African Journal of Pharmaceutical Research & Development

Vol. 9 No.1; pp. 24-26 (2017)

BIOLOGICALLY ACTIVE METABOLITES OF AN ENDOPHYTIC FUNGUS ISOLATED FROM *VERNONIA AMYGDALINA*

Okezie UM[1], Eze PM[1*], Okoye FBC[2*], Ikegbunam MN[1], Ugwu MC[1], Esimone CO[1]

[1]Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka-Nigeria

[2]Department of Pharmaceutical and Medicinal Chemistry, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka-Nigeria

ABSTRACT

An endophytic fungus was isolated from the leaves of *Vernonia amygdalina.* The fungus was subjected to solid state fermentation on rice medium and the metabolites were extracted using ethyl acetate. The fungal extract was screened for antimicrobial activity and the bioactive compounds of the extract were detected using high-performance liquid chromatography-diode-array detection (HPLC-DAD) analysis. The antimicrobial assay was carried out using agar diffusion assay method against *Staphylococcus aureus, Salmonella typhi, Escherichia coli*, *Pseudomonas aeruginosa, Bacillus subtilis, Entorococcus faecalis, Aspergillus niger and Candida albicans.* The fungal extract, at a concentration of 1 mg/mL, inhibited all test bacteria except *S. aureus*. No antifungal activity was recorded against *A. niger and C. albicans.* The HPLC-DAD analysis of the extract revealed the presence of four bioactive compounds 4-hydroxyphenyl acetic acid, *p*-methoxycoumaric acid, indole-3-acetic acid, and acropyrone. 4-hydroxyphenyl acetic acid, which is a known antimicrobial agent, was the most abundant compound in the extract. Results of this study suggest that endophytic fungi associated with *V. amygdalina* could be a promising source of novel antimicrobial compounds.

KEYWORDS: ***Vernonia amygdalina*, Endophytic fungi, Secondary metabolites, Antimicrobial activity, HPLC-DAD Analysis.**

INTRODUCTION

Vernonia amygdalina is a tropical plant belonging to the family Asteraceae. It is commonly known as bitter leaf due to its characteristic bitter taste. The plant grows in Nigeria and in other tropical parts of Africa where it is consumed as a vegetable or used ethnomedicinally. Extracts and compounds isolated from *V. amygdalina* have been reported to possess several pharmacological properties including cytotoxic/anticancer [1,2,3,4,5,6], antimicrobial [7,6,9], antimalarial [10], and antioxidant [11] activities. The plant has also been reported to be effective against amoebic dysentery [12] and gastrointestinal disorders [13].

Recent studies on the endophytic fungal populations of Nigerian medicinal plants have confirmed the enormous potentials which abound in these organisms as sources of novel bioactive molecules [14,15,16,17,18,19,20,21,22]. Also, endophytes from *V. amygdalina* have been shown to possess potentials in the discovery of novel bioactive compounds [23,24].

In our effort to further explore Nigeria's biodiversity for novel biologically important molecules, we isolated an endophytic fungus associated with *V. amygdalina*

***Corresponding author:** ezep2004@hotmail.com*

collected from Agulu, Anambra state, Nigeria, and identified some of its bioactive secondary metabolites.

MATERIALS AND METHODS

Figure 1: Leaves of *Vernonia amygdalina*

Isolation of endophytic fungus, Fermentation and extraction of metabolites

The isolation of the endophytic fungus, fermentation and extraction of metabolites was carried out as previously described [21,22]. Fresh leaves of *V. amygdalina* were collected from Agulu, Anambra state, Nigeria. Selected healthy plant leaves were washed thoroughly in running tap water and processed as follows: the leaves were cut into 1 cm fragments and 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 placed on Petri plates containing malt extract agar (MEA) supplemented with chloramphenicol. The plates were then incubated at 28^{o}C temperature and fungal growths from the leaf fragments were monitored. Hyphal tips from distinct colonies emerging from leaf segments were sub-cultured onto fresh MEA plates to obtain pure colonies.

Solid state fermentation of the endophytic fungus was carried out in 1L Erlenmeyer flask containing autoclaved rice medium (100 g of rice and 200 mL of distilled water). The flask was inoculated with 3 mm diameter agar blocks containing the fungi and incubated at 28°C for 21 days. At the completion of fermentation, the secondary metabolites were extracted in ethyl acetate and then concentrated under vacuum at 40^{o}C using a rotary evaporator.

Antimicrobial assay

Antibacterial and antifungal screening of the fungal extracts was carried out using the agar well diffusion method previously described [21,22]. The extracts were tested against laboratory strains of *Staphylococcus aureus*, *Bacillus subtilis, Enterococcus faecalis, Escherichia coli, Salmonella typhi*, and *Pseudomonas aeruginosa, Candida albicans* and *Aspergillus niger*. A concentration of 1 mg/mL was prepared for all the fungal extracts by dissolving the extracts in 100% DMSO. A volume of 20 mL of molten Mueller Hinton Agar (MHA) and Sabouraud Dextrose Agar (SDA) (in the antibacterial and antifungal tests respectively) were poured into sterile Petri plates (90 mm) and allowed to set. Standardized concentrations (McFarland 0.5) of overnight cultures of test isolates were swabbed aseptically on the agar plates and holes (6 mm) were made in the agar plates using a sterile metal cork-borer. A volume of 20 µL of the extract and controls were put in each hole under aseptic condition, kept at room temperature for 1 h to allow the agents to diffuse into the agar medium and then incubated accordingly. For positive controls, gentamicin (10 µg/mL) and miconazole (50 µg/mL) were used in the antibacterial and antifungal tests respectively; while 100% DMSO was used as the negative control in both tests. The MHA plates were then incubated at 37^{o}C for 24 h, and the SDA plates were incubated at room temperature (28^{o}C) for 3days. The inhibition zones diameters (IZDs) were measured and recorded. The size of the cork borer (6 mm) was deducted from the values recorded for the IZDs to get the actual diameter. This procedure was conducted in triplicate, and the mean IZDs calculated.

High-Performance Liquid Chromatography-Diode-Array Detection (HPLC-DAD) Analysis

HPLC-DAD analysis was carried on the fungal extract with a Dionex P580 HPLC system coupled to a photo-diode array detector (UVD340S, Dionex Softron GmbH, Germering, Germany). The separation column (125 mm × 4 mm; length × internal diameter) was pre-filled with Eurospher-10 C18 (Knauer, Germany). A linear gradient of nanopure water (adjusted to pH 2 by addition of formic acid) and methanol was used as eluent. The fungal extract (2 mg) was reconstituted with 2 mL of HPLC grade methanol. The mixture was sonicated for 10 min and thereafter centrifuged at 3000 rpm for 5 mins. Then, 100 µL of the dissolved

sample was then transferred into a HPLC vial containing 500 µL of HPLC grade methanol. Detection was at 235 nm. The absorption peaks for the extract were analyzed by comparing with those in the HPLC-ultraviolet (UV)/visible database, which contains over 1600 registered compounds.

RESULTS AND DISCUSSION

An endophytic fungus (BL-MR1) was isolated from the leaves of *V. amygdalina*. Results of the antimicrobial assay of the fungal extract (Table 1) reveal that at 1 mg/mL, the extract showed mild antimicrobial activity only against the bacterial test isolates with IZDs ranging from 0 − 8 mm. The fungal extract showed no antifungal activity against *C. albicans* and *A. niger.*

The extract of the endophytic fungus from *V. amygdalina* represent a dependable source of bioactive compounds, evidenced by the wide range of compounds with diverse biological properties present in these extracts. The HPLC-DAD analysis of the extracts revealed the presence of 4-hydroxyphenyl acetic acid, *p*-methoxycoumaric acid, indole-3-acetic acid, and acropyrone)**.** The HPLC chromatogram, UV-spectra and chemical structures of detected compounds are presented in Figure 2.

Table 1: Inhibition zones diameters (IZDs) produced against test organisms in the antimicrobial assay of the crude extract of endophytic fungus (BL-MR1) isolated from *V. amygdalina*

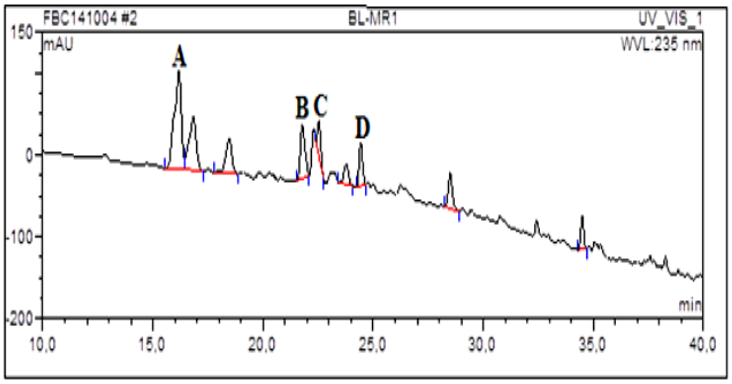
Test Organisms	Concentration (1 mg/mL)	Positive control	Negative control
		Gentamicin (10 µg/ml)	DMSO
S. aureus	0	12	0
E. coli	4	17	0
P. aeruginosa	5	12	0
S. typhi	8	16	0
B. subtilis	6	14	0
E. faecalis	7	12	
		Miconazole (50 µg/ml)	DMSO
C. albicans	0	23	0
A. niger	0	21	0

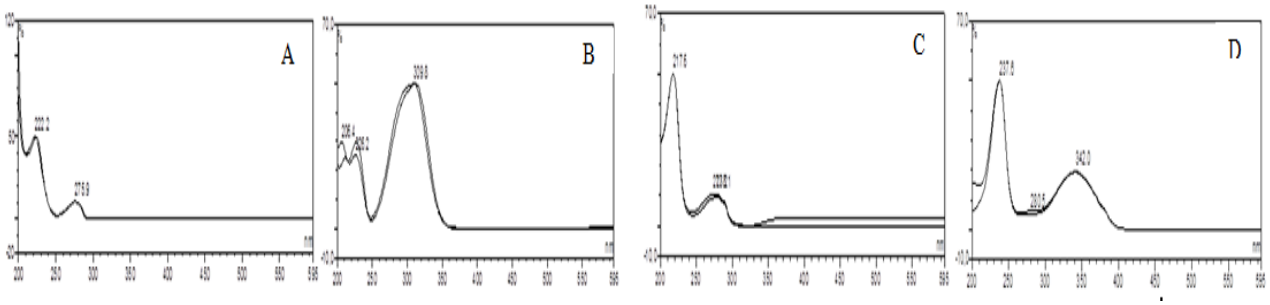
The HPLC-DAD analysis of the extract revealed the presence of four compounds with biological activities that are either antimicrobial, cytotoxic, anti-inflammatory or antioxidant. These compounds include 4-hydroxyphenyl acetic acid, p-methoxycoumaric acid, indole-3-acetic acid, and acropyrone.

Acropyrone has been previously isolated from *Acronychia pedunculata* and has been reported to show cytotoxic activity [25, 26]. 4-Hydroxyphenyl acetic has been has been reported to show nematicidal and antimicrobial activities [27,28,29]. Indole-3-acetic acid has been has been reported to show cytotoxic/anticancer, antioxidant, and anti-inflammatory activities [30-32]. *p*-Methoxycoumaric acid (p-Methoxycinnamic acid) a cinnamic acid derivative, has been reported to show antihyperglycemic [33].

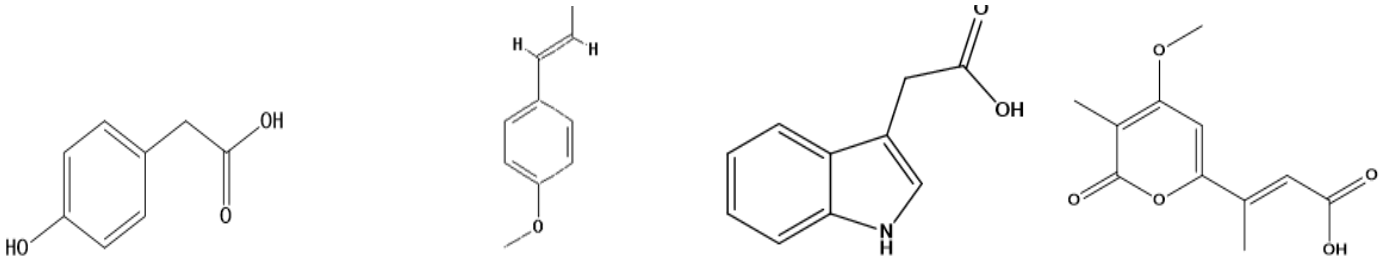
The extracts of endophytic fungi isolated from *V. amygdalina* showed antimicrobial activity. The detected compound 4-hydroxyphenyl acetic acid with known antimicrobial activity [27, 29], was the most abundant compound in the extract as it showed the most prominent peak (A) in the HPLC chromatogram of the extract (Figure 2). This compound may have contributed greatly to the antimicrobial activity recorded by the fungal extract against the test bacterial and fungi.

The HPLC is remains a major analytical tool for the identification of the various constituents of crude mixtures, such as the endophytic fungal extracts. However, the use of the HPLC as the sole tool of identifying the bioactive metabolites in the crude extracts is limited, as only compounds whose UV-spectra are already in the HPLC spectral library can be detected. As a result of this limitation, the undetected compounds or compounds whose spectra had no library hit/match may represent important or novel bioactive compounds [21,22]. It is therefore recommended that further studies be carried out employing other more sensitive analytical tools such as mass spectrometry and/or NMR to validate the findings of this research.





4-Hydroxyphenyl acetic acid (A) ***p*-*Methoxycoumaric* acid (B)** **Indole-3-acetic acid (C)** **Acropyrone (D)**



$C_8H_8O_3$. 152.15 g·mol^{-1} $C_{10}H_{10}O_3$. 178.187 g·mol^{-1} $C_{10}H_9NO_2$. 175.19 g·mol^{-1} $C_{11}H_{12}O_5$, 224 g.mol^{-1}

4-Hydroxyphenyl acetic acid (A) ***p*-Methoxycoumaric acid (B)** **Indole-3-acetic acid (C)** **Acropyrone (D)**

Figure 2: HPLC chromatogram, UV Spectra, and chemical structures of detected bioactive compounds: A (4-Hydroxyphenyl acetic acid), B (*p*-Methoxycoumaric acid), C (indole-3-acetic acid) and D (Acropyrone)

CONCLUSION

The results of this study suggest that endophytic fungi associated with *V. amygdalina* could be a potential source of novel antimicrobial compounds and other biologically important molecules.

ACKNOWLEDGEMENTS

The authors are grateful to Prof. Dr. Peter Proksch of the Institute of Pharmaceutical Biology and Biotechnology, Heinrich-Heine-University, Düsseldorf, for his express permission to use his facilities for the HPLC-DAD analysis.

CONFLICT OF INTEREST

Authors declared no conflict of interests in te conduct and reporting of this study

REFERENCES

1. Sweeney CJ, Mehrotra S, Sadaria MR, Kumar S, Shortle NH, Roman Y, Sheridan C, Campbell RA, Murray DJ, Badve S, Nakshatri H. The sesquiterpene lactone parthenolide in combination with docetaxel reduces metastasis and improves survival in a xenograft model of breast cancer. Mole. Cancer Ther. 4 (6), 2005:1004-1012.
2. Song YJ, Lee DY, Kim SN, Lee KR, Lee HW, Han JW, Kang DW, Lee HY, Kim YK. Apoptotic potential of seequiterpene lactone ergolide through the inhibition of NF-κB signaling pathway. J. Pharmacol. 57 (12), 2005: 1591–1597.
3. Izevbigie, EB, Bryant JL, Walker A. Natural Inhibitor of Extracellular Signal-Regulated Kinases and Human Breast Cancer Cells. Exp Biol & Medicine. 229, 2004: 163–169.
4. Opata MM, Izevbigie EB. Aqueous *V. amygdalina* Extracts Alter MCF-7 Cell Membrane Permeability and Efflux. Int. J. Environ. Res. Public Health. 3 (2), 2006: 174–179.
5. Kupchan SM, Hemingway RJ, Karim A, Werner D. Tumor inhibitors. XLVII. Vernodalin and vernomygdin, two new cytotoxic sesquiterpene lactones from *Vernonia amygdalina*. J. Org. Chem. 34 (12), 1969: 3908.
6. Jisaka M, Ohigashi H, Takegawa K, Huffman MA, Koshimizu K. Antitumoral and antimicrobial activities of bitter sesquiterpene latones of *Vernonia amygdalina*, a possible medical agent used by wild chimpanzees. Biosci Biochem. 57 (5), 1993: 833.
7. Adetunji CO, Olaniyi OO, Ogunkunle ATJ. Bacterial activity of crude extracts of *Vernonia amygdalina* on clinical isolates. Journal of Microbiology and Antimicrobials. 5(6), 2013: 60-64.
8. Uzoigwe CI, Agwa OK. Antimicrobial activity of *Vernonia amygdalina* on selected urinary tract pathogens. African Journal of Microbiology Research. 5(12), 2011:1467-1472.
9. Akinpelu DA. Antimicrobial activity of *Vernonia amygdalina* leaves. Fitoterapia. 70, 1999:232–234
10. Challand S, Willcox M. A clinical trial of the traditional medicine *Vernonia amygdalina* in the treatment of uncomplicated malaria. J Altern Complement Med. 15 (11), 2009: 1231–7.
11. 11 Erasto P, Grierson DS, Afolayan AJ. Evaluation of Antioxidant activity and the fatty acid profile of the leaves of *Vernonia amygdalina* growing in South Africa. Food Chemistry. 104, 2007: 636–642.
12. Moundipa FP, Kamini G, Melanie F, Bilong FC, Bruchhaus I. In vitro amoebic activity of some medicinal plants of the Bamun region (Cameroon) Afr. J. Tad. Cam. 62, 2000:113–121.
13. Akah PA, Ekekwe RK. Ethnopharmacology of some of the asteraceae family used in the Nigerian traditional medicine. Fitoterapia. 66, 1995:352–355.
14. Okoye FBC, Nworu CS, Akah PA, Esimone CO, Debbab A, Proksch P. Inhibition of inflammatory mediators and reactive oxygen and nitrogen species by some depsidones and diaryl ether derivatives isolated from *Corynespora cassiicola*, an endophytic fungus of *Gongronema latifolium* leaves. Immunopharmacology and Immunotoxicology. 35(6), 2013: 662-668.
15. Okoye FBC, Lu S, Nworu CS, Esimone CO, Proksch P, Chaldi A, Debbab A. Depsidone and Diaryl Ether Derivatives from the Fungus *Corynespora cassiicola*, an Endophyte of *Gongronema latifolium*. *Tetrahedron Letters*. 54, 2013: 4210–4214.

16. Okoye FBC, Nworu CS, Debbab A, Esimone CO, Proksch P. Two new Cytochalasins from an endophytic fungus, KL-1.1 isolated from Psidium guajava leaves. Phytochemistry Letters. 14, 2015: 51-55.
17. Ojimba NK, Eze PM, Abba CC, Okoye FBC, Esimone CO. Investigation of Secondary Metabolites of Endophytic Fungi from *Loranthus micranthus* Linn and *Citrus jambhiri* Lush for their Antioxidant Activity. Planta Medica 2015: 81 - PM_190. DOI:10.1055/s-0035-1565567.
18. Chen H, Daletous G, Okoye FBC, Lai D, Dai H, Proksch P. A new cytotoxic cytochalasin from the endophytic fungus *Trichodama harzianum*. Natural Products Communications. 10(4), 2015: 585-587.
19. Ebada SS, Eze P, Okoye FBC, Esimone CO, Proksch P. The Fungal Endophyte *Nigrospora oryzae* Produces Quercetin Monoglycosides Previously Known Only from Plants. Chemistry Select. 1(11), 2016: 2767-2771.
20. Abba CC, Nduka I, Eze PM, Ujam TN, Abonyi DO, Okoye FBC. Antimicrobial Activity of Secondary Metabolites of Endophytic *Aspergillus Species* Isolated from *Loranthus micranthus.* African journal of pharmaceutical research and development. 8(2), 2017:136-140.
21. Akpotu MO, Eze PM, Abba CC, Nwachukwu CU, Okoye FBC, Esimone CO. Metabolites of Endophytic Fungi Isolated from *Euphorbia hirta* growing in Southern Nigeria. Chemical Science Review and Letters. 6(21), 2017:12 – 19.
22. Akpotu MO, Eze PM, Abba CC, Umeokoli BO, Nwachukwu CU, Okoye FBC, and Esimone CO. Antimicrobial activities of secondary metabolites of endophytic fungi isolated from *Catharanthus roseus*. *Journal of Health Science.* 7(1), 2017: 15-22.
23. Khiralla A, Mohamed L, Thomas J, Mignard B, Spina R, Yagi S, Laurain-Mattar D. A pilot study of antioxidant potential of endophytic fungi from some Sudanese medicinal plants. Asian Pacific Journal of Tropical Medicine. 8(9), 2015: 701–704.
24. SJ, Tiong OK. Bacterial endophytes of therapeutically important strobilanthescrispa (L.) Bremek and *Vernonia amygdalina* Del. JPBMS. 14, 2012:1–3.
25. Ito C, Matsui T, Ban Y, Wu TS, Itoigawa M. Acetophenones Isolated from *Acronychia pedunculata* and their Anti-proliferative Activities. Nat Prod Commun. 11(1), 2016: 83-86.
26. Kouloura E, Halabalaki M, Lallemand M, Nam S, Jove R, Litaudon M, Awang K, Hadi HA, Skaltsounis A. Cytotoxic Prenylated Acetophenone Dimers from *Acronychia pedunculata*. J Nat Prod. 75, 2012: 1270-1276.
27. Zuo WJ, Jin PF, Dong WH, Dai HF, Mei WL. Metabolites from the endophytic fungus HP-1 of Chinese eaglewood, Chinese Journal of natural Medicines. 12(2), 2014: 151-153.
28. Ohtani K, Fujioka S, kawano T, Shimada A, Kimura Y. Nematicidal activities of 4-hydroxyphenylacetic acid and oidiolactone D produced by the fungus *Oidiodendron* sp. Journal of biosciences. 66(1-2), 2011: 31-34.
29. Chapla VM, Zeraik ML, Leptokarydis IH, Silva GH, Bolzani VS, Young MCM, Pfenning LH, Araújo AR. Antifungal Compounds Produced by *Colletotrichum gloeosporioides,* an Endophytic Fungus from *Michelia champaca.* Molecules. 19, 2014: 19243-19252.
30. Wardman P. Indole-3-acetic acids and horseradish peroxidase: a new prodrug/enzyme combination for targeted cancer therapy, Curr Pharm Des. 8(15), 2002: 1363-1374.
31. Jeong YM, Oh MH, Kim SY, Li H, Yun HY, Baek K.J, Kwon NS, Kim WY, Kim DS. Indole-3 acetic acid/horseradish peroxidase induces apoptosis in TCCSUP human urinary bladder carcinoma cells. Pharmazie, 65(2), 2010:122-126.
32. Jones LH, Abdalla DSP, Freitas JC. Effects of indole-3-acetic acid on croton oil and arachidonic acid-induced mouse ear edema, Inflammation Research, 44(9), 1995: 372-375.
33. Adisakwattana S, Roengsamran S, Hsu WH, Yibchok-anun S. Mechanisms of antihyperglycemic effect of p-methoxycinnamic acid in normal and streptozotocin-induced diabetic rats. Life Sciences. 78(4), 2005: 12:406–412