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Evaluation of the Antimicrobial, Antioxidant and the Staining Property of *Cnestis ferruginea* Ethanoic Fruit Extract

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ABSTRACT: Plants contain variety of bioactive compounds known to have chemotherapeutic value some of these plants also have dyes. Against the background, this study is aimed at determining the nutritional composition, antimicrobial, antioxidant and staining properties of *Cnestis ferrugenea* fruit. Plant fruits were collected and processed using soxhlet extraction technique. Phytochemical analysis was determined using standard laboratory method. Phenolic content estimation of plant fruits was determined using folin-ciocalteu (F.C) method. Antioxidant was estimated using 2,2 diphenyl-1-picrylhydrazyl (DPPH) . *Cnetis ferrugenea* fruit extract had anti-fungi activity but low antibacterial activity. Phytochemical analysis of plant fruit shows the presence of alkaloid, saponin, tannin, flavonoid and cardiac- glysoside. Nutritional compositions are total ash, moisture, crude fibre, lipid, carbohydrate and protein. MIC of fruit extract is 6.25 and has IC_{50} value 15966.02 with pH of 4.6. *Cnetis ferruginea* ethanoic fruit extract stained fungal isolates but didn't stain bacteria isolate. Studied fruit extract is a good source of carbohydrate, crude fibre and moisture content, it has antioxidant, antifungal and staining properties but low antibacterial properties. Free radicals are central cause of disease, knowledge of antimicrobial and antioxidant properties of plants extract will help pharmaceutical company formulate products that will combat the menace of free radicals. Some medicinal plants possess natural dyes.

Keywords: *Cnestis ferruginea,* Antimicrobial, Antioxidant, Nutritional composition, Staining property.

Introduction

Due to increase in development of resistance of microorganism to antimicrobial drugs in recent times, search for plants with antimicrobial activities has gain increasing importance, globally the last two decades has witnessed an unprecedented increase of drug resistant to pathogenic organism as well as the appearance of undeniable side effect of certain antibiotics (Akunyili *et al.,* 1991; Anegbeh *et al.,* 2006). Free radicals are central cause of disease. Knowledge of the antimicrobial and antioxidant properties of plant extract will help pharmaceutical companies formulate products that will combat the menance of multidrug resistant of clinical isolates and reduce the incidence of diseases associated with free radicals thereby reducing the rate mortality and mobidity. Some medicinal plants possess natural dyes, (Raja *et al*., 2013) staining of microorganism has been an important aspect of microbiology and its assist in identification and characterization of organisms, Dyes from which stains are made are either natural or synthetic product, most dyes used for bacterial and fungal stain are synthetic (Ochei and Kolhatkar, 2008). However, synthetic dyes cause skin allergies and other harms to human body on exposure and produce toxic waste (Avwioro *et al.,* 2005), Goodarzian and Ekrami (2010). The use of non-allergic and non-toxic stain has become a matter of importance due to the increased environmental awareness in order to avoid some harzadous synthetic ones, there is paucity of information in the possibility of the use of use of plants' extract from *Cnestis ferruginea* fruit as substitute for staining of microorganism. Findings from the

use of extract as stain will provide useful alternative that will be readily accessible and cheap which will translate to lower cost of laboratory stains for better management of patients.

Materials and methods

Sampling technique: Cnestis ferruginea fruit was obtained from Obiarukwu town in Ukwuani Local Government Area of Delta State.
Plant identification and authentication: Plants collected were identified and authenticated by Plant Taxonomists (Prof. J.F. Bamidele and Dr. H.A. Akinibosun) at Plant Biology and Biotechnology Department University of Benin using their local names and standard texts. Samples of plants were deposited in the herbarium of the Department of Plant Biology and Biotechnology University of Benin. Their Voucher numbers are as follows: UBH369 *(Cnestis ferruginea*).
Ethical approval: Ethical approval was sought for and obtained from the Ethical Committee, Hospitals Management Board, Benin City, Edo State and Ethical Committee, Faculty of Health Sciences and Technology, Nnamdi Azikiwe University, Nnewi Campus, Nnewi.
Study area: The study was carried out in Nnewi Campus of the Nnamdi Azikiwe University, Awka, Anambra State. Nnewi which is located on latitude 6° 01′ 10.63″ N and Longitude 6° 55′ 2.24″ E, is the second largest city in Anambra State, South Eastern Nigeria. Their population is 193,987 dwellers. The ethnic group in the area is majorly Igbo and the people are known for trading and manufacturing of cars and motorcycles.
Study site: Analysis of the plants' parts was carried out in the Faculty of Pharmaceutical Sciences premises, Nnamdi Azikiwe University, Awka, Nigeria. Fully automated Soxhlet solvent extraction technique was used for the extraction of the plant fruits using ethanol. After extraction, the solvent was removed and concentrated using a rotary evaporator yielding the extracted compound. The filtrates were evaporated using rotary evaporator and were finally concentrated to dryness using water bath at a temperature of 50 °C (Dada and Ikuerowo, 2009). The crude extracts were weighed after extraction, placed in an air-tight and water-proof container and kept in a refrigerator at 4 °C. Solvent-solvent fractionation technique was done using ethylacetate, n-hexane and n-butanol. The whole fractions were concentrated using water bath and then preserved in the refrigerator at 4 °C.
Preparation of Cnestis ferruginea fruit extract solution: One gram (1 g) of *C. ferruginea* fruit extract was dissolved in 5 mililiters (5 ml) of seventy percent (70%) alcohol to give a concentration of 200 mg/ml of *C. ferruginea* solution. In order to increase colour intensity from light red to deep red colour, 0.2 ml of HCl was added to the solution. It was filtered with Whatman filter paper No 1 and stored in a clean universal bottle ready for use.
Qualitative analysis of constituents: This was as reported by Okwu and Omodamiro (2005) and followed in the analysis for test for tannins, saponins, flavonoids**,** cardiac, glycosides and terpenes; Quantitative analysis of the constituents; Determination of alkaloid (Harbone, 1973); Determination of saponin content (AOAC 2000); Determination of tannin content (AOAC, 2000); Determination of flavonoid content (Boham and Kocipai, 1994). Test for antioxidant property using 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity was carried out using spectrophotometric method of Mensor *et al.* (2001). Ten (10) strains of both Gram negative and Gram positive bacteria (*Morganella morganii, Pseudomonas aeruginosa, Klebsiella pneumoniae, Bacillus subtilis, Staphylococcus aureus, Streptococcus pneumonia Escherichia coli, Proteus mirabilis, Staphylococcus aureus* (ATCC 25923) and *Salmonella typhi* (ATCC 14028) and three fungal strains (*Aspergillus niger, Candida albican*s and *Candida albicans* (ATCC 10231) were used in this study. Antimicrobial activity of the ethanolic fruit extract of *C. ferruginea* fruit determined by agar diffusion method (Perez *et al.*, 1990). Ciprofloxacin (5 µg/ml) and Miconazole (50 µg/ml) were used as positive controls in the antibacterial and the antifungal evaluation respectively; while DMSO was used as the negative control. Minimum Inhibitory Concentration (MIC) is defined as the lowest concentration of the antimicrobial agent that inhibits bacterial growth. The MICs of the plants' extracts on the test isolates were determined by the agar dilution method as described by Russell and Furr (1977).
Cnestis ferruginea fruit extract preparation: One gram (1 g) of *C. ferruginea* fruit extract was dissolved in ten mililiters (10 ml) of seventy percent (70%) alcohol and 0.2 ml of HCl was added to increase the intensity of the colour. It was filtered with Whatman filter paper No 1 and stored in a clean universal bottle ready for use.
Staining of bacteria isolates: Smears of *S. aureus, E. coli, P. aeruginosa, P. mirabilis, K. pneumoniae* were made on twenty-five sets of clean grease free slide and heat fixed (Beishir, 1987; Cheesbrough, 2000; Baker *et al.*, 2001). Gram staining reagents (crystal violet, Lugols iodine and acetone) were used except for the counter stain (neutral red). These slides were counterstained with the solutions made from extracts of *Cnestis f*erruginea fruit and Control slides were also prepared and stained by Grams method using neutral red as counter stain (Prescott *et al.*, 1999).

Staining of fungi: The fungi isolates were stained as previously described (Beishir, 198; Harrigan and McCancee, 1990). Briefly, a drop of the prepared solution of the C. ferruginea extract was placed on a clean grease free microscope slide. Using a sterile wire loop, a colony of *C. albican* from SDA was transferred into the drop of extract solution. This was emulsified and a cover slip placed over the mixture and subsequently viewed under the microscope using X10 magnification. Slide culture technique was used to grow *Aspergilus niger*. Staining of the *Aspergilus niger* was done by aseptically removing the cover slip from the slide culture apparatus and placing it on a drop of *C. ferruginea* solution resting on a clean grease free microscope slide. The preparation was allowed to stand for 3 minutes and later viewed for morphogical details with the X40 magnification lens of a microscope. Control slide was prepared and stained with lactophenol cotton blue.
Statistical analysis: Data obtained were analysed using student t-tests and ANOVA as well as Pearson Correlation with the statistical software INSTAT. Statistical significance was set at $P < 0.05$. Bivariate analysis of mean zone diameter was done with student t–test. Analysis of antimicrobial effect of *C. ferruginea* fruit extract on microbial isolates was done with ANOVA while the relationship between antioxidant activity of extract and the total phenolic content was determined with Pearson Correlation.

Results

Phytochemical analysis of C. ferruginea revealed the presence of alkaloids, tannin flavonoid and cradiac glycosid only. Saponins, Steroids and Terpenes were absent (Table 1).

Table 1: Qualitative analysis of phytochemical composition of ethanolic *C. ferruginea* fruit extract

Plant Part/Extract	Alkaloid	Saponin	Tanin	Flavonoid	Steroids	Terpenes	Cardia glycosides
Cnestis ferruginea	++	–	++	+	–	–	++

C. ferruginea plant analysed in this study had a mean total phenolic content and pH value of 971.47 + 15 mgGAE/g and 4.8 respectively. Analysis of IC_{50} content of study plant showed a value of 154117.6 µg/ml. (Table 2).

Table 2: Plant extract and the corresponding IC_{50} and pH value

Plant Extract	pH	IC_{50} (ug/ml)	Total Phenolic Content (mgGAE/g extract)
Ethanolic solvent	4.8	154117.6	971.47 + 15

Of all the bacterial isolates studied, *M. morgani*, *B. subtilis* and *S. pneumonea* were most susceptibible to 200 mg/ml *C. ferruginea* extract with a mean zone diameter of 3.3±1.15 recoreded for each isolate. The isolates that were least susceptible to the *C. ferruginea* (200 mg/ml) extract were *K. pneumoniae*, *S. aureus* and *P. auroginosa*, which had a common mean zone diameter of 2.0±0.93. Although a generally higher antibacterial activity was observed on all isolates with the use of ciprofloxacin ((5 ug/ml ciprofloxacin) as compared to *C. ferruginea* extract, its activity was only found to be statistically significant with respect to *E coli* (P=0.027) and *P. mirabilis* only (0.0152).
The antifungal activity of *C. ferruginea* extract was found to be markedly higher than that recorded for bacterial isolates. The mean antifungal activity of *C. ferruginea* extract against *C albican* and *A. niger* were 9.3±1.528 and 5.3±1.155 respectively. Although, the control antifungal agent used in this study (50 ug/ml miconazole) fared better against the two fungi, statistics did not show any significant difference in the performance of the control antifungal agent and our study extract ($P > 0.05$) (Tables 3 and 4).

Table 3: Mean zone diameter of bacterial isolates to 200 mg/ml of ethanolic extract of *C. ferruginea* fruit extract

Bacterial isolates	No	200 mg/ml *Cnestis ferruginea fruit* Mean zone diameter (mm ± SD)	5 ug/ml ciprofloxacin Mean zone diameter (mm ± SD)	P. Value
M. mongani	3	3.3 ± 1.15	19.8 ± 0.72	0.34
P. aeruginosa	3	2.0 ± 0.93	17.3 ± 1.15	0.39
K. pneumoniae	3	2.0 ± 0.93	10.56 ± 1.25	0.35
B. subtilis	3	3.3 ± 1.15	10.56 ± 1.25	0.19
S. aureus	3	2.0 ± 0.93	9.6 ± 059	0.27
S. pneumonia	3	3.3 ± 1.15	12.6 ± 1.55	0.50
E coli	3	2.5±0.12	19.8 ± 0.72	0.027
P. mirabilis	3	2.5± 0.23	17.3± 1.85	0.0152
S. aureus (ATTC 25973)	1	5.3 ± 1.15	0.0	N\A
S. typhi (ATTC 14028)	1	3.6 ± 1.555	12.6 ± 1.577	0.37

Table 4: Mean zone diameter of fungi isolates to 200 mg/ml of ethanolic extract of *C. ferruginea* fruit extract

Fungi isolates	No	200 mg/ml *Cnestis ferruginea fruit* Mean zone diameter (mm ± SD)	5 ug/ml ciprofloxacin Mean zone diameter (mm ± SD)	P. Value
C albican	3	9.3 ± 1.528	18.3 ± 1.528	0.5
A. Niger	3	5.3 ± 1.155	9.0 ± 1.000	0.43
C. albican (ATTC 10231)	1	12.3 ± 1.128	20.3 ± 1.528	0.35

The growth of all bacteria isolates was inhibited with 200 ug/ml *C. ferruginea* extract solution. However, the minimum inhibition concentration (MIC) of *M. morgana*, *B. subtilis*, *S pneumonia*, and *P. mirabilis* was found to be 100 ug/ml. The most sensitive bacteria isolate of the lot was *E. coli* which had a minimum inhibition concentration of 50 ug/ml (Table 5).

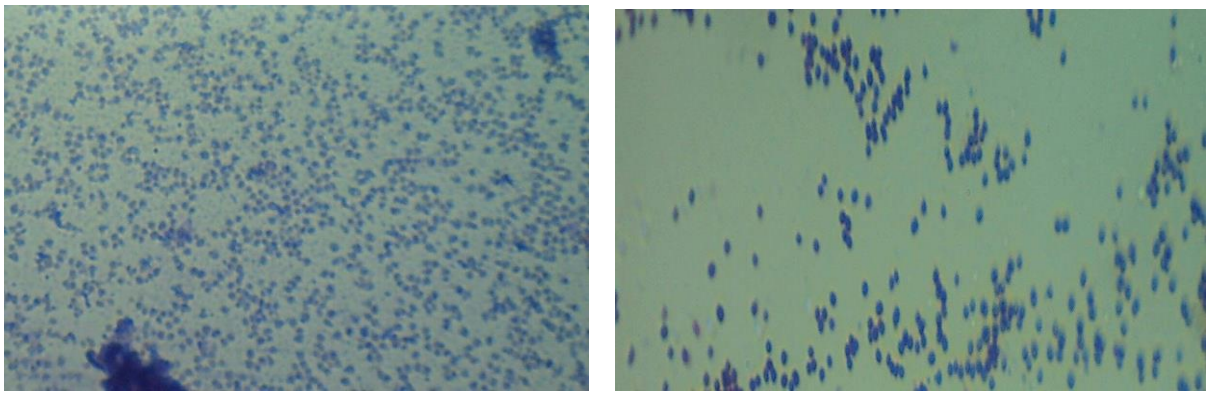
Table 5: Minimum inhibitory concentrations of C. ferruginea ethanolic plant extract determined against tested bacterial and fungal isolates

Isolates	Concentrations of *C. ferruginea* extract (mg/ml)							
	200	100	50	25	12.5	6.25	3.125	1.56
M. mongani	-	-	+	+	+	+	+	+
P. pseudomonas	-	+	+	+	+	+	+	+
K. pneumonia	-	+	+	+	+	+	+	+
B. subtilis	-	-	+	+	+	+	+	+
S. aureus	-	+	+	+	+	+	+	+
S. pneumonia	-	-	+	+	+	+	+	+
E. coli	-	-	-	+	+	+	+	+
P. mirabilis	-	-	+	+	+	+	+	+
S. aureus (ATCC 25923)	-	-	-	-	-	-	-	-
S. typhi	-	-	-	-	-	-	-	-
C. albican	-	-	-	-	+	+	+	+
A. niger	-	-	-	+	+	+	+	+
C. albicans (ATCC 10231)	-	-	-	-	-	-	-	-

Key: + Growth; - No growth

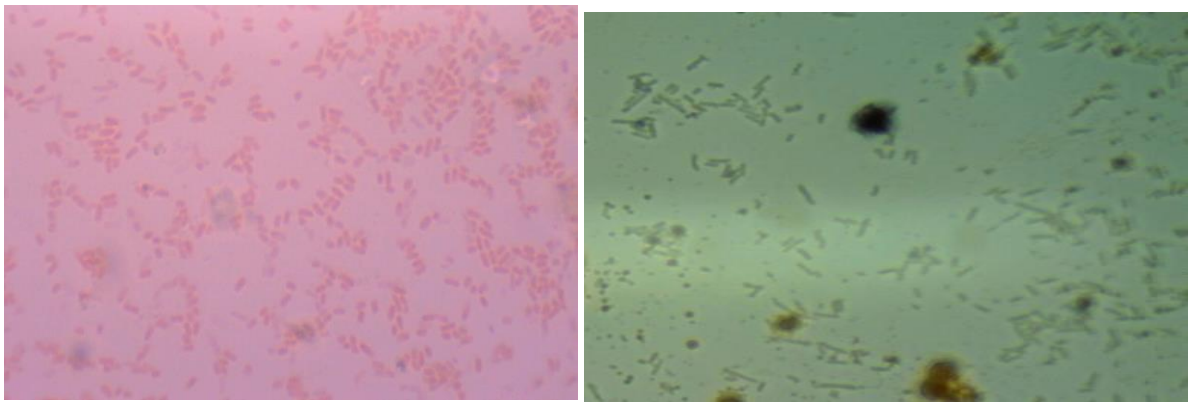
Generally, *C. albican* and *A. niger* isolates used in this study were found to be more susceptible to *C. ferruginea* extract than bacteria isolates as they recorded an MIC of 25 ug/ml and 50 ug/ml respectively (Table 5). All bacteria and fungi isolates were inhibited by 200 ug/ml *C. ferruginea* extract solution. Of all bacteria isolates, *Escherichia coli* isolate was found to be most sensitive with an MIC of 50 ug/ml of extract. *Candida albican* had a higher MIC (25 mg/ml) than *Aspergilus niger* which had an MIC of 50 mg/ml).

C. ferruginea extract had a poorer staining activity (Plate 1B) on *Staphylococus aureus* than conventional Gram counter stain (Plate 1A). Uptake of *C. ferruginea*, by isolate of *Escherichia coli* as seen in (Plate 1D), was generally poorer than that observed with the use of conventional Neutral red (plate 1C). Compared to conventional gram counter stain (Plates 1A and 1C), *C. frruginea* ethanolic extract had a poor staining performance on *Staphylococus aureus* (Plate 1B) and *Escherichia coli* (Plate 1D).



A **B**

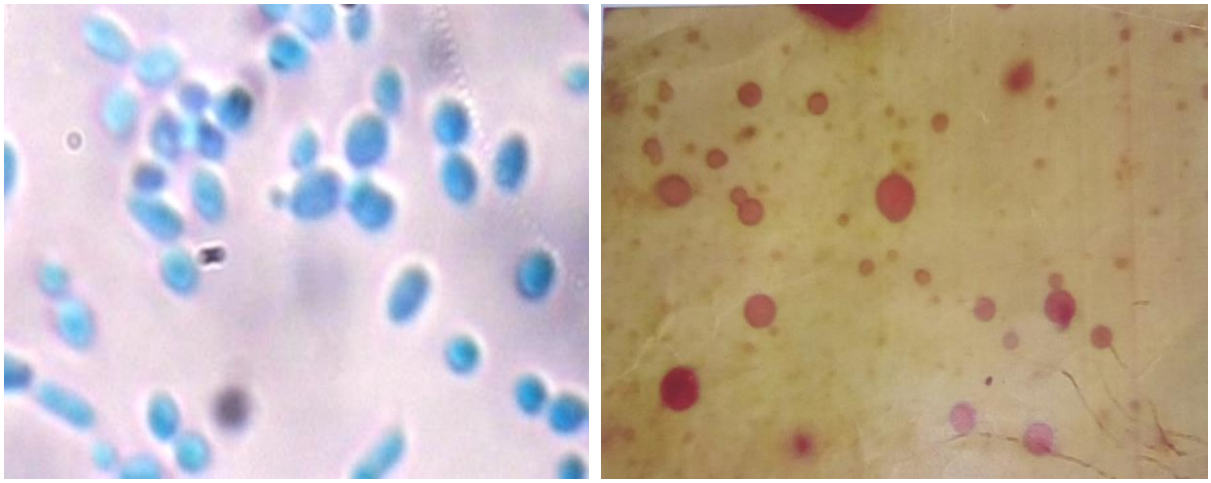
Plate 1A: *Staphylococus aureus* stained with gram staining reagents (control) **B:** *Staphylococus aureus* counterstained with *C. ferrugenea* ethanolic extract solution



C **D**

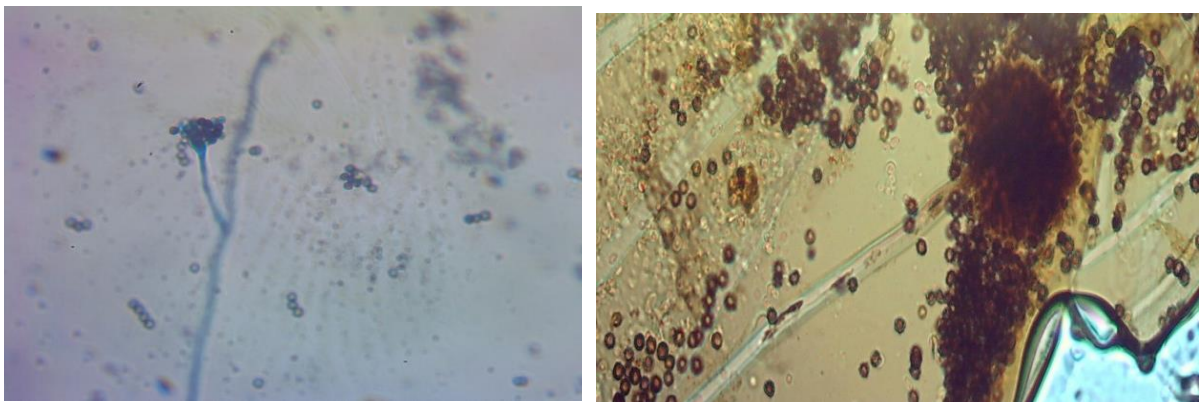
Plate 1C: *Escherichia coli* stained with gram staining reagents and counterstained with neutral red (control). **D:** *Escherichia coli* counterstained with *C. ferrugenea* ethanolic extract solution

However, a generally good staining uptake and differentiation was observed with the *C. ferruginea* ethanolic extract on fungi isolates (*Candida albican* (Plate 2B) and *Aspergilus niger* (Plate 2D) which compared favourable with conventional Lactol Phenol Cotton Blue stain on the fungi isolates respectively (Plate 2A and Plate 2C).



A **B**

Plate 2A: *Candida albicans* stained with Lactophenol cotton blue (control). **B:** *Candida albicans* stained with *C. ferruginea fruit* ethanolic extract



C **D**

Plate 2C: *Aspergilus niger* stained with Lactophenol cotton blue (control). **D:** *Aspergilus niger* stained with C. ferruginea fruit ethanolic extract

Discussion

There is paucity of information on the antimicrobial activity of *C. ferruginea* fruit in literature. Against this background this study was conducted to evaluate the phytochemical content of *C .ferruginea* fruit as well as its antimicrobial activity on selected bacterial and fungi isolates.

Phytochemical analysis of *C. ferruginea* fruit extract in this study revealed the presence of alkaloids, cardiac glycosides, flavonoids and tannins. Similar constituents were detected in root and stem extract of *C. ferruginea* in a study conducted in Nigeria by Emenor *et al.* (2005). The presence of alkaloids, tannins and flavonoids suggests that *C. ferruginea* fruit have antibacterial, healing and anticancer activities (Raina *et al.*, 2014). The presence of these constituents in the fruit extract of *C. ferruginea* in this study also indicates that it has good pharmacological and therapeutic values. In herbal medicine and some literatures, the fruit extract has diverse therapeutic uses against infections like snakebite, dysentery, syphilis, gonorrhea, cough, dysmenorrhea, ovarian troubles and aphrodisiac. The root and fruit extracts however prevent abortion, constipation, fever and pain (Gill, 1992; Okafor and Ham, 1999). The fruit extract is used locally for the treatment of tooth-ache, mouth and skin infections (Boakye-Yiadom and Konning, 1975).

C. ferruginea fruit extract in this study demonstrated good antioxidant activity with an IC_{50} value and total phenolic content of 154117.6 ug/ml and 971.47 + 15 mgGAE/g respectively. To the best of our knowledge, no documented study has evaluated the antioxidant activity of *C. ferruginea* fruit. However, one study which examined the antioxidant activity of *C ferrugniea* focused on the seed and recorded an IC_{50} value and total phenolic content of 77.4 ug/ml and 31.7 mgGAE/g respectively (Ita, 2017). The extract of *C. ferruginea* fruit in this study was observed to have a generally low antibacterial activity against both Gram negative and Gram positive bacterial isolates.

Findings from a study conducted by Emenor *et al.* (2015) on stem extract of *C. ferruginea* on bacterial isolates, a generally low activity was observed against Gram negative bacterial isolates. Similarly, low sensitivity of Gram negative was reported on aqueous stem extract of *C. ferruginea* by Ndukwu *et al.* (2005) and Akharaiyi *et al.* (2012). In this study *C feruginea* fruit extract was found to inhibit isolates of *S. aureus* and most Gram negative isolates, albeit to a very small degree. Of the bacteria isolates tested, the extract was found to have the most activity on *E coli* isolates. A generally higher antimicrobial activity was elicited by extract on fungi isolates, with no significant different observed between its performance and that of conventional miconazole antifungal agent as control.

Conclusion

C. ferruginea fruit extract used in this study contain some bioactive compounds namely: flavonoids, alkaloids, tannins, and cardiacglycosides. The fruit extract also demonstrated good antioxidant properties and showed good antimicrobial activity especially against fungi isolates. There is need to explore the potentials of these plants in traditional medicine and pharmaceutical industries.

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