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# Screening of endophytic fungal metabolites from *Cola nitida* leaves for antimicrobial activities against clinical isolates of *Pseudomonas aeruginosa*

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## Abstract

Endophytic fungi of selected Nigerian plants are important sources of bioactive products with enormous potentials for the discovery of new drug molecules for drug development. *Pseudomonas aeruginosa* is one of the major causes of healthcare-associated bacterial infections, leading to increased mortality and morbidity. In this study, isolated endophytic fungi from *Cola nitida* were screened for anti-pseudomonas properties. Endophytic fungi associated with healthy leaves of *C. nitida* were isolated using standard methods. Fungi were identified through their morphological, cultural and microscopic characteristics. The fungi were subjected to solid-state fermentation and secondary metabolites extracted using ethyl acetate and concentrated under vacuum. The crude extracts were screened for antimicrobial activity against clinical and laboratory strains of *Pseudomonas aeruginosa* using the agar diffusion method. The bioactive components of the fungal extracts were identified using High-Performance Liquid Chromatography-Diode Array Detector (HPLC-DAD) analysis. Three endophytic fungi; *Acremonium* sp., *Aspergillus* sp. and *Trichophyton* sp. were isolated. At 1 mg/ml, extracts of the three fungi displayed antipseudomonal activity with inhibition zone diameter ranging from 6 - 4 mm. HPLC-DAD analysis revealed the presence of compounds, such as 4-hydroxyphenyl acetate. indole-3-acetic acid, and protocatechuic acid among others in the fungal extracts. The findings in this study reveal that endophytic fungi associated with *C. nitida* possess promising antipseudomonal properties. This finding can open new doors for the discovery of new agents against *P. aeruginosa.*

**Keywords:** Endophytic fungi, *Cola nitida*, antimicrobial activity, secondary metabolites, *Pseudomonas aeruginosa*

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## Introduction

Endophytes refer to microorganisms that grow intercellularly and asymptomatically within living tissues establishing a mutual relationship with the host plant (1). Currently, endophytes are viewed as an outstanding source of bioactive natural products, because many of them are occupying millions of unique biological niches growing in so many unusual environments.

The mortality due to drug-resistant strains of *Pseudomonas aeruginosa* infections is increasing. It has become increasingly clear that resistance development in *P. aeruginosa* is multifactorial, with mutations in genes encoding porins, efflux pumps, penicillin-binding proteins, and chromosomal $\beta$-lactamase, all contributing to resistance to $\beta$-lactams, carbapenems, aminoglycosides, fluoroquinolones and sulphonamides (2-5). Due to the emergence of MDR pathogens, it is of ultimate importance to develop new antimicrobial agents that will inhibit or completely kill this microorganism.

Almost all plant species habour one or more endophytic organisms (6). *Cola nitida* (Vent.) Schott & Endl. plant is popular in African traditional medicine and belongs to the family *Sterculiaceae* (7). They are reputed to exhibit several potent pharmacological activities such as antioxidant, antibacterial, antifungal, antiviral, and anti-inflammatory properties (8, 9).

The resistance exhibited by *P. aeruginosa* may be as a result of excessive antibiotic administration, leading to the accumulation of antibiotic resistance, cross-resistance between antibiotics and the appearance of multidrug-resistant *P. aeruginosa*. Presently, an intensive search is ongoing for newer, cheaper and more effective pharmaceutical products to deal with health disorders, and scientists believe that endophytic fungi possess novel compounds that are active pharmaceutical substances. The aim of this study, therefore, is to isolate endophytic fungi from *C. nitida,* extract their secondary metabolites and screen them for antipseudomonal activity.

## Materials and Method

### Isolation and purification of endophytic fungi

Fresh leaf samples of *Cola nitida* were collected from mature healthy plants from Nsukka, in Enugu State located in South-Eastern Nigeria. The plant leaves were washed thoroughly in running tap water and then cut into small fragments (about 1 $cm^2$). The leaf fragments were surface-sterilized by immersion in 2% sodium hypochlorite solution for 2 min, 70% ethanol for nearly 2 min, before a final rinse in sterile water for 5 min. The leaf fragments were transferred into malt extract agar (MEA) plates supplemented with chloramphenicol. The Petri plates were then incubated at a temperature of 27°C and fungal growths from the leaf fragments were monitored. The efficacy of surface sterilization was tested by pressing the sterilized segments on to the surface of the MEA medium and removed immediately. The absence of growth of any fungi on the medium after incubation confirmed that the surface sterilization procedure was effective (10). Hyphal tips from several distinct colonies emerging from the leaf segments were sub-cultured on fresh MEA plates to obtain pure colonies.

### Identification of fungi isolates

For the characterization of the morphology of fungal isolates, slide preparations from cultures were stained with lactophenol cotton blue reagent and examined with a bright-field and phase-contrast microscope. Morphological identification of the fungal isolates was according to the standard taxonomic key which includes colony diameter, texture, colour, margin character and the dimensions, colony reverse and microscopic characteristics including conidiophore, the structure of hypha and conidia (11).

### Fermentation and extraction of secondary metabolites

Solid-state fermentation was carried out as previously described by Okoye *et al.* (12) in 1000 ml Erlenmeyer flasks containing 100 g of rice media (200 ml of water was added to the rice and then autoclaved at 121°C at 15 psi for 30min). The flasks were inoculated with 3 mm diameter agar blocks containing pure fungi endophyte and incubated at 28°C for 21 days. The culture media and the mycelia were extracted in ethyl acetate and separated by filtration. The organic phase was vacuum-concentrated at 40°C under reduced pressure using a rotary vacuum evaporator to obtain the extracts.

### Antipseudomonal Assay

#### *Test organisms*

Various clinical isolates of *P. aeruginosa* were obtained from orthopedic wound infection, urine, sputum, and a vaginal swab. The samples for isolation were obtained from the National Orthopedic Hospital in Enugu, Nigeria. An isolate obtained from the laboratory stock culture was also used for the study. The identities of the organisms were confirmed at the Pharmaceutical Microbiology Laboratory of Nnamdi Azikiwe University, Agulu, using standard morphological and biochemical characteristics of the organism.

#### *Bioassay procedure*

Preliminary antimicrobial screening of the endophytic fungal extracts for antipseudomonal activity was carried out using the agar well diffusion assay method as described by Onyegbule *et al.* (13). Working concentrations (1 mg/mL) of the fungal extracts were prepared by dissolving the extracts in dimethyl sulphoxide (DMSO 100% v/v). Standardized broth cultures of test bacterial isolates (*P. aeruginosa*) were spread aseptically onto the surface of Mueller Hinton Agar (MHA) using sterile cotton swabs. All culture plates were allowed to dry for about 5 min and agar wells were made by using a sterile cork-borer (6 mm in diameter). These wells were respectively filled with 20 µL of the fungal extracts and controls. The plates were then kept at room temperature for 1 h to allow the agents to diffuse into the agar medium and incubated accordingly. Gentamicin (10 µg/mL) was used as the positive control, while DMSO (100% v/v) was used as the negative control. The MHA plates were then incubated at 37°C for 24 h. The inhibition zones diameters (IZDs) were measured and the size of the well (6 mm) was deducted from the values obtained to get the actual IZDs. This was conducted in triplicate and the mean IZDs were calculated and recorded.

### High-Performance Liquid Chromatography (HPLC) analysis

HPLC analysis was carried on the fungal extracts as described by Eze *et al.* (14). A Dionex P580 HPLC system coupled to a photodiode array detector (UVD340S, Dionex Softron GmbH, Germering, Germany) was used in the analysis. The separation column (125 × 4 mm; length × internal diameter) was prefilled with Eurospher-10 C18 (Knauer, Germany) and a linear gradient of nanopore water (adjusted to pH 2 by addition of formic acid) and methanol was used as eluent. A weight of 2 mg of each fungal extract was reconstituted with 2 mL of HPLC grade methanol, and the mixture sonicated for 10 min, and thereafter centrifuged at 3000 rpm for 5 min. A volume of 100 µL of the dissolved sample was then transferred to a vial containing 500 µL of HPLC grade methanol, and the vial was put in the HPLC machine for analysis. The detection was at 235 nm. The absorption peaks of the fungal extracts were analyzed by comparing it with those in the HPLC-UV/Vis database.

### Data processing and analysis

Data were expressed as mean ± standard deviation (SD) for three parallel experiments. The collection of information, anal-


**Figure 1.** Macroscopic morphologies of the pure endophytic isolates.
(Cn1 - *Acremonium* sp., Cn2 - *Aspergillus* sp., Cn3 - *Trichophyton* sp.)

ysis of data and graph was made using Microsoft Excels 2016 software. Descriptive statistics were performed using SPSS version 20.

## Results

A total of three (3) endophytic fungi were isolated from leaf segments of the *C. nitida* plant and labeled Cn 1-3. In **Fig. 1**, the three isolates exhibited different characteristic colonies and microscopic morphology on MEA. All isolates were identified based on their morphological and microscopical characters as belonging to different genera, namely Cn1 (*Acremonium* sp.), Cn2 (*Aspergillus* sp.) and Cn3 (*Trichophyton* sp.) (**Table 1**).

**Identification of principal secondary metabolites using HPLC**

A list of the detected compounds contained in the individual fungal extracts and their previously reported biological activities is shown in **Table 2**. From the result obtained, four (4) known compounds were identified namely Protocatechuic acid, Indole-3-acetic acid, P-hydroxybenzoic acid, and 4-hydroxyphenyl acetate acid.

The chromatogram and structures of Cn3 fungal extracts are presented in **Fig. 2a and b**. It reveals the peaks and structures for protocatechuic acid and P-hydroxybenzoic acid.

**Antipseudomonal assay**

At 1 mg/ml, extracts of the three fungi displayed varying antipseudomonal activity against the different *P. aeruginosa* clinical isolates, with inhibition zone diameter ranging from 6 - 3 mm (**Fig. 3**). Laboratory isolate (*Ps.a* A) and urine isolates (*Ps.a* C) were susceptible to 100% of the fungi extracts understudy, at 1 mg/ml concentration. The extract of Cn1 exhibited the best antipseudomonal activity against 80% of the tested *P. aerugino-*

**Table 1.** Cultural morphology and microscopic features of the endophytic fungi isolates from *C. nitida*

| Isolates | Form | Elevation | Margin | Colour | Reverse | Growth Rate | Texture | Hyphae | Spores | Diameter (mm) | Suspected Organism |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Cn1 | Circular | Flat | Curled | Orange | Light orange | Slow/ moderate | Slimy and moist | Septed | Micro conidia | 75 | *Acremonium* (15) |
| Cn2 | Circular | Raised | lobate | White | Colourless with stripes | Moderate | Thick cottony and Dry | Septed | Micro conidia | 88 | *Aspergillus* II (16) |
| Cn3 | Circular | Nmbonate/ Flat | Entire | White | Absent or pale brown-ish-yellow with age | Moderate | Cottony Smooth | Septed | Micro conidia | 60 | *Trichophyton* (17) |

**Table 2.** The antimicrobial activities of compounds detected in the fungal extracts

| Fungi Extract | Identified Compound | Reported Biological Activities |
|---|---|---|
| Cn1 | 4-hydroxyphenyl acetic acid | Antimicrobial (18, 19) |
| | Indole-3-acetic acid | Antimicrobial (20, 21) |
| Cn2 | P-hydroxybenzoic acid | Antimicrobial (22, 23) |
| Cn3 | Protocatechuic acid | Antimicrobial (23-26) |
| | P-hydroxybenzoic acid | Antimicrobial (22, 23) |

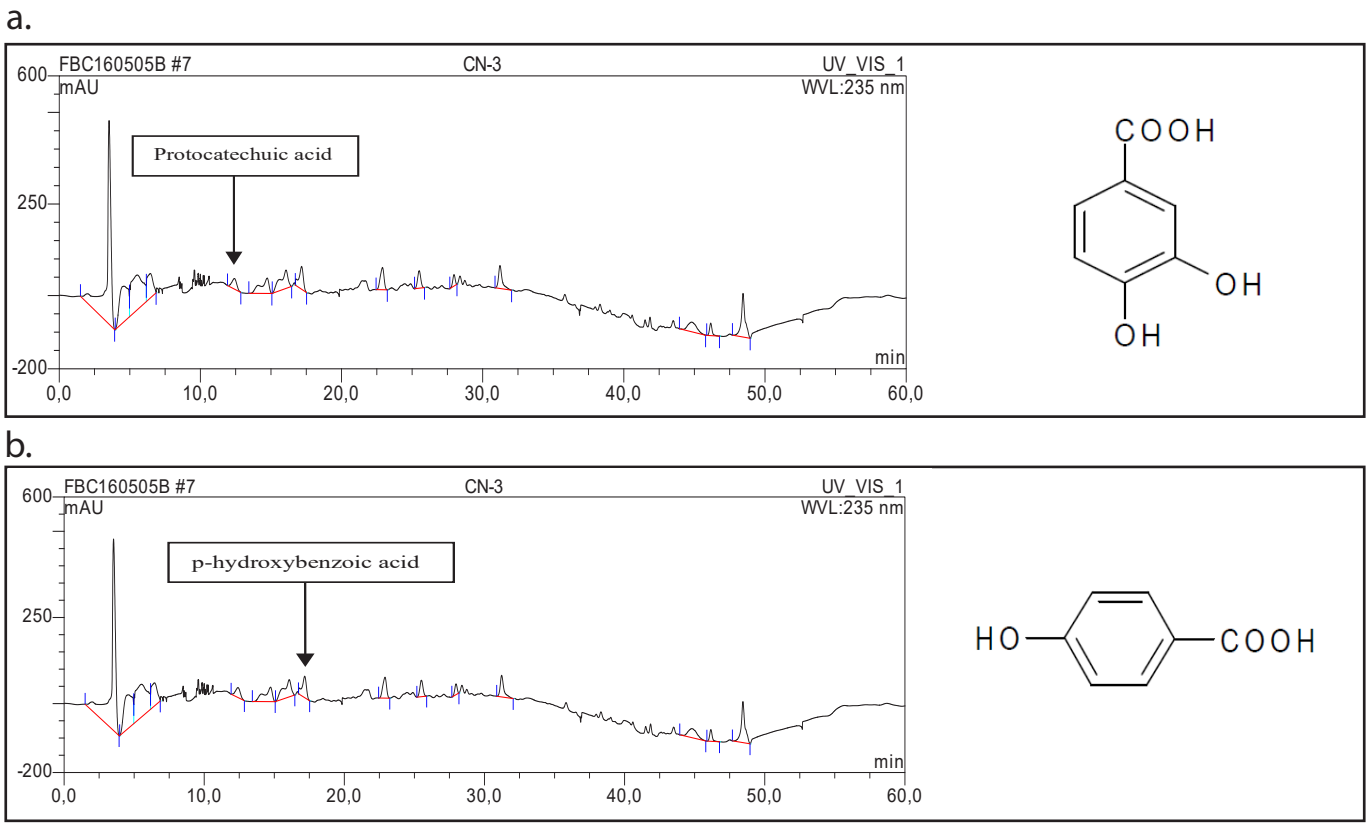


**Figure 2. a:** HPLC chromatogram of Cn3 extract showing protocatechuic acid; its UV spectrum and structure.
**b:** HPLC chromatogram of Cn3 extract showing p-hydroxybenzoic acid; its U V spectrum and structure.

*sa* isolates. It was observed to be resisted by only *P. aeruginosa* isolated from a vaginal swab. The mean values of the zones of inhibition obtain are statistically highly significant as $P < 0.001$.

## Discussion

Endophytic fungi of selected plants are important sources of bioactive compounds with enormous potential for the discovery of new drug molecules. All fungi endophytes isolated from the plants in this study have been previously isolated as endophytes of plants. *Aspergillus* sp., *Acremonium* sp., (27) and *Trichophyton* sp. (28), have all been reported as endophytes isolated from various host plants. We therefore, conclude that all isolated fungi are endophytes.

The antipseudomonal activity displayed by the endophytic

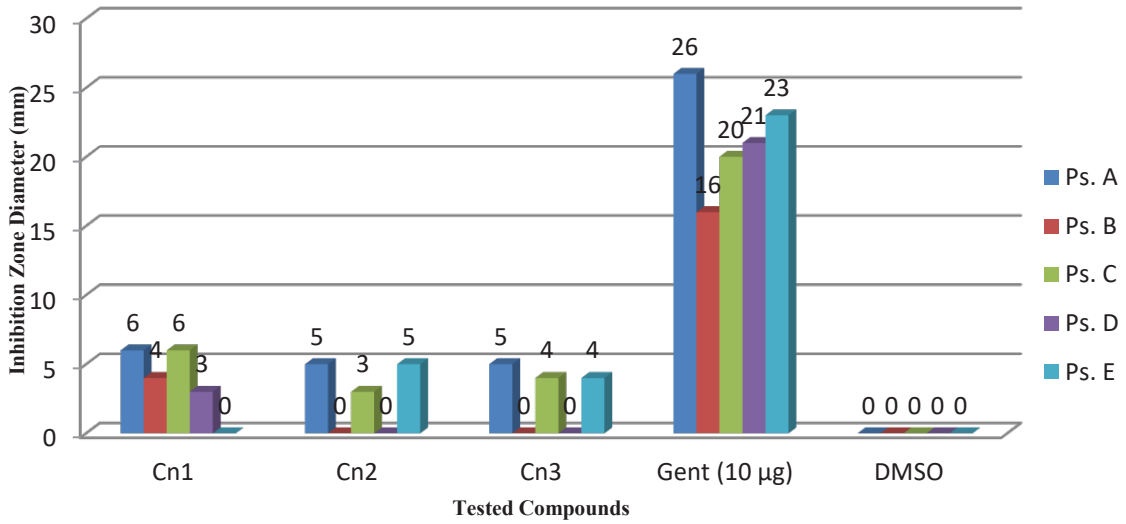


**Figure 3.** Antipseudomonal zones of inhibition of the fungal extracts against various clinical isolates (IZD in mm). Values represent the mean of experiments in triplicates.
*Ps.a* A = Laboratory isolate, *Ps.a* B = Orthopedic wound infection, *Ps.a* C = Urine, *Ps.a* D = Sputum isolate, *Ps.a* E = Vaginal swab Isolate.

fungal extracts in this study can be attributed to the antimicrobial compounds present in the extracts. A list of detected bioactive compounds present in the individual fungal extracts with antimicrobial activities is shown in **Table 2**. From the result obtained, four (4) known compounds were identified as Protocatechuic acid, Indole-3-acetic acid, P-hydroxybenzoic acid, and 4-hydroxyphenyl acetic acid. These compounds have been reported to be produced by fungi of endophytic origin (29-31) and reported to possess antimicrobial activities (18-26).

The antipseudomonal activities of the endophytic fungal extracts in this study showed a varying effect against the different *P. aeruginosa* clinical isolates (**Fig. 3**). Orthopedic wound (*Ps.a* B) and sputum (*Ps.a* D) isolates were observed to be strongly resistant to the fungi extracts of Cn2 and Cn3, being susceptible only to the extract of Cn1 ( IZD of 4±0 mm). While laboratory (*Ps.a* A) and urine (*Ps.a* C) were susceptible to 100% of the fungi extracts under study. There are several reports that *P. aeruginosa* wound isolates are highly resistant when compared to isolates from other sources (32, 33). This can also be evidenced by the lower IZD obtained in the case of the control gentamycin (**Fig. 3**). An emerging problem with *P. aeruginosa* infection is that this pathogenic microbe exhibits a high degree of resistance to a broad spectrum of antibiotics. One striking highlight of this study was that a higher percentage (80%) of the *P. aeruginosa* isolates were found to be susceptible to the extract of Cn1. This may be due to the presence of 4-hydroxyphenyl acetic acid and indole-3-acetic acid identified in the extract of Cn1, as revealed by the HPLC results (**Table 2**). A proper observation shows that only the extracts of Cn1 contained both 4-hydroxyphenyl acetic acid and indole-3-acetic acid, one could propose that both principal compounds combined could produce synergistic mechanism responsible for their activity against the highly resistant wound isolate. Moreover, 4-hydroxyphenyl acetic acid is a phenolic compound and phenolics have been reported to possess antimicrobial properties (22). This is an important observation and suggests the extract of Cn1 as a potential source of novel agents against multiple drug-resistant *P. aeruginosa* species.

Overall, results obtained suggest that *C. nitida* harbor many species of endophytic fungi which are potential sources of novel antibacterial compounds. The presence of bioactive compounds has been reported to confer resistance to plants against bacteria, fungi, and pests (34), and therefore explains the antibacterial activity demonstrated by the endophytic fungi extracts in this study. Also, compounds responsible for the observed antimicrobial activity may have a structural analogue to previously established drugs known to show such effective anti-*Pseudomonas* activity (35). This can be ascertained through the structural elucidation of the identified compounds, and on further purification and characterization, it may be considered as promising compounds for the development of an anti-*Pseudomonas* drug.

## Conclusions

Endophytic fungal extracts of *C. nitida* exhibited potential for anti- *Pseudomonas* activity. This could be attributed to the presence of principal compounds identified in the fungal extracts in this study. This plant and its associated endophytic fungi could be a promising source of bioactive compounds of natural origin for the treatment of infections caused by different strains of *Pseudomonas aeruginosa*.


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### Conflict of interest statement

The authors declare that there is no conflict of interest regarding the publication of this article.

### Ethical compliance

Ethical clearance (S/313/IU) was granted by the joint Committee on Human Research Publications and Ethics of National Orthopedic Hospital Enugu, Nigeria. Approval number is IRB/IIEC NUMBER: S/313/IU.

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