

Original Article

**In vitro Insights into Prospect of *Cnestis ferruginea* Pulp Extract as an Antimicrobial Agent**

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**Abstract:** Sensitivity study of *Cnestis ferruginea* pulp extract with respect to the reference commercial antibiotics was tested against four bacteria (*Staphylococcus aureus*, *Bacillus subtilis*, *Proteus mirabilis*, and *Klebsiella pneumoniae*) and three fungi (*Trichoderma rubrum*, *Aspergillus niger*, and *Candida albicans*) strains isolated in man with a view to elucidating the inhibitory potency of the plant pulp' extract using an agar well diffusion assay. The investigation discovered that the extract had 71% inhibitory activities against the microbial isolates tested either in sterilized or non-sterilized form and in the two different concentrations examined, which compared favourably with reference antibiotics used. The optimal performance of the pulp extract against the test organisms could be due to the presence of the metabolites such as alkaloids, flavonoids, steroids, saponins, and polyphenols found in ample form in the extract. This study has revealed the possible utilization of *Cnestis ferruginea* in the treatment of wound, urinary infections and in the management of oral-related infections.

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**INTRODUCTION**

Herbal medicines have been widely used and form an integral part of primary health care of many countries (Nair *et al.*, 2005; Jaafreh *et al.*, 2019). Nearly all culture and civilizations from ancient times to the present day have depended fully or partially on herbal medicines because of their effectiveness, affordability, low toxicity, and acceptability (Akharaiyi and Boboye, 2010). The health benefits of medicinal plants are attributed in parts, to their unique phytochemical compositions (Okwe and Iroabuchi, 2009; Pulipati *et al.*, 2016; Oyeleke *et al.*, 2021) and these secondary metabolites are known to confer protective properties on plants and by and large, have made significant contribution in maintaining human health (Oluwafemi and Debiri, 2008; Igwe, 2007; Jaafreh *et al.*, 2019) and protect humans against diseases (Ohaeri, 2005; Oyeleke *et al.*, 2021).



Fig. 1 Habit photograph of *Cnestis ferruginea*.

Among these plants of therapeutic and health benefits is *Cnestis ferruginea* (Fig. 1). It is a perennial shrub found mainly in the savannah region of tropical West Africa. The plant is about 3.0-3.6 m high with densely, rusty brown, pubescent branches, indeciduous leaves with more or less alternate or sometimes opposite, ovate to narrowly oblong leaflets and orange-red fruits. The ovoid follicles are 1-5 in fruit, often united at base and contains one seed each (Garon *et al.*, 2007; Atere and Ajao, 2009).

*C. ferruginea* has been acclaimed in herbal medicine and some literatures to have diverse therapeutic uses such as management of bronchitis, tuberculosis, snakebite, dysentery, syphilis, and gonorrhoea (Yakubu and Olaide, 2012; Ahmed, 2017). The plants have been reported to have anti-microbial (Ahmed, 2017; Lewis and Elvin-Lewis, 2003; Akharaiyi *et al.*, 2012a), antistress and laxative (Yakubue *et al.*, 2011; Ishola and Ashorobi, 2007), antioxidant (Oke and Hamburger, 2002; Akharaiyi *et al.*, 2012a), analgesics and anti-inflammatory (Ishola *et al.*, 2011; Ahmed, 2017), aphrodisiac (Yakubu and Olaide, 2012; Ahmed, 2017), hepatoprotective (Akharaiyi *et al.*, 2012b; Ahmed, 2017) activities. Cytotoxic (Atere and Ajao, 2009), hypoglycemic and acute toxicity (Ahmed, 2017; Adisa *et al.*, 2010) and hematological (Olayemi *et al.*, 2013) effects of the plant' extract had also been documented. The fruit pulp is used as a tonic, and to treat whooping-cough, sore throat, and tuberculosis, sore throat. The pulp juice is used as an eye drop for conjunctivitis, healing wounds, mouthwash, and to clean teeth (Ahmed, 2017). Studies have shown that the plant's extract was able to perform those wonders because it contained alkaloids, flavonoids, saponins, anthraquinones and tannins among other bioactive metabolites (Yakubu *et al.*, 2011; Adisa *et al.*, 2010; Ahmed, 2017).

The indiscriminate use of synthetic antibiotics in recent years has led to emergence of drug resistant human pathogenic microorganisms (Jaafreh *et al.*, 2019). Again, the antibiotics

commonly used are sometimes associated with adverse effect on the host, which include immune-suppression, hypersensitivity, and allergic reaction (Khan and Shah, 2013). Therefore, research for development of new antimicrobial agents from plant origins is a time honour because natural products are known to be therapeutic agents without any side effect that are associated with synthetic antimicrobials, non-narcotic, and mostly available and affordable health care for the poor (Jaafreh et al., 2019). It is clear from published research works that the raw juice of *C. ferruginea* is effective against many common pathogenic bacteria and against those that have become resistant to antibiotics and even toxin production by some pathogenic strains can be prevented by the extract (Ohaeri, 2005). The vitamin C content and the anti-oxidants present in *C. ferruginea* work together to prevent the oxidation of cholesterol. This prevents the build-up of plaques (Ohaeri, 2005). Despite the abundance of this plant in virtually all the regions of Nigeria, there appears to be dearth research conducted on the effectiveness of its pulp extract against enteric pathogenic organisms common in the country. The study was therefore conducted to evaluate the susceptibility of some notable enteric pathogenic micro-organisms known to possess multiple drug resistant to the commercial antibiotics, to the juice extracted from *C. ferruginea* pulp. Results of studies on complementary and alternative medicine practices suggest that introduction of plant extracts from *C. ferruginea* microbial therapy may significantly decrease this emerging burden of drug resistance. The findings from this study would therefore serve as a pointer to the development of therapeutic herbal drugs from this plant of hidden health benefits.

## MATERIALS AND METHODS

**Plant collection, identification and extract preparation:** Healthy-looking pulps of the plant (*C. ferruginea*) used in this study were collected from Akerekete forest in Otan-Ayegbaju, Nigeria and identified at the Department of Pharmacognosy, Igbinedion University, Okada, Nigeria and authenticated at Forest Reserve Institute of Nigeria. They were rinsed with distilled water and ripped open to remove the seeds. The juice was aseptically extracted and filtered. One part of the filtrate was sterilized and labelled as 'S', the other part, not sterilized was labelled as 'NS'. Each fraction was divided into two portions; one part each was re-constituted with dimethyl sulphoxide (DMSO) to 50%. The four fractions were poured separately into different sterile bottles and kept at 4 °C in a refrigerator for further analyses.

**Phytochemical analysis:** The extract was tested qualitatively for the presence of phytochemicals such as alkaloids, saponins, tannins, cardiac glycosides, flavonoids, polyphenols, terpenoids, phlobotannins, anthraquinones, and steroids using standard methods (Harborne, 1998; Trease and Evans, 2006). Those that tested positive are reported below:

**Alkaloids test:** Five grams of the plant extract was stirred with 5 mL of 1% aqueous hydrochloric acid on a steam bath and filtered. The filtrate was divided into three portions and were subjected to the following tests.

The first portion was treated with few drops of Dragendoff's reagent. A blue-black turbid solution obtained served as a preliminary evidence of alkaloids.

To the second portion, 2 drops of Meyer's reagent were added. A creamy white precipitate indicated the presence of alkaloids.

The last portion was treated with 2 drops of Wagner's reagent. A reddish-brown precipitate indicated the presence of alkaloids.

**Saponins test:** Five grams of the extract was shaken with distilled water in a test tube. Frothing which persisted on warming was taken as preliminary evidence for the presence of saponins.

**Tannins test:** Five grams of the extract was stirred with 100 mL distilled water and filtered. Ferric chloride reagent was added to

the filtrate. A blue-black precipitate obtained determined the presence of tannins.

**Flavonoids test:** Approximately 5 mL of ammonia solution was added to aqueous filtrate of the test sample followed by addition of concentrated H<sub>2</sub>SO<sub>4</sub>. A yellow coloration observed confirmed the presence of flavonoids.

**Cardiac glycosides (Keller-killiani test):** Five grams of the extract was dissolved in 2 mL glacial acetic acid containing a drop of ferric chloride solution. This was underplayed with 1 mL concentrated H<sub>2</sub>SO<sub>4</sub>. Formation of a brown ring at the interface indicated glycosides.

**Terpenoids (Salkowski test):** Approximately 5 mL of the extract was mixed with 2 mL of chloroform, and concentrated H<sub>2</sub>SO<sub>4</sub> (3 mL) was carefully added. A reddish brown coloration at the interface confirmed positive result for the presence of terpenoids.

**Steroids:** About 5 mL of the extract was mixed with 2 mL of conc. H<sub>2</sub>SO<sub>4</sub> carefully so that the acid form a lower layer. A reddish-brown colour at the interphase indicated the presence of steroidal ring.

**Phenols:** About 2 mL aliquot of the filtrate was placed in a test tube and diluted with distilled water. A greenish colour indicated the presence of phenols.

**Test Organisms:** The test organisms, four clinical bacterial strains (*S. aureus*, *B. subtilis*, *P. mirabilis*, and *K. pneumonia*) and three fungi strains (*T. rubrum*, *A. species*, and *C. albicans*) isolated from human urine, faeces, and septic wounds were collected from University of Benin Teaching Hospital, Benin City, Nigeria.

**Antimicrobial studies:** Agar cup diffusion method described by Hojjati and Beirami-Serizkani (2020) was employed for antimicrobial screening. An overnight culture of each microbial isolate was standardized to contain approximately 10<sup>6</sup> cfu mL<sup>-1</sup>. Exactly 1 mL each of the microbial suspension was dispensed aseptically into plated Mueller-Hinton agar. The cultured medium was allowed to stand for 1 hr for the isolates to be properly established in the seeded medium. Thereafter, a sterile cork borer (5 mm) was used to make equal wells in the seeded plates. Each well was aseptically filled with the respective pulp's extracts (S, NS, S50, NS50) while avoiding splashes and overfilling. Sterile 5% aqueous DMSO was used as negative control while Nystatin and Gentamycin (10 mg mL<sup>-1</sup>) standard antibiotics were used as positive control for fungi and bacteria, respectively. The plates were incubated at 37 °C for 24 hr (bacteria) and 25 °C for 48 hr (fungi) and the zone of inhibition, which is an index of the degree of sensitivity, was measured. The procedure was repeated in triplicates.

**Acute toxicity test:** Exposure of tadpoles to the extract solutions was conducted similar to the protocol outline by Alhou et al. (2016). Briefly, the tadpoles were collected from a pool of water in a ditch dug around the Department of Pharmaceutical Science, Igbinedion University, Okada, Nigeria. They were about 20–30 days old with 16–30 mm in length. Four different media with the following concentrations: 10, 25, 50, 100% were prepared from the fruit extract and ten (10) tadpoles were selected and introduced into each medium. Sterile water was used for the preparation of the media and also served as a control. The mortality of the tadpole was monitored each day for the next 96 hr of exposure to the extract solutions.

## Statistical analysis

All determinations were performed in triplicate, and data were presented as means ± standard deviations using SPSS statistics software (IBM, version 23.0). One-way analysis of variance (ANOVA), followed by Duncan's multiple range test were carried out to analyze data, and the difference was considered significant at P = 0.05.

**Table 1:** Result of qualitative phytochemicals of the extract of *C. Ferruginea*

Phytochemical Abundance	Tannins	Phenols	Steroids	Flavonoids	Alkaloids	Glycosides	Saponins	Terpenoids
	+	++	++	+++	+++	+	++	+

Legend: + = present in trace; ++ = present moderately; +++ = present fully.

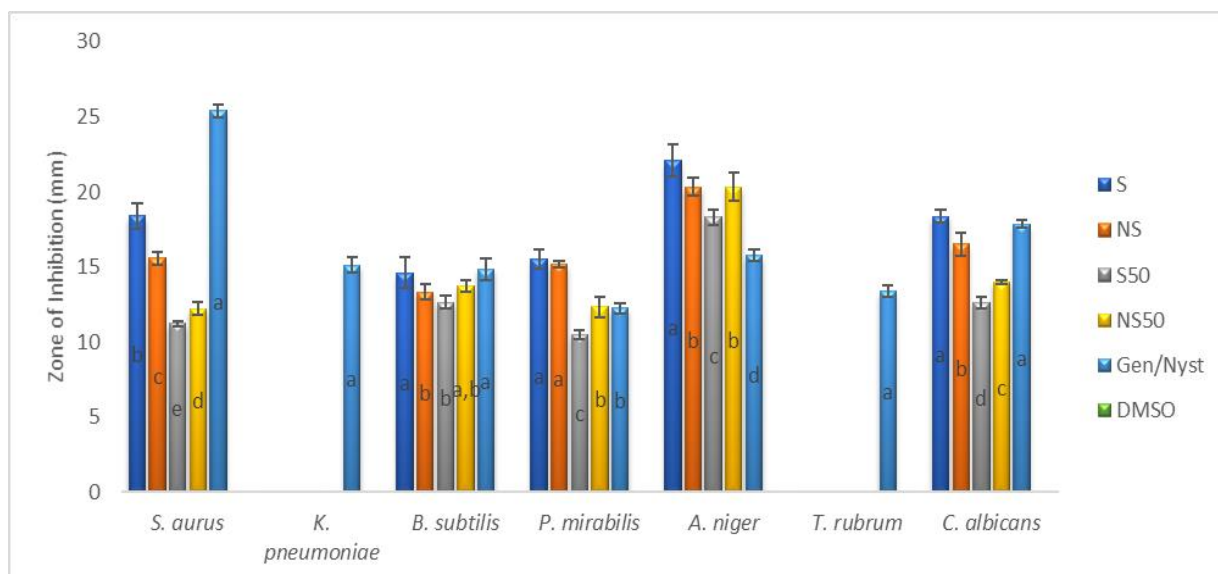


Fig.2: Mean inhibitory zone (mm) as expressed by pulp extract of *C. ferruginea* compared with reference antibiotics.

## RESULTS AND DISCUSSION

### Organoleptic properties

The organoleptic properties of the *Cnestis ferruginea* extract were cloudy white in colour, astringent in taste and mild in odour.

### Preliminary phytochemical constituents

Qualitative phytoconstituents of the plant's pulp extract were present in varied proportions (Table 1). This study revealed that the extract contained saponins, tannins, alkaloids, and flavonoids in substantial quantity. These bioactive components might have conferred the anti-microbial property (Singh et al., 2012; Pulipati et al., 2016; Jaafreh et al., 2019; Oyeleke et al., 2021) to *C. ferruginea* pulp. Isolated flavonoids in plant extracts have been confirmed to possess antifungal and antibacterial activities (Manimozhi et al., 2012; Oyeleke et al., 2021). Research has also established that highly oxidized phenols present in the pulp are inhibitory to micro-organisms (Sivakumar and Sunmathi, 2016; Jaafreh et al., 2019). The major functions of other bioactive compounds in the plant's pulp had been discussed in our previous study (Ajala et al., 2013; Olagunju et al., 2016). The development of phytomedicine of antimicrobial importance against man and animals' harmful microbes are now appearing rewarding. Plant-based antimicrobials have enormous therapeutic potentials with lesser side effects that are often associated with synthetic antimicrobial agents (Akharaiyi and Boboye, 2010; Khan and Shah, 2013; Jaafreh et al., 2019). Majority of the phytochemical compounds identified in the pulp extract of this plant had been reported to be of therapeutic importance (Akharaiyi et al., 2012; Ahmed, 2017).

### Antimicrobial efficacies

The antibacterial activities of different media (S, NS, S50, and NS50) were compared with the standard antibiotics, Gentamycin. Apart from the positive control, the sterilized form of the pure extract (S) exhibited the highest activity against *S. aureus* while 'NS50', the least. There was no statistical different ( $p = 0.05$ ) when the different media were tested against *B. subtilis* and the reference antibiotic. With exception of *P. mirabilis*, the extract had a significant activity (at  $p = 0.05$ ) against the test organisms in sterilized form compared to unsterilized medium. When the sterilized and unsterilized extracts were diluted 50 per cent, it was observed that there was a noticeable decrease in the activity against the test organisms. This is an indication that the extract performed better in undiluted form.

Data are means of triplicate determinations; S, NS, S50 & NS50 are sterilized 100% extract, unsterilized 100% extract, sterilized 50% extract and unsterilized 50% extract, respectively; Gentamycin/Nystatin for bacteria and fungi positive control, respectively and DMSO for negative control.

The antifungal activities of the different extracts were also compared with a standard anti-fungal agent, Nystatin. The highest activity was observed against *A. niger* with 'S', followed by 'NS' and 'NS50'. There existed statistical different (at  $p = 0.05$ ) between the different media and the reference antifungal agent used. The result indicated that the plant pulp extract was more potent than Nystatin against *A. niger*. The extract showed highest activity against *A. niger* among the microorganisms tested with the widest zone of inhibition in each case, either in sterilized, unsterilized or diluted form. This suggests that *C. ferruginea* pulp extract would be highly effective in controlling ailments caused by this organism. Also, either in sterilized, unsterilized or diluted form, the extract was more potent against *A. niger* than the reference antibiotic.

Further sensitivity tests showed that the extract from *Cnestis ferruginea* fruit had inhibitory potency against the tested enteric microorganisms. The fruit extract has a significant antibacterial activity against *S. aureus*, an important human pathogen with known history of multiple drug resistance (Manimozhi et al., 2012). The extract also showed potential antibacterial activity against *B. subtilis*, which could be attributed to the presence of flavonoids (Manimozhi et al., 2012) in sufficient quantity (Table 1). The effectiveness of the fruit extract with respect to the reference antibiotic against bacteria such as *S. aureus* and *B. subtilis* signifies that the extract could be used for healing wounds (Sunil et al., 2008). Also, the inhibitory effect of the extract against *P. mirabilis* is an indication that the plant's pulp can be used in the treatment of urinary tract infection associated with this organism (Ogundare and Oladejo, 2014).

However, *K. pneumoniae* was not susceptible to the extract compared to Gentamycin, a well-known broad spectrum antibacterial agent. This organism and similar kinds of Gram-negative bacteria are known for their multiple drug resistance towards antibiotics. This is attributed to their morphological constitution in which such bacteria have thicker cell wall made up of phospholipid membrane; this makes it impermeable for antimicrobial agents (Sivakumar and Sunmathi, 2016). Generally, the inhibition zone of the *C. ferruginea* pulp extract against selected bacteria was significantly narrower than that of Gentamycin ( $p = 0.05$ ), except against *P. mirabilis*. The antibiotic sensitivity test of Gentamycin against both the Gram-positive (*S. aureus* and *B. subtilis*) and Gram-negative (*K. pneumoniae* and *P. mirabilis*) bacteria proved its broad spectrum. Several similar antibacterial activities of plants' extracts had been documented (Ogundare and Oladejo, 2014; Manimozhi et al., 2012; Sunil et al., 2008; Singh et al., 2012; Khan and Shah, 2013; Pulipati et al., 2016; Sivakumar and Sunmathi, 2016).

The pulp extract also showed antifungal activity against *A. niger* and *C. albicans*; hence, could be used in the management of human infection caused by these organisms. However, *T. rubrum* was resistant to the extract. The inability of the pulp extract against this organism might be due to the lack of specific active component in the plant extract in which *T. rubrum* is susceptible to. The antifungal activity of the extract was similar to *Jatropha multifida* latex studied by Olagunju et al. (2016) and leaf extract of *Alternanthera sessilis* and *Alternanthera philoxeroides* by Sivakumar and Sunmathi (2016). The pulp extract had an antifungal efficacy against *C. albicans* which was significantly similar to that of Nystatin ( $p = 0.05$ ). This property demonstrated its usefulness as an alternative medicine to the broad-spectrum reference antibiotic. Although the plant extract and the reference antibiotic had an inhibition against *A. niger*, its inhibitory efficiency either in sterilized, unsterilized or diluted form demonstrated its utilization in preference to Nystatin.

#### Acute toxicity study

The acute lethal toxicity test of *C. ferruginea* pulp extract against tadpoles were measured and expressed as per cent of mortality. As shown in Table 2, the mortality rate showed a dose-dependent effect for the tadpoles and increased as the concentration of the extract increased in the entire tadpole media. This trend was similar to a previous report (Alhou et al., 2016). The tadpoles showed a sign of distress for some hours after exposure to the 10% extract solution but became active later in the first day; indicating that the extract solution had lost

its potency. This could have been the reason why no death was recorded at this concentration for the entire period of the test. This was similar to the control treatment. In the first day of tadpoles' exposure to the different extract concentrations, no death was also recorded except with the crude extract where two tadpoles death were recorded. Generally, tadpole' deaths were recorded at concentrations equal to or greater than 25%. The highest mortality was recorded in 100% crude extract within the 48 hr of exposure. An increase in the exposure time beyond 72 hr caused a total cidal effect on the organism.

#### CONCLUSION

The pulp extract of this medicinal plant demonstrated inhibitory activity against three bacterial isolates (*S. aureus*, *B. subtilis* and *P. mirabilis*) as verified by the *in vitro* experiments. This is an indication that *C. ferruginea* could be possibly used as an alternative herbal medicine for the treatment of wound and urinary infections. The further inhibitory efficacy of the extract against tested fungi (*C. albicans* and *A. niger*) signifies that it could be used in the management of oral-related infections. The plant pulp extract could therefore be incorporated in toothpaste formulation to enhance its germicidal potencies. Structural elucidation of the active components of this plant pulp of numerous and hidden health benefits may pave the way for the discovery of candidate templates for eventual drug design and formulation.

**Table 2:** Mortality rate of tadpoles exposed to the varied concentrations of *C. ferruginea* fruit extract between 24 and 96 hr.

Concentration (%)	Exposure Time (hr)				Total Mortality	Total Survival	% Mortality
	24	48	72	96			
10	0	0	0	0	0	10	0
25	0	0	1	2	3	7	30
50	0	1	2	2	5	5	50
100	2	5	3	--	10	0	100
Control	0	0	0	0	0	10	0

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