Antimicrobial and antioxidant studies of the methanol leaves extract of *Cnestis ferruginea* Dc (connaraceae)

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Abstract

The leaves of *Cnestis ferruginea* is used in the treatment of various ailment and diseases. This work is aimed at investigating the antimicrobial and antioxidant studies of the extract of *Cnestis ferruginea* DC (Connaraceae) leaves. The methanol extract was evaluated for the presence of phytochemicals. The methanol extract was evaluated for antimicrobial activities against *Staphylococcus aureus, Escherichia coli, Bacillus subtilis, Pseudomonas aeruginosa, Candida albicans,* and *Aspergillus spp*. Thereafter, antioxidant activities were also carried out with DPPH (2,2- Diphenyl-1-picryl hydrazyl) and FRAP (Ferric reducing antioxidant power) methods using ascorbic acid as standard for DPPH and FRAP. The total phenolic content was determined using garlic acid and total flavonoids content was determined using quercetin as their standard respectively. The results indicate the presence of phytochemicals such as, tannins, alkaloids, terpenoids, saponins, cardiac glycosides, anthraquinones, and flavonoids. The results also indicate that all the organisms were sensitive to the various extract concentrations used (100 – 6.25 mg/mL). The minimum inhibitory concentration demonstrated by the organisms include, *E. coli* (12.5mg/mL), *B. subtilis* (6.25mg/mL), *S. aureus* (12.5mg/mL), *P. aeruginosa* (6.25mg/mL), *C. Albicans* (100mg/mL), *and A. spp* (100mg/mL). The mean % inhibition for DPPH increases with increasing concentrations compared to FRAP that decreases with increasing concentrations. The total phenolic and flavonoids content of the crude extract of *C. ferruginea* leaves are 82.89 $\pm$ 0.06 mg/GAE/g and 57.09 $\pm$ 0.04 mg/QAE/g respectively. This study shows that the plant extract exhibited good

antimicrobial and antioxidant activities against the test microorganisms.

Keywords: Antimicrobial, Antioxidant, DPPH, FRAP, *Cnestis ferruginea*, Connaraceae**,**

Introduction

Plants with medicinal value are known as the origin of different kinds of bioactive compounds (Olufunmiso *et al.,* 2018) with varied pharmacological and therapeutic activities. The therapeutic strength of these medicinal plants has been a major cause of discussion over the years. The remission of deadly diseases together with the adverse effects of chemically synthesized drugs, suggest the need to move from modern medicine to traditional medicine. Because they contain no synthetic ingredients and are derived entirely from natural plants, traditional medicines appear to have no negative side effects. They are more affordable, easy to obtain and don't require prescriptions, strengthen the overall immune system, have low chances of addiction, and cost very little to harvest and produce unlike chemically synthesized drugs (Ahmed, 2017). In traditional medical systems of medicine, the use of these medicinal plants as drugs has been of great importance in the development of new chemical entity (NCE) for modern medicine (Nwodo *et al.,* 2015). Notwithstanding the current trend of synthesis as a preferred method of drug development, the strength of medicinal plant extracts to yield new chemical entity (NCE) for treatment and prevention remains extremely good (Sharma *et al.*, 2015). These synthesized drugs provide our desire medications in the required dose and provide quick relief from specific symptoms.

Resistance to antibiotics by microorganisms from all continent of the globe has given antimicrobial resistance a global dimension (Chetan *et al.,* 2018). In part, this is due to the evolving ability of microorganisms including pathogens to aquire and spread resistance genes via plasmids and also inadequate use of antibiotics. As a direct consequence of this, infectious diseases have gotten more difficult to treat, and in certain cases, treatment has become completely ineffective. This has led to a rise in both morbidity and mortality. In spite of the success of contemporary antimicrobial therapy, during the course of the last twenty years, not a single category of antibiotics has been able to stop this threat. As a direct result of this, the search for innovative therapeutic alternatives that attack infectious organisms that are resistant to several drugs is ongoing worldwide.

Oxidation of free radicals in the body normally leads to several complications, hence causing potential diseases such as; Cancer, Alzheimer's disease, parkinson's disease, and accelerating aging (Lateef *et al.*, 2021). Creation of reactive oxygen species (ROS) through oxidative stress and cellular metabolism adds to the etiology, pathogenesis, and progression of a few diseases including; inflammations, cancer, and cardiovascular diseases (Borel *et al.*, 2022). These ROS can be inactivated by antioxidants and allow for protection from oxidative harm and accordingly viewed as essential therapy and preventive agents against disease development. Saponins, flavoniods, and flavones are secondary metabolites with antioxidant and antiradical properties widely distributed in plants (Ashutosh *et al.*, 2020). When free radicals are affected negatively, they result in many human diseases (Dalaram, 2018). The natural defense of the human organs against these free radicals is not always enough mainly due to the continuous exposure to free radicals from external sources in the modern world (Dong-Ping *et al.,* 2017). In this manner, screening of natural products for novel antioxidant agents is a need (Bee *et al.,* 2018).

Essential secondary metabolites present in plant are responsible for the various biological activities. Terpenoids are utilized in a wide variety of consumer goods, from perfumes and cosmetics to cleaning supplies and food and drink (Waltz, 2021). Saponins have the potential to be useful in the creation of cosmetics and pharmaceuticals due to their surfactant-like properties and their amphipathic nature (Maribelle, 2021). This property allows saponins to interact with components of cell membranes, such as cholesterol and phospholipids. Antimalarial, antiasthmatic, anticancer, cholinomimetic, vasodilatory, antiarrhythmic, analgesic, antibacterial, and antihyperglycemic are just some of the pharmacological properties exhibited by alkaloids (Ahmed, 2021). Various alkaloids also exhibit psychotropic and stimulating properties. Phenolic acids which consist of flavonoids (Hai-Yan *et al.*, 2020), tannins, e.t.c can exert antioxidant activity by scavenging hydroxyl radical, superoxide radical anion, several organic radicals, peroxyl radical, peroxynitrite and singlet oxygen, among others and they are important compounds to change cell signaling pathways. Cardiac glycosides are medicines for treating heart failure and certain irregular heartbeats. Derivatives of anthraquinones have been used since centuries for medical applications, for example, as laxatives and antimicrobial and

anti-inflammatory agents. Current therapeutic indications include constipation, arthritis, multiple sclerosis, and cancer.

This research was designed to evaluate the antimicrobial and antioxidant properties of *Cnestis ferruginea* leaves extract on bacterial and fungal isolates. The study aimed to determine their minimum inhibitory concentration (MIC), the phenolic and flavonoids content of the leaf extract, and the phytochemical properties of the leaf extract so as to offer informed recommendation on its use for disease management and to curb antibiotic resistance cases.

Fig 1. Picture of *C. ferruginea* in it's habitat.

Materials and Methods

Collection and preparation of plant material

The fresh leaves of *Cnestis ferruginea* were collected by dabbling process (hand-plucking), at Ugono in Abraka, Ethiope East, Delta State in February 2022 and were identified and authenticated by Dr. Ikpefan Emmanuel Oise of the Department of Pharmacognosy and Traditional Medicine, Faculty of Pharmacy, Delta State University, Abraka with a voucher number DSU-P078. Thereafter, the fresh leaves were air-dried under shade at room temperature because they contain volatile compounds.

The dried leaves were blended to coarse powder with the aid of a grinding machine. A total of 600 g of the powdered leaves was extracted with 70% methanol using cold maceration method. After three (3) days, the extract was filtered and the resultant filtrate was concentrated to dryness using rotary evaporator maintained at 40°C. The concentrated extract was weighed and stored in a refrigerator.

Preliminary phytochemical screening of the plant extract

The phytochemical screening of the plant extract obtained from C. ferruginea leaves

was carried out in accordance with conventional procedures (Ukwubile CA *et al*, 2020; Ukwubile *et al.,* 2019; Ukwubile *et al.,* 2017; Ikpefan and Ayinde,2013) in order to identify the presence of a variety of secondary metabolites.

Antimicrobial evaluations of *C. ferruginea* leaves extract

Test organisms used

Nutrient broth cultures of *Escherichia coli* JCM 20135, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 25783, *Candida albicans* ATCC 10231, *Bacillus subtilis* JCM 1465^{T}, and *Aspergillus spp* ATCC 1015 were prepared using standard procedures. These microorganisms were obtained from Department Of Pharmaceutical Microbiology, Delta State University, Abraka. Nutrient agar, Muella Hinton agar, and Sabouraud Dextrose agar were prepared.

Sensitivity test of leaves extract of *C. ferruginea*

The "Agar well diffusion" method as described by Mela *et al.,* (2020) was used with little modification. A 20mL quantity of the sterilized nutrient agar was poured into each petri-dish and allowed to set. Then using the swab sticks provided, the standard cell (bacteria) cultures were used to swab the surface of the nutrient agar. In the agar that had been set, many holes were drilled with a cork borer measuring 6 millimeters in diameter, and drops of the extract of *C. ferruginea* leaves in various concentration were added. Drops of ciprofloxacin and ketoconazole (positive control) and 70% methanol (negative control) were also added to their respective bored holes. The plates were allowed to stand for 30 minutes, incubated (37°C for 24 hours) and examined for zones of inhibition.

Minimum inhibitory concentration

The method of Jayachithra *et al.,* (2022) was used with little modification. The minimum inhibitory concentration (MIC), the least concentration of the fraction that inhibits the growth of a microorganism was determined using different concentration of the crude plant extract (100 – 3.125 mg/mL) through two-fold serial dilution. A 20mL quantity of the sterilized nutrient agar was used to dispense the 100mg/mL of the crude plant extract into a petri-dish. This was repeated for the other concentrations and allowed to solidify. Using a sterile wire loop, three (3) parallel streaks of the test cultures (bacteria and fungi) were made on the solidified nutrient agar and crude plant extract. Each

microorganism was labeled accordingly underneath the plates and were allowed to stand for some time at room temperature.

Determination of Total Phenolic and Total Flavonoids content

(i) Total Phenolic Content

The amount of phenol in the methanol extract of *C.ferruginea* leaves were determined by Folin - Ciocalteu reagent method with some modifications. A total of 1.5mL of 10% Folin - Ciocalteu reagent and 1mL of 2% solution of Na2CO3 were added to the 1mL of plant extract. The resulting content was kept at room temperature for 15minutes, and the absorbance of the content was measured at 765nm using a UV spectrophotometer. Garlic acid was used as the standard (1mg/mL) and the total phenolic content was estimated.

(ii) Total Flavonoids Content

Aluminium chloride colorimetric technique was used with some modifications to ascertain the flavonoids content. A total of 1mL of the plant extract, 3mL of methanol, 0.2mL of 10% aluminium chloride, 0.2mL of 1M potassium acetate, and 5.6mL of distilled water were mixed together. The resulting content was kept at room temperature for 30minutes, and the absorbance of the content was measured at 420nm using a UV spectrophotometer. Quercetin was used as the standard (1mg/mL) and the total flavonoids content was estimated.

Evaluation of antioxidant potentials of extract of *C. ferruginea* leaves

(i) 2,2 - Diphenyl -1- Picryl Hydrazyl (DPPH)

The method of Hcini *et al.,* (2021) was used with little modification. The DPPH solution was prepared in methanol and 0.5mL of the DPPH was subsequently added to 2mL of the plant extract at various concentrations (1000mg/mL, 500mg/mL, 250mg/mL, 125mg/mL, and 62.5mg/mL) and the contents were vortexed for 10 seconds and allowed to stand for 20 minutes at room temperature. Their absorbance were recorded at 517nm using UV Spectrophotometer. The result of their absorbance was compared with 70% methanol (control solution) and ascorbic acid (standard solution). These measurements were performed in triplicate and the mean percentage inhibition as well as the standard error of mean (SEM) were calculated using the following equation;

$$\text{Percentage Inhibition (\% INH)} = \frac{\text{Abs of control - Abs of test sample}}{\text{Abs of control}} \times 100$$

Where; Abs = Absorbance; INH = Inhibition

(ii) Ferric Reducing Antioxidant Power (FRAP)

The method of Dong-Ping *et al.,* (2017) was adopted with little modification. The method is based on the reduction of the Fe (III) - TPTZ complex to the ferrous form at low pH. The fresh working solution (FRAP reagent) was prepared by mixing 25mL of 0.3M acetate buffer, 2.5mL of TPTZ solution, and 2.5mL of FeCl3. 6H20 solution and then warmed (pre-heated) at 37°C before use. A properly diluted sample (0.1mL) was added to 4.0mL of FRAP reagent to form a mixture. The content was incubated in the dark for 10 minutes at 37°C and its absorbance was measured at 593nm against the blank that was prepared using distilled water. The results obtained from triplicate analyses were expressed as aqueous solution of ferrous sulfate (FeSO4. 7H20) and derived from the calibration curve of the standards (62.5 ~ 1000mg/mL). The mean percentage inhibitions were obtained with the same procedure.

Statistical Analysis

The data obtained were evaluated using GraphPad Prism 7.0. One-way analysis of variance (ANOVA) was used in data analysis and were represented as Mean $\pm$ Standard Error of Mean (SEM).

Results

Percentage yield

The 600 g of the powdered plant material of *C.ferruginea* yielded 72g of the extract which is equivalent to 12 % of the dried mass (Table 1)

Table 1: Result of weight and percentage yield of sample

Sample	Weight in gram	Percentage yield
Crude extract	72	12%

Results of phytochemical screening of extract of *C. ferruginea*

The phytochemical screening of the extract of *C. ferruginea* showed the presence of all metabolites tested for (Table 2)

Table 2: Phytochemicals detected in the methanol extract of *C. ferruginea* leaves

Phytochemicals	Observation
Alkaloids	+
Saponins	+
Terpenoids	+
Tannins	+
Cardiac glycosides	+
Anthraquinones	+
Flavonoids	+

(+) = Present, (-) = Absent

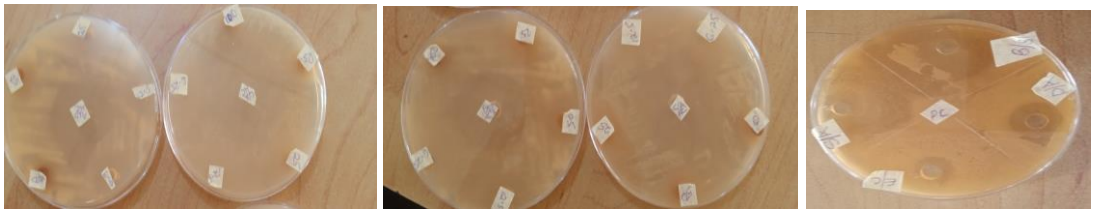
Results of the antimicrobial activity of extract of *C. ferruginea*

The results indicate that all the organisms were sensitive to the various extract concentrations used (100 – 6.25 mg/mL). Although lower zones of inhibition were shown in higher concentrations of the crude extract as indicated in the control drug for bacteria than the lower concentrations of the crude extract. Higher zone of inhibitions were indicated in the control drug for fungi than the crude extract (Table 3).

Table 3: Zones of inhibition of the methanol extract of *Cnestis ferruginea* leaves on selected organisms

Sample	Conc.	Mean zone of inhibition (mm) ± SEM					
	(mg/mL)	*E. coli*	*B. subtilis*	*S. aureus*	*P. aeruginosa*	*C. albican*	*Aspergillus*
Extract	100	9.00±0.50	8.5±1.50	9.5±0.50	12.0±0.50	11.5±0.50	5.5±0.5
	50	15.0±0.50	13.0±1.00	16.0±0.50	16.0±0.5	12.5±1.50	11.0±0.50
	25	17.0±0.50	16.5±0.50	18.0±0.5	14.0±0.50	12.5±1.50	13.0±1.0
	12.5	19.0±0.50	15±1.00	20.5±0.5	17.5±0.50	13.3±0.75	14.5±0.5
	6.25	22.0±0.5	17.0±2.0	20.0±1.00	19.0±0.5	13.0±1.0	14.0±0.5
Negative control		–	–	–	–	–	–
Positive control		9.0±0.50	14.0±0.50	11±0.50	11.5±0.50	31.5±2.50	30.5±1.50

Values are Mean ± SEM, n = 3



A B **C**

2. Some plates showing bacteria (A, B) and fungi (C) treated plates.

Results of the MIC

The result showed that *E. coli* and *S. aureus* recorded MIC at 12.5 mg/mL while *C. Albicans and A. spp* at 100mg/mL. The highest MIC values were recorded by *B. subtilis* and *P. aeruginosa* at 6.25mg/mL (Table 4)

Table 4: Showing the minimum inhibitory concentration of the methanol extract of *C. ferruginea* leaves

	Concentrations (mg/mL)					
Organisms	**100**	**50**	**25**	**12.5**	**6.25**	**3.125**
E. coli	_	_	_	_	+	+
B. subtilis	_	_	_	_	_	+
S. aureus	_	_	_	_	+	+
P. aeruginosa	_	_	_	_	_	+
C. albicans	_	+	+	+	+	+
Aspergillus	_	+	+	+	+	+

Results of the total phenolic and flavonoid contents of *C. ferruginea* leaves extract

The total phenolic and flavonoids content estimated in the crude extract of *C. ferruginea* leaves are 82.89 $\pm$ 0.06 mg/GAE/g and 57.09 $\pm$ 0.04 mg/QAE/g respectively (Table 5)

5: Total Phenolic and Total Flavonoids content in the crude extract of *Cnestis ferruginea* leaves

Extract	**Total Phenolic content (mg/GAE/g)**	**Total flavonoids content (mg/QAE/g)**
	82.89 ± 0.06	57.09 ± 0.04

Results for antioxidant activity

The absorbance of *C. ferruginea* leaves in the DPPH scavenging activity was determined to be 0.133 at 62.5 mg/mL, 0.116 at 125 mg/mL, 0.065 at 250 mg/mL, 0.052 at 500 mg/mL, and 0.031 at 1000 mg/mL. The mean % inhibition for DPPH scavenging activity increases with increasing concentrations (Table 6)

Table 6: DPPH scavenging activity of the extract of *Cnestis ferruginea* leaves

Concentration (mg/mL)	Mean Absorbance	Mean Inhibition (%)
1000	0.031	88.77 0.72
500	0.052	81.36 ± 0.41
250	0.065	76.58 ± 1.14
125	0.116	58.30 ± 0.31
62.5	0.133	52.21 ± 0.86

Values are Mean ± SEM, n = 3. **N.B:** Absorbance of the control = 0.279

The absorbance of *C. ferruginea* leaves in the FRAP assay was determined to be 0.0217 at 62.5 mg/ml, 0.0237 at 125 mg/ml, 0.0248 at 250 mg/ml, 0.0367 at 500 mg/ml, and 0.0479 at 1000 mg/mL. The mean % inhibition for FRAP decreases with increasing concentrations (Table 7)

Table 7: Ferric reducing antioxidant power of the extract of *Cnestis ferruginea* leaves

Concentration (mg/mL)	Mean Absorbance	Mean Inhibition (%)
1000	0.0479	86.00 ± 0.19
500	0.0367	89.27 ± 0.10
250	0.0248	92.73 ± 0.12
125	0.0237	93.07 ± 0.10
62.5	0.0217	93.65 ± 0.10

Values are Mean ± SEM, n = 3. **N.B:** Absorbance of the control = 0.342

Discussion

The results from this study confirm that the extract of *Cnestis ferruginea* leaves are sources of terpenoids, flavoniods, and tannins which represent excellent sources of antioxidant and antimicrobial drugs. This is in collaboration with the findings of Claudia

Cafarchia *et al.,* (2022) and Kumar *et al.,* (2019). Interestingly, the presence of alkaloid and anthraquinone derivatives in the leaves of *C. ferruginea* also demonstrate a good source of antimicrobial drugs as reported by Juliana *et al.,* (2016).

The antimicrobial property of the crude extract of *C. ferruginea* leaves on the different test organisms were shown. At various concentrations, the different test organisms were susceptible to the extract of *C. ferruginea* leaves. The zones of inhibition on the agar plates indicated that the plant extract possesses antimicrobial property on the different test organisms which include; gram -ve bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*), gram +ve bacteria (*Staphylococcus aureus* and *Bacillus subtilis*) and fungi organisms (*Candida albicans* and *Aspergillus spp*). Although lower zone of inhibitions were shown in higher concentrations of the crude extract as indicated in the control drug for bacteria than the lower concentrations of the crude extract. This was demonstrated by Patterson *et al.,* (2015) that higher methanol concentrations increasingly inhibit growth of microorganisms as shown in the negative control with no zone of inhibitions. Higher zone of inhibitions were indicated in the control drug for fungi than the crude extract. This demonstrates that the extract of *Cnestis ferruginea* leaves have low antifungal activities because of it's low flavoniod content as showed in Table 5. This is in collaboration with Mohammed *et al.,* (2020).

The results obtained from antioxidant studies show antioxidant properties in the crude extract of *C. ferruginea* leaves. Although, the total phenolic content was higher than total flavonoids content, because phenolics are the largest group of phytochemicals that account for most antioxidant properties in plants or plant products (Petya *et al.,* 2021). When compared to the standard (Ascorbic acid), the results of the DPPH radical scavenging activity demonstrated a commensurate increase in antioxidant property with increasing concentration. This was demonstrated by Sushant Aryal *et al.,* (2019) that the higher the contents of total phenol or total flavoniod, the stronger the antioxidant capacity.

The absorbance of *C. ferruginea* leaves in the FRAP assay was determined to be 0.0217 at 62.5 mg/mL, 0.0237 at 125 mg/mL, 0.0248 at 250 mg/mL, 0.0367 at 500 mg/mL, and 0.0479 at 1000 mg/mL. At a concentration of 1000 mg/mL, ascorbic acid exhibited an absorbance of 0.342 (the reference value). By interacting with a ferric tripyridyltriazine

(Fe3+-TPTZ) complex to form a colored ferrous tripyridyltriazine (Fe2+-TPTZ), the FRAP assay quantifies an antioxidant's reducing capacity (Iris et al., 2018). In most cases, substances having reducing characteristics are those that operate by donating a hydrogen atom, thus terminating the chain reaction of free radicals (Kumar et al., 2018). Due to the formation of Fe2+-TPTZ complex, the absorbance of C. ferruginea leaves extract obviously increased with increasing concentrations. The absorbance at each concentration was compared to that of the reference solution, and the results showed that Cnestis ferruginea leaves had an antioxidant effect and may remove free radicals from rapidly dividing or meristematic cells (Michael et al., 2018).

Conclusion

The extract of *C. ferruginea* leaves showed significant antimicrobial property against the test organisms used. The idea that antimicrobial chemicals may be extracted from plants, which will lead to the creation of phytomedicines that are effective against bacteria, has the potential to be very profitable. Therefore, isolation and purification of phytochemicals from these plants may yield significant novel antimicrobials, as plant based antimicrobials have enormous therapeutic potential and they can serve the purpose without any adverse effects that are often associated with synthetic compounds. The results from the study confirm that the extract from *C. ferruginea* leaves displayed relatively high antioxidant properties that correlated with a high phenolic content. These observations may qualify this plant as "genuine" adaptogen and may help account for some of its claimed medicinal properties.

Conflict of Interest

The authors declare no conflict of Interest.

Author's Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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