0.16-8.45%. The isolates were identified as *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Proteus spp*. The results of the antimicrobial sensitivity revealed varying zones of inhibitions; however, it increased as the concentration of the extract increased. The observed highest zones of inhibitions were 33.86 mm for the fruit ethanolic extract and 30.56 mm for the aqueous extract of the leaves against *Proteus* and *Staphylococcus aureus,* respectively.

***Keywords*:** Phytochemicals, GC-MS, Antimicrobials, resistance, clinical isolates, *Cnestis ferruginea*

(Received 01 July 2019; Accepted 17 July; Date of Publication 18 July 2019)

1. INTRODUCTION

Microorganisms resistant to antibiotics have been isolated from all continent of the globe giving antimicrobial resistance a global dimension. More worrisome is the fact that it is spreading at an alarming rate and no class of antibiotics is spared this menace. This is due in part, to the evolution ability of microorganisms including pathogens to acquire and spread resistance genes via plasmids and also improper use of antibiotics. Despite advances in key technologies and disciplines such as medicine, chemistry and molecular biology such as genomics, combinatorial chemistry and rational drug design, very few antibiotics have made it to the commercial market after Vancomycin in 1972. *Staphylococcus* has been reported in 2011 to be resistant to Ceftaroline introduced in 2010.

These developments have prompted the search for newer antibiotics to bridge the gap between the number of effective antibiotics and spread of resistance. Some of these alternatives include the recent use of pathogens such as *Bdellevibrios* and like organisms and as well as the use of medicinal plants. Medicinal plants include plants or their organs which contain in their parts, phytochemicals that have been shown to have therapeutic uses or are used as drug precursors. It is therefore not surprising that a good number of medicinal plants form the core of traditional medicine for centuries.

The therapeutic use of medicinal plants probably predates written human history. According to the world Health Organization (WHO), an estimated 80% of the populace of developing nations preferentially use herbal extracts viz-a-viz their bioactive components for therapeutic purposes for several reasons. These include insufficient supply of conventional antimicrobial drugs, exorbitant cost of drugs, toxic side effect of drugs and the dwindling effectiveness of some presently used antibiotics. These plants contain phytochemical or bioactive components that have been shown by various studies to possess therapeutic roles including antimicrobial activities.

One of such plants that have therapeutic value is *Cnestis ferruginea* which belongs to the family called Connaraceae. It flourishes in Nigeria and it is called different names by different ethnic groups. The fruit contains a soft, juicy, a little bit bitter and acidic pulp that is used as teeth whitener. Other uses of the plants include snakebite antidote, eye drops, to cure migraine, gonorrhoea, and madness, and as well as a laxative for sore throat, pyorrhea, and oral infections. Furthermore, it has been shown to contain phytochemical such as phenol, alkaloids, saponins, flavonoids, cardiac glycosides, anthraquinones, tannins and reducing sugar. Furthermore, they have been shown to possess excellent anticonvulsant, antioxidant, hepatotropic effect, aphrodisiac, antidepressant, anti-fertility activities.

Thus, the aim of this research was to screen for phytochemicals present in the fruits and leaves of *C. ferruginea* and the antimicrobial activity of extracts of aqueous and ethanolic extracts on human pathogenic bacteria.

2. MATERIALS AND METHODS

2. 1. Collection of plant parts

The plant (leaves and fruits) were collected from Obong University community in Obong Ntak, Etim Ekpo LGA, Akwa Ibom State, Nigeria and were identified by Prof. Rose U.B. Ebana as *Cnestis ferruginea*. The samples were taken immediately to the laboratory for more analysis.

2. 2. Collection and identification of clinical isolates

The bacterial isolates used in our study were collected from the Microbiology Laboratory of the University of Uyo Teaching Hospital (UUTH). The isolates were indentified using standard morphological and biochemical tests procedures described previously. The isolates were identified as *Staphylococcus aureus*, *Proteus species*, *Pseudomonas aeruginosa* and *Escherichia coli*.

2. 3. Preparation of fruits and leaves samples

The freshly collected fruits were pounded using a clean mortar and pestle and allowed to air dry within the room temperature (20 – 25 ºC). After drying, the fruits were then grounded to powdery form using a clean mortar and pestle. The fruit powder were labelled and stored in sterile sample bottles at room temperature away from moisture. On the other hand, the freshly collected leaves were first air-dried and then made into tiny pieces using a clean stainless knife and then oven dried for 2 hours at 60 ºC. After which, they were then grounded into a powered using a clean mortar and pestle. The leaves powder were labelled and stored similarly.

2. 4. Preparation of aqueous and ethanol extracts of leaves and fruits

This was done as previously described. Briefly, 10g each of the freshly prepared powdered leaves and fruits were dissolved in 100 ml of ethanol (75%) and 100 ml sterile distilled water in separate clean beakers, swirled gently, beaker wrapped around with an aluminum foil paper and allowed to stand overnight at 25 ±2 ºC. Following overnight soaking, it was filtered and the filtrate gently heated in water bath at 60 ºC to allow the ethanol and water to evaporate completely leaving behind slurries. The slurries were then stored in sterile samples bottles until required for further.

2. 5. Phytochemical screening

The extracts were first screened for the presence of phytochemicals using the crude methods previously reported. The phytochemicals screened for the presence or absence of certain phytochemical and these included alkaloids, tannins, saponins, flavonoids, glycosides, polyphenol, anthraquinones, hydroxylmethyl anthraquinones and reducing compounds. In order to ascertain their concentration, the extracts were subjected to gas chromatography coupled to mass spectrophotometer.

2. 6. GC-MS analysis

This was performed on the fruits and leaves extracts of our study plant strictly following the instructions of the manufacturer. This was done using the Clarus 500 Erkin-Elmers Gas Chromatography equipped and coupled to a mass detector Turbo mass gold 5.1 spectrophotometer.

2. 7. Antimicrobial susceptibility test

The was carried out using the agar disk diffusion methods of Erikson (1960) and CLSI (2006). This was using various manually bored sterilized discs made from Whatman filter paper impregnated with various concentrations (0.1, 0.3, 0.5 mg/ml) of both the ethanolic and aqueous slurries for 15minutes and were placed gently using a sterile forcep on the Mueller Hinton Agar

plates inoculated with the test organisms. These were then incubated overnight at 37 ºC for 24 hours. After incubation, the sizes (diameter) of the zone of inhibitions on the various concentrations were measured in millimeter using a transparent meter rule.

2. 8. Determination of minimum inhibitory concentration (MIC)

The MIC of the plant extracts were determined using pour plate technique. Different concentrations were prepared by weighing out 0.1g, 0.05g and 0.025g of each of the extracts and dissolved in 1ml of water separately. Sterilized 6mm discs were soaked in the various concentrations and allowed to air dry. These were then incubated with the pour plates of the test organisms at 37 ºC for 24 hours. The plates were then examined for inhibition and the lowest inhibitory concentration was taken as the MIC for the extract and test organism.

2. 9. Statistical analysis

Replicate readings obtained for the antimicrobial sensitivity analysis for both extracts of the leaves and fruits were subjected to analysis of variance and the results expressed as mean ± standard deviation at 95 % level of significance.

3. RESULTS

The results obtained are presented in the tables below. The isolates were confirmed to be *Escherichia coli*, *Staphylococcus aureus*, *Proteus spp* and *Pseudomonas aeruginosa* after morphological, Gram reaction and biochemical tests.

The results of the antimicrobial sensitivity are as presented in Tables 1 and 2. Table 1 shows the antimicrobial activities of both extracts for the fruits of our study plant. The results indicate that all the isolates were sensitive to the various extract concentrations used and the higher concentrations of both extracts of the fruit, the higher zones of inhibitions for the isolates tested. For *E. coli*, the highest zone recorded was 31.11±1.00mm followed by 29.10 ±2.08 mm for ethanol and aqueous extracts, respectively with the highest concentration of 0.5mg/ml. Similar results were also obtained with 0.5mg/ml for *S. aureus*, *Proteus* species and *P. aeruginosa* which gave 32.35 ±1.53 and 28.11 ±1.34, 33.86 ±2.21and 15.83 ±2.01, and 30.29 ±0.00 and 29.29 ±2.10 mm, respectively for both ethanol and aqueous extracts.

As shown in Table 2 and compared to the extracts of the leaves, *Proteus* species was not sensitive to the ethanol extracts but was to the aqueous extract. Overall, the test isolates were more sensitive to the extracts of the fruits than that of the leaves even at the highest concentration used in our study. *S. aureus* was the most sensitive across all concentrations for both isolates and also gave the highest inhibitions of 29.31 ±0.22 and 30.56 ±1.10 for ethanol and aqueous extracts, respectively with the highest concentration of 0.5 mg/ml. Tables 3 and 4 show the results of the minimum inhibitory concentrations of the leaves and fruits extracts. Consistently, the MIC of the leaves and fruits extracts was 0.5 mg/ml for each of the test isolates.

Tables 5 and 6 show the results of the GC-MS analyses of both leaves and fruits extracts. Both tables reveal that a total of 14 similar phytochemical compounds each and these were present in varying concentrations. The phytochemical were terpenes, phytosterol, oxalate, steroid, tannin, phenol, saponin, alkaloid, coumarin, anthocyanins, flavonoids, phytate, cardiac glycosides, and

cyanogenic glycoside. The least abundant phytochemicals in the fruits and leaves of our study was cardiac glycosides with concentrations of 0.01 and 0.35%, respectively. The highest phytochemical compound in the fruits was phenol followed by terpenes with concentrations of 15.01 and 10.64 %, respectively. This was then followed by alkaloid and phenol in the leaves with concentrations of 31.62 and 20.59%, respectively.

Table 1. Antimicrobial Activities of the fruits extract of *C. ferruginea.*

Isolates	**Ethanol**			**Aqueous**		
	0.1	0.3	0.5	0.1	0.3	0.5
E. coli	13.50±2.08[a]	17.68±1.00[a]	31.11±1.00[a]	14.72±0.02[a]	18.67±0.02[a]	29.10±2.08[a]
S. aureus	14.50±1.57	20.38±1.53	32.35±1.53	15.80±0.55	19.36±1.88	28.11±1.34
Proteus sp	12.24±1.72	19.44±0.65	33.86±2.21	8.24±1.11	10.32±1.11	5.83±2.01
P. aeruginosa	10.20±1.73	19.28±2.00	30.29±0.57	15.62±0.66	21.38±2.84	29.29±2.10

[a]Represents significant Mean ± SD that were significant ($p < 0.05$) across both column and rows, respectively.

Table 2. Antimicrobial Activities of the leaves extract of *C. ferruginea.*

Isolates	**Ethanol**			**Aqueous**		
Isolates	0.1	0.3	0.5	0.1	0.3	0.5
E. coli	11.20±0.23[a]	13.07±0.91[a]	20.23±2.10[a]	12.40±0.00[a]	17.27±0.51[a]	25.38±0.33[a]
S. aureus	14.20±1.72	17.82±0.22	29.31±0.22	15.50±2.19	18.2±1.10	30.56±1.10
Proteus sp	-	-	-	13.52±1.11	17.77±0.21	28.48±2.15
P. aeruginosa	11.12±0.63	15.00±0.63	19.28±2.13	12.24±1.22	18.32±0.00	26.30±0.00

[a]Represents significant Mean ± SD that were significant ($p < 0.05$) across both column and rows, respectively.

Table 3. Minimal inhibitory concentration (MIC) for the fruits of *C. ferruginea.*

Isolates	**Ethanol**			**Aqueous**		
	0.05	0.1	0.025	0.05	0.1	0.025
E. coli	-	±	+++	-	±	+++

S. aureus	-	±	+++	-	±	+++
Proteus sp	-	±	+++	-	±	+++
P. aeruginosa	-	±	+++	-	±	+++

Keys: -, ± and +++ represent growth, no growth and no growth at all

Table 4. Minimal inhibitory concentration (MIC) for the leaves of *C. ferruginea.*

Isolates	**Ethanol**			**Aqueous**		
	0.025	0.05	0.1	0.025	0.05	0.1
E. coli	-	±	+++	-	±	+++
S. aureus	-	±	+++	-	±	+++
Proteus sp	-	-	-	-	±	+++
P. aeruginosa	-	±	+++	-	±	+++

Keys: -, ± and +++ represent growth, no growth and no growth at all

Table 5. GC-MS analysis of the phytochemical components of the fruits of *C. ferruginea.*

S/N	**Compounds**	**% Conc. units**
1.	Terpenes	10.64
2.	Phytosterol	1.33
3.	Oxalate	0.93
4.	Steroid	2.05
5.	Tannin	5.16
6.	Phenol	15.01
7.	Saponin	1.68
8.	Alkaloid	5.43
9.	Coumarin	0.03
10.	Anthocyanins	2.89
11.	Flavonoids	0.62

12.	Phytate	4.21
13.	Cardaic glycosides	0.01
14.	Cyanogenic glycoside	0.05

Table 6. GC-MS analysis of the phytochemical components of the leaves of *C. ferruginea*

S/N	*Compounds*	*% Conc. units*
1.	Terpenes	7.29
2.	Phytosterol	1.46
3.	Oxalate	1.09
4.	Steroid	0.16
5.	Tannin	12.34
6.	Phenol	20.59
7.	Saponin	2.83
8.	Alkaloid	31.62
9.	Coumarin	0.73
10.	Anthocyanins	1.51
11.	Flavonoids	8.45
12.	Phytate	15.18
13.	Cardaic glycosides	0.35
14.	Cyanogenic glycoside	0.92

4. DISCUSSION

Medicinal plants have fulfilled the health needs of about 80% of the populace of developing countries according to WHO. The usefulness of the medicinal plants depends on the phytochemicals such as alkaloids, saponins, glycosides, flavonoids, polyphenol, anthraquinones. Some of these health effects include include being used as antimalaria, antibacterial, antifungal, antididental plaque, antidiabetics, antitrypanasomal.

Enemor *et al.* (2015) [26] worked with the stem of *C. ferruginea* and found six phytochemicals including saponin, flavonoid, cardiac glycoside, anthroquinone, tannin and

reducing sugar. In our study, these compounds were also present in the leaves and fruits of the study plant. Furthermore, they also showed that the ethanol extract of stem had excellent antimicrobial activity against multidrug resistant bacteria isolates from retailed meats in Awka, South Eastern Nigeria. They zones of inhibitions was within the range of 3-18 mm while the MIC and MBC ranged from 3.2 to 6.3 mg/ml. The zones they reported were smaller compared to what we obtained in our study. However, the MIC values were higher than our reported MIC of 0.5 mg/ml.

To ascertain these phytochemicals, a GC-MS analysis which is more reliable was carried out on the fruits and leaves of *C. ferruginea.* The results revealed a total of 14 phytochemical components with varying amounts of abundance in the fruit and leave of the plant. These phytochemicals include terpenes, phytosterol, oxalate, steroid, tannin, phenol, saponin, alkaloid, coumarin, anthocyanins, flavonoids, phytate, cardaic glycosides and cyanogenic Glycoside. For the fruit, phenol was the most abundant component, followed by terpenes, alkaloid and tannin. While for the leave, the most abundant phytochemical was alkaloid followed by phenol, phytate and tannin.

Antimicrobial sensitivity results showed that all the isolates were sensitive apart from the ethanolic extract of the leaves against *Proteus sp* which showed resistance against all concentrations used. Comparatively, the fruit extracts showed better inhibitory activity than those of the leaves. The highest zone of inhibition recorded was 33.86 ±2.21 mm for the fruit extracts while it was 30.56 ±1.10 mm for the extracts of the leaves. Our zones were higher than those of an earlier study which reported zones that ranged from 10.00 to 18.67 mm and 10.33 and 19.00 mm, respectively for extracts of *D. eludis* and *G. kola*, respectively. Furthermore, our zones are higher than that previously reported for the edible vegetables *Lasianthera africana* and *Dennettia tripetala against bacterial isolates* which gave zones that ranged from 8.70 - 25.00 mm and 8.30 - 14.70 mm, respectively. Our zones were still higher than those reported earlier for *Acanthus montanus, Aspilia africana* and *Desmodium velutinum*urinary tract pathogens.

In an earlier study, the antioxidant, antimicrobial and FTIR analysis of the methanolic root extracts of *C. ferruginea* and ethanol extract of *C. limon* were carried out. Although we only evaluated the fruits and leaves of C. *ferruginea*, similar phytochemicals were observed and these included alkaloids, flavonoids, saponins, tannins, glycosides, and anthraquinones in addition using crude methods of screening. Furthermore, they showed that each of the components showed antimicrobial as well as antioxidant activities.

In tradition medicine, the study plant is used in many ways. The aqueous preparation is used to treat and manage a number of infections. In our study, excellent antimicrobial activity was observed for the aqueous extract especially against *S. aureus*. In an earlier study using bioassay- guided purification showed that antimicrobial activity was due to the presence water-soluble compounds. These fractions were hydroquinone and caffeic acid methyl ester that gave MIC values of 63 >200 mg/mL, respectively.

5. CONCLUSION

The present study has confirmed the antimicrobial or medicinal usefulness of the fruits and leaves of *Cnestis ferruginea*. The study has also estimated quantitatively, the amount of crude phytochemicals present in the fruit and leaves of *C. ferruginea*. The extracts showed

excellent antimicrobial activities the clinical isolates used in this study that need to be exploited further in future studies.

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