Activity-Guided Isolation and Identification of Metabolites from Endophytic Fungus using HPLC-DAD, Mass Spectrometry and Multidimensional NMR Spectroscopy

Nazifi S. Ibrahim[a,b], Craig P. Butts[b], Ahmed A. Yakasai[a*]

a: Department of Pure and Industrial Chemistry, Bayero University, P.M.B. 3011, Kano, Nigeria.

b: School of Chemistry, University of Bristol, BS8, 1TS, Bristol, United Kingdom.

Email: nsibrahim.chm@buk.edu.ng

Supported by

BAYERO UNIVERSITY KANO

University of BRISTOL

tetfund TERTIARY EDUCATION TRUST FUND

ABSTRACT

Endophytic fungi *Cladosporium spp.* isolated from healthy leaves of *Psidium guajava* was cultured in potato dextrose broth (PDB), tenellin production medium (TPM) and Rice medium (RM). The antioxidant activity of the crude ethyl acetate extracts was observed in DPPH and ABTS followed by further fractionation on C18 column (water acetonitrile gradient). Activity-guided purification and HPLC-DAD analysis of the active fractions revealed the presence of three pairs of alkaloids as the major constituents. The structure of the isolated metabolites was determined by various spectroscopic techniques, including NMR, UV, and MS. The results of the 1D and 2D (including ^{15}N) NMR spectra as well as MS fragmentations, phenazine alkaloids were identified as; phenazine-1-ol **(1)**, phenazine-1-carboxylic acid **(2)** and phenazine-1-carboxamide **(3)**. The 15N and HMBC experiments provided fast resolution of the aromatic proton assignments and the type of phenazine derivatives isolated. This study reveals the first-time isolation of these metabolites from *Cladosporium spp.*, an endophytic fungus obtained from the leaves of an ethnomedicinal plant *Psidium guajava*. Gene tracking and modification will enable bulk isolation of these major constituent for further bioactive study.

1. BACKGROUND

- Endophytic fungi are nonpathogenic microorganisms that are ubiquitous in every plant species.
- They Colonize living, internal tissues of plants without causing any effects (Symbiotic relationship).
- Since the discovery of Taxol, Penicillin and Vincristine from endophytic fungal cultures, these class of microorganisms have been greatly explored as an alternative metabolite isolation pathway.
- Endophytic fungi are reservoir for numerous untapped bioactive secondary metabolites. Antimicrobial, insecticidal, cytotoxic and anticancer active compounds have been successfully discovered from the different endophytic fungus.
- Guava (*Psidium guajava* Linn.) Family Myrtaceae is important plant used traditionally for medicinal purposes. Different classes of natural products have been reported from this plant justifying its wide medicinal applications.
- Our current research studies mainly target the isolation of an endophytic fungi from the leaves of *Psidium guajava* and selecting best culture method for the isolation of bioactive metabolites.

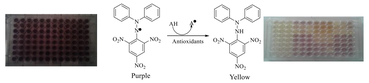
2. EXPERIMENTAL

Isolation and Identification of endophytic fungi → *Fermentation of endophytic fungi in PDB, TPM and RM at RT* → *Extraction of mycelia and broth using EtOAc and MeOH* → ***Analysis of Each Extract***
- *Yield of Extract*
- *Molecular Diversity (HPLC)*
- *Bioassays (cytotoxicity, radical scavenging)*

→ *Large scale fermentation of the active strain* → *Extraction and Purification of extract* Chromatographic techniques (silica CC, sephadex LH-20, PTLC, HPLC) → *Identification of Compounds* ^{1}H-NMR, ^{13}C-NMR, COSY, HSQC, HMBC, ^{15}N HSQC (where applicable), ^{15}N HMBC, HRES-MS, IR and UV.

Antioxidant Activity

DPPH Radical Scavenging Assay



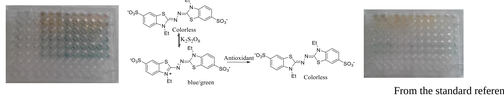
Absorbances were measured at 517 nm

$$I\% = [(A_{blank} - A_{sample})/A_{blank}] \times \frac{}{100}$$

IC_{50} by probit analysis using SPSS 20

Standards: Ascorbic Acid (AA), Buytlated Hydroxy Toluene (BHT) and Tannic Acid (TA)

ABTS Radical Scavenging Assay

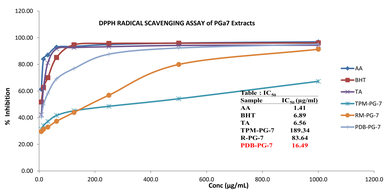


Absorbances were measured at 734 nm

From the standard reference curve, Trolox/Gallic acid equivalence were calculated

3. PRELIMINARY RESULTS

Antioxidant Activity of Fungal Extracts



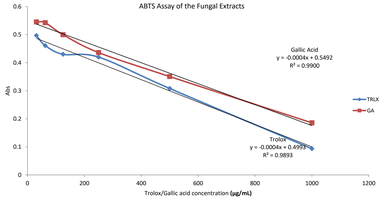


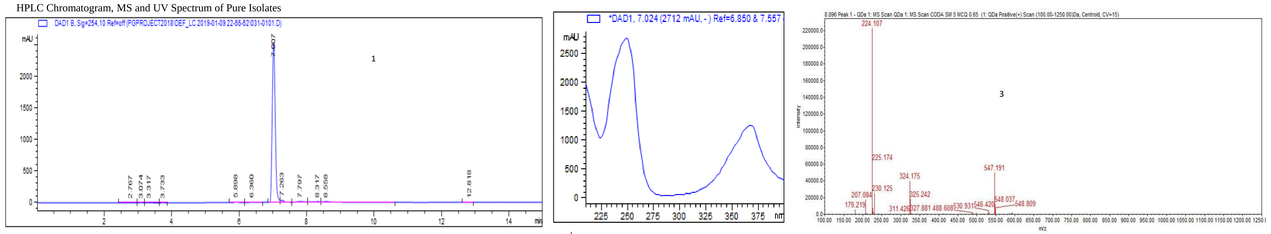
Table 1: Antioxidant activity by ABTS methods of the extracts (1mg/mL) expressed as Trolox and gallic acid equivalent mg/g.

Extract	Trolox Equivalents (mg TE/g)	Gallic Acid Equivalents (mg GAE/g)
PDB	739.736 ± 0.003	846.167 ±0.03
TPM	77.667 ± 0.021	47.083 ± 0.016
RM	403.708 ± 0.003	466.083 ± 0.04

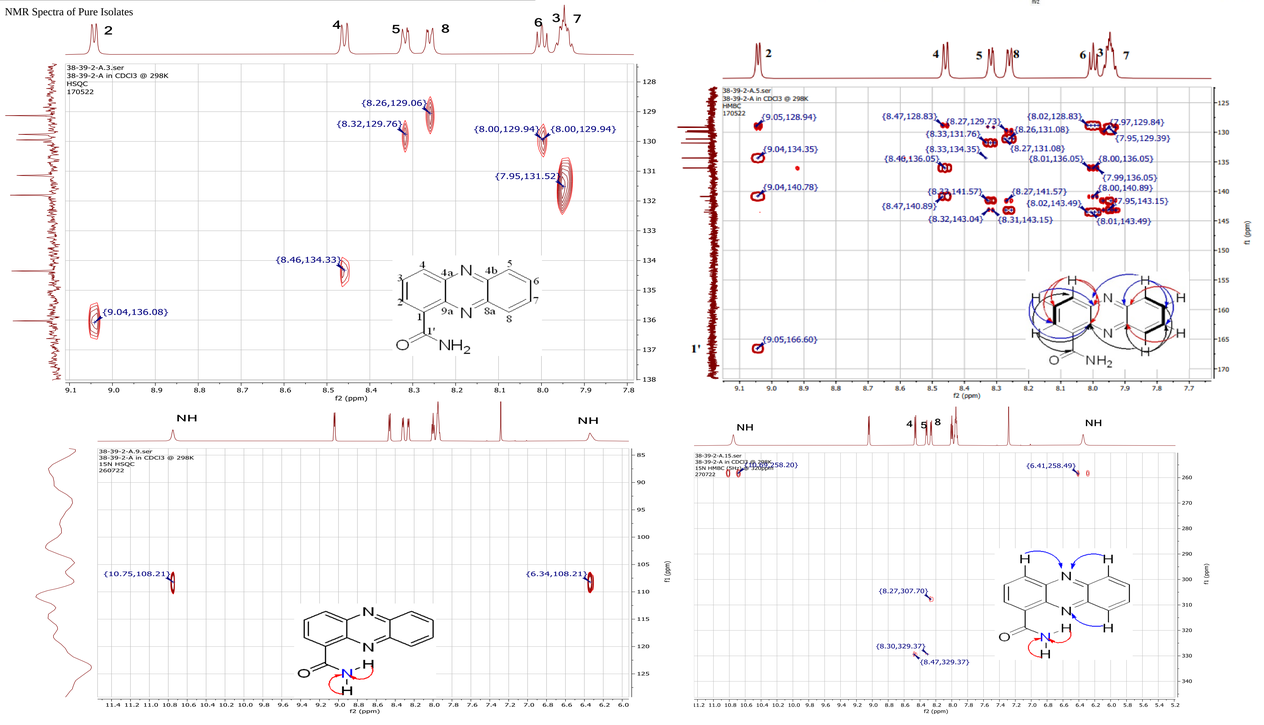
Values are the mean of three replicates ± standard deviation

4. CHARACTERIZATION RESULTS

HPLC Chromatogram, MS and UV Spectrum of Pure Isolates

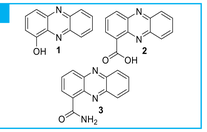


NMR Spectra of Pure Isolates



5. SUMMARY

- ✓ This is the first report of the endophytic fungus *Cladosporium spp.* from healthy leaves of *Psidium guajava*.
- ✓ Fermented fungal strain in PDB medium afforded phenazine-1-ol **(1)**, phenazine-1-carboxylic acid **(2)** and phenazine-1-carboxamide **(3)** as the major metabolites.



6. FUTURE WORK

- ➢ Tracking down the fungal gene responsible for the biosynthesis of these metabolites
- ➢ Genetic modification of the fungus to achieve selective production of these metabolites
- ➢ Enzyme inhibition and other bioactivity study of the isolated metabolites

7. REFERENCES

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