Trop J Nat Prod Res, May 2018; 2(5):209-213 **ISSN 2616-0684 (Print)**
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Secondary Metabolites from *Curvularia sp,* An Endophytic Fungus Isolated from the Leaves of *Picralima nitida* Durand and Hook (Apocynaceae)

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ARTICLE INFO

Article history:
Received 05 March 2018
Revised 25 April 2018
Accepted 05 May 2018
Published online 10 May 2018

ABSTRACT

Nature has remained a major source of pharmacologically active compounds used for the treatment of new and existing diseases, or as lead molecules for the development of synthetically derived analogues. This research was carried out to determine the secondary metabolites from the extract of *Curvularia sp*, an endophytic fungus associated with the leaves of *Picralima nitida*. The endophytic fungus was isolated and purified from the leaves of the plant material, using the conventional methods. The identity of the fungus was determined by molecular biology characterization. A combination of chromatographic methods (high performance liquid chromatography and column chromatography), spectroscopic techniques (1-D and 2-D nuclear magnetic resonance spectroscopy and mass spectrometry), dereplication and literature data comparison were used to isolate, purify and identify the secondary metabolites in the fermentation extract of the endophytic fungus. Three previously reported compounds, acropyrone (**1**), 4-hydroxyphenylacetic acid (**2**) and indole-3-acetic acid (**3**) were isolated for the first time from *Curvularia Sp* extract. Compounds **1-3** were tested for microbial and cytotoxic properties. The compounds showed no antimicrobial properties at 1 mg/mL, no observable cytotoxic properties at the dose of 10 µg/mL and no antimycobacterial properties at the dose tested. Although there was no significant bioactivity, the result further confirms the usefulness of endophytic fungi as a secondary metabolite factory for the production of drugs and drug precursors in the laboratory.

Keywords: Endophytic fungus, secondary metabolites, *Curvularia sp*, structure elucidation, antimicrobial activity

Introduction

The continuous emergence of new diseases, development of multi-drug resistance by pathogenic microorganisms and toxicity of existing treatment options have resulted in an increasing demand for new and safer bioactive compounds in all aspects of medicine .[1] Nature has consistently provided mankind with a diverse range of pharmacologically active compounds that continue to be utilized as drugs for the treatment of new and existing diseases or as lead molecules for the development of synthetically derived drugs. Drug molecules and precursors have been sourced from plants, fungi, bacteria, endophytes (bacteria, fungi) etc.[2] The remedies from these natural sources have been the mainstay of orthodox and traditional medicine.[2]

The importance of endophytes as a potential source of pharmaceutical leads has been demonstrated. Many endophytic fungi have been shown to produce novel bioactive metabolites with antimicrobial, anti-inflammatory, anticancer and antiviral properties.[3] The discovery of taxol-producing fungi[4] further buttressed the significance of endophytes as a source of pharmaceutical leads. Taxol, a brand name for paclitaxel, is an anticancer agent indicated in the treatment of many types of cancer including breast cancer.[5]

Subsequently, cytotoxic quinone dimer, torreyanic acid[6] isolated from the endophytic fungus, *Pestalotiopsis microspora* associated with the tree *Torreya taxifolia*, novel cytocalasin alkaloids with anticancer properties[7,8] and podophyllotoxins obtained from fungal endophyte *Trametes hirsute*[9] among many other bioactive metabolites were reported.

Picralima nitida is an understory plant the various parts of which are used for different medicinal purposes in sub-Saharan African countries.[10] Uzor and co-workers[11] had established that some of the secondary metabolites isolated from plants are not the product of plant metabolism but rather synthesized by the endophytes inhabiting the plants. Bearing this in mind, this work seeks to investigate and identify the secondary metabolites produced by an endophyte *Curvularia sp.* isolated from *P. nitida*.

Materials and Methods

Collection of plant materials

Branches from *Picralima nitida* were collected from the terrestrial forest of Igbo-Ukwu in Aguata Local Government Area, in Anambra State, Nigeria on the 12th of June, 2014. The plant material was identified by Mrs. A. U. Emezie, a plant technologist in the Department of Pharmacognosy and Traditional Medicine, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Anambra State, Nigeria. This was authenticated by a plant taxonomist Mr. A. O. Ozioko of Bioresources Development and Conservation, Program (BDCP) Center, Nsukka, Enugu State. The plant sample was deposited in the herbarium of the Department of Pharmacognosy and

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Citation: Nwachukwu CU, Ngwoke KG, Eze PM, Eboka CJ, Okoye FBC. Secondary Metabolites from *Curvularia sp*, An Endophytic Fungus Isolated from the Leaves of *Picralima nitida* Durand and Hook (Apocynaceae). Trop J Nat Prod Res. 2018; 2(5):209-213. doi.org/10.26538/tjnpr/v2i5.2

Traditional Medicine as voucher specimen (PCG474/A/027). Freshly collected healthy leaves from the branches were then used for the fungi isolation.

Isolation of endophytic fungi from plant leaves

Fresh leaves of the plant were rinsed sequentially with fresh water under a running tap and distilled water. Surface sterilization was achieved by immersing the leaf samples in 70% ethanol for 2 min, rinsed with distilled water, immersed in 1% sodium hypochlorite for 2 min after which the leaves were soaked in 70% ethanol for 2 min and then rinsed with fresh sterilized distilled water for 5 min. The leaves were cleaved aseptically into small segments (approximately 2 x 2 mm) from the lamina with sterile blades. They were then dried under laminar air flow chamber.[12,13]

Using sterilized forceps each leaf segment was placed on Petri dishes containing Malt Extract Agar (MEA) supplemented with chloramphenicol to suppress bacterial growth. The plates were then incubated at 27ºC and monitored periodically until mycelia growth were observed from the leaves. Each hyphal tip from distinct colony was transferred onto fresh malt extract plates to obtain pure cultures of each fungal isolate.

Identification of fungus

Taxonomic identification of all fungal strains was achieved by DNA amplification and sequencing of the fungal internal transcribed spacer (ITS) region. Total fungal genomic DNA was extracted and purified directly from fresh, axenic mycelia using fungal DNA extraction and purification kits. The molecular identification was performed according to a molecular biologic protocol as described by Kjer.[14]

Fermentation, extraction and isolation

Solid state fermentation was carried out in 1 L Erlenmeyer flasks containing 100 g of rice and 110 mL of tap water. This was autoclaved at 121ºC for 1 h and allowed to cool. Each flask was inoculated with 3-5 mm diameter agar blocks containing test fungi and incubated at 27°C for 21 days.[8] At the end of 21 days, the fermentation was stopped with the addition of 500 mL of ethyl acetate. The solid media was then cut into small pieces to allow for proper extraction and the flask was allowed to extract for 24 h with intermittent shaking. The ethyl acetate was evaporated and dried in a vacuum evaporator at 40ºC to yield crude extract under reduced pressure. The crude ethyl acetate extract (1.5 g) was subjected to VLC using binary solvent combinations of hexane:ethyl acetate and dichloromethane: methanol in increasing order of polarity. VLC fraction F13 (114 mg) was further subjected to semi-preparative HPLC separation using Hitachi semi-preparative chromatograph (Merck/Hitachi, Germany) equipped with pump (L-7100), photodiode array detector (UV-L7400), column (Eurosphere 100-C18; 10 µm; 300 x 8 mm; Knauer), pre-column (Eurosphere 100-C18; 10 µm; 30 x 8 mm; Knauer), printer (Chromato-Integrator D-2000) with methanol:water as the eluting solvent in a gradient mode. The composition of the crude extract and fractions as well as the purity of the isolated compounds, were monitored by using the analytical HPLC (Dionex, Germany) equipped with: pump (P580A LPG), autosampler (ASI-100), photodiode array detector (UVD 340S), column oven (STH 585), column (Eurosphere, 100-C18; 5 µM; 125 x 4 mm; with integrated pre-column) and Chromeleon 6.30 software.

Structure elucidation of compounds isolated from fungus

The structure elucidation of the isolated compounds followed a standard scheme. Firstly, the data obtained from analytical HPLC were compared with the in-house substance library regarding their retention times at the standard gradient programme and their UV spectra. Comparable hit indicated the class to which the compound belongs. From LC-MS (HPLC:Agilent 1100 series equipped with pump, photodiode array UV-detector, autosampler and injector, Column (Eurosphere 100-C18; 5 µM; 227 x 2 mm; Knauer)- MS spectrometer Finnigan LCQDeca, Thermoquest, Vacuum pump (Edwards 30, BOC), Xcalibur software, version 1.3) (Agilent, Germany) measurement, the mass of the compound and from ^{1}H-NMR measurement using DPX 300, ARX 400, 500 NMR (Bruker, Germany), substructures were compiled.

With this information together with the identity of the fungus, a literature search using the dictionary of natural products (DNP, Chapman and Hall, 2005-2009), Antibase, SciFinder, was performed to finally identify the secondary metabolites.

Antimicrobial assay

Antimicrobial screening of the crude fungal extracts, fractions and compounds was carried out using the agar well diffusion assay method as previously described.[15] A concentration of 1 mg/mL of the fungal extracts/fractions was prepared by dissolving the extracts in dimethyl sulphoxide (DMSO). A range of concentration between 1 mg/mL and 12.5 µg/mL were obtained by two-fold serial dilutions. Standardized broth cultures (0.5 McFarland turbidity standard) suspension of each of the test isolates of test bacterial strains (*Staphylococcus aureus*, *Salmonella typhi*, *Bacillus subtilis* and *Escherichia coli* and fungal strains (*Aspergillus niger* and *Candida albicans*) were spread aseptically onto the surface of Mueller Hinton Agar (MHA) and Sabouraud Dextrose Agar (SDA) plates respectively by 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/fractions and controls. The plates were then kept at room temperature for 1 h to allow the agents to diffuse into the agar medium. Ciprofloxacin (5 µg/mL) and miconazole (50 µg/mL) were used as positive controls in the antibacterial and antifungal evaluations respectively; while DMSO was used as the negative control. The MHA plates were then incubated at 37ºC for 24 h, and the SDA plates were incubated at 25-27ºC for 2-3 days. 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 and recorded.

Also, the antimicrobial activity of pure compounds isolated from the fungal extracts was determined using the method described above.

Cytotoxicity assay

Tetrazolium MTT (3, [4,5- dimethylthiazolyl-2]-2,5-diphenyltetrazolium bromide assay was performed following the method described by Carmichael *et al*.[16] and % cell viability was deduced by spectrophotometric determination of accumulated formazan derivative in treated cells at 560 nm in comparison to control cells.[17] L5178Y mouse lymphoma cells were grown in Eagle's minimal essential medium supplement with 10% horse serum in roller tube culture. The medium contained 100 units/mL penicillin and 100 units/mL streptomycin. The cells were maintained in a humidified atmosphere at 37°C with 5% CO_2. Of the test samples to be analysed in the bioassay, stock solutions of crude extracts (10 µg/mL) in ethanol (96% v/v) were prepared. Exponentially growing cells were harvested, counted and diluted appropriately. Of the cell suspension, 50 µL containing 3750 cells were pipetted into 96- well microtiter plates. Subsequently, 50 µL of a solution of the test samples containing the appropriate concentration was added to each well. The test plates were incubated at 37°C with 5% CO_2 for 72 h. A solution of MTT was prepared at 5 µg/mL in phosphate buffered saline (PBS; 1.5 mM KH_2PO_4, 6.5 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl; pH 7.4) and from this solution, 20 µL were pipetted into each well. The yellow MTT penetrated the cells and in the presence of mitochondrial dehydrogenases, MTT was transformed to its blue formazan complex. After an incubation period of 3 h 45 min at 37°C in a humidified incubator with 5% CO_2, the medium was centrifuged (15 min at 210 x g) with 200 µL DMSO and the cells were lysed to liberate the formazan product.

After thorough mixing, the absorbance was measured at 520 nm. The colour intensity could be correlated with the number of healthy living cells and cell survival was calculated using the formula:

$$\text{Survival \%} = \frac{\text{Absorbance of untreated cells} - \text{Absorbance of culture medium}}{\text{Absorbance of treated cells} - \text{Absorbance of culture medium}} x100$$

All experiments were carried out in triplicate.

As negative controls, media with 0.1% (v/v) ethanol were included in all experiments.

Anti-tubercular assay

Growth inhibition of *Mycobacterium tuberculosis* was tested using a metabolic activity assay employing the resazurin dye reduction method as described by Patil *et al*.[18] *M. tuberculosis* cells were grown

aerobically at 37°C in Middlebrook 7H9 media supplemented with 0.5% (v/v) glycerol, 0.05% (v/v) tyloxapol, and 10% (v/v) ADS enrichment (5%, w/v, bovine serum albumin fraction V; 2%, w/v, glucose; 0.85%, w/v, sodium chloride). Bacteria were pre-cultured until log-phase (OD 600 nm ~1) and then seeded at 1×10^5 cells per well in a total volume of 100 µL in 96-well round-bottom microtiter plates. The test substances were then added to achieve a working concentration of 10 µg/mL for the fungal extracts. The microtiter plates were then incubated at 35°C for 6 days. For viability determination, 10 µL of resazurin solution (100 µg/mL, Sigma-Aldrich) was added per well and incubated for about 8 h. Then cells were fixed by addition of formalin (5%, v/v) for 30 min, and fluorescence was measured using a microplate reader (excitation 540 nm, emission 590 nm). Residual growth was calculated relative to rifampicin-treated (0% growth) and DMSO-treated (100% growth) controls.

Antioxidant assay

The free radical scavenging activity of all the extract and compounds was evaluated by 1, 1-diphenyl-2 picryl-hydrazyl (DPPH) according to the method by Shen *et al*.[19] with modification.

A volume of 50 mL of 0.2 mM solution of DPPH in methanol was prepared by weighing 3.94 mg of DPPH to 50 mL of methanol. Also, 2 mL of 0.2 mM solution of DPPH was added to 2 ml of the sample and quercetin respectively and dissolved in methanol (1 mg/mL, 1000 µg/mL). These final reaction mixtures result in a 2-fold dilution of both the extract and DPPH concentrations bringing them to a final concentration of 0.1 mM for the DPPH solution and 500 µg/ml for the samples.

The mixtures were shaken vigorously and allowed to stand at room temperature for 30 min. Then the absorbance was measured at 517 nm using a UV-VIS spectrophotometer.

Lower absorbance values of reaction mixture indicate higher free radical scavenging activity.

The capability of scavenging the DPPH radical was calculated by using the following formula.

$$\text{DPPH scavenging effect (\% inhibition)} = \frac{\text{A0} - \text{A1}}{\text{A0}} x100$$

Where, A_0 is the absorbance of the control and A_1 is the absorbance in the presence of all of the extract samples and reference. All the tests were performed in triplicates. The mean value and standard deviation were determined and recorded.

Also, the antioxidant activities of some of the pure compounds isolated from the fungal extracts were determined using the method described above.

Results and Discussion

The isolated fungal endophyte was identified by molecular characterization to belong to the *Curvularia sp*. The DNA sequence of the ITS1 region is shown in Table 1.

A combination of chromatographic and spectroscopic methods was used to isolate and identify the secondary metabolites from the extract. Three compounds (**1**, **2** and **3**) were identified based on the UV, mass and ^{1}H-NMR spectra. Spectra data are shown in tables 2, 3 and 4.

Compound **1** was isolated from the VLC fraction (DCM: MeOH 80:20) of ethyl acetate crude extract of *Curvularia sp*. as a yellow powder. It exhibited UV maxima at 237.1 nm and 341 nm. Its molecular weight was determined as 224 g/mole based on the ESI-MS quasi peak at m/z 225.1 $(M+H)^+$ upon positive ionization and 223.1 $(M–H)^-$ upon negative ionization. These suggested a molecular formula of $C_{11}H_{12}O_5$. The UV maxima observed is typical of an alpha pyrone which suggest the presence of pyrone as the basic structure.

The ^{1}H-NMR showed the presence of three CH_3 signals. These include a methoxy singlet observed at δ 4.04 (H_3-12), a methyl singlet at δ 2.43 (H_3-11), and another methyl singlet at δ 1.95 (H_3-10). The assignments of these methyl signals were based on their relative deshielded positions, the methoxy being most deshielded followed by methyl attached to aromatic, then that attached to olefinic carbon. Two proton signals resonated downfield at δ 6.85s (H-5) and δ 6.70s (H-8) the desheilding was due to the anisotropic effect of the olefinic system. The assignment of H-8 was based on a long range COSY correlation of its signals with that of H_3-10. The presence of the carboxyl unit was rationalized based on the observed fragment at *m/z* 179.0 $[M\text{-}COO^-]^-$ in the negative mode which corresponds to the loss of ion of carboxylate. Analysis of the MS and NMR data in comparison with those reported in the literature[20] led to the elucidation of compound **1** as acropyrone.

Compound **2** (4- Hydroxyphenylacetic acid) was isolated from the VLC fraction (DCM:MeOH 80:20) of ethyl acetate crude extract of *Curvularia sp*. as a beige powder. It exhibited UV maxima at 221.9 and 275.9 nm. This is typical of a simple phenolic compound. Its molecular weight was determined as 152 g/mole based on the molecular ion peak $[M+H]^+$ of 153 upon positive ionization. The fragment peak at 107 suggests the loss of carboxylic group. This therefore, suggests a formula of $C_8H_8O_3$.

The ^{1}H-NMR showed the presence of aromatic proton signals of the AA'BB' system at δ_H δ 7.11 (m) (H-2/6), and δ 6.75 (m) (H-3/5). The ^{1}H-NMR also showed deshielded methylene signal at δ 3.50 (s, 2H) (H-7) rationalized as the CH_2 group of the acetic acid. Analysis of the MS and NMR data in comparison with literature[21,22] led to the elucidation of compound **2** as 4-hydroxyphenylacetic acid.

Compound **3** was isolated as a colourless solid from the VLC fraction (DCM:MeOH 80:20) of ethyl acetate crude extract of *Curvularia sp*. The characteristic shape of the UV spectrum showed absorption maximum at 217.6 nm and 279.1 nm. This suggested the presence of indole as the basic structure.

The ESI-MS spectrum gave the base peak M/Z = 130.2 indicating the molecular fragment which has lost a carboxylic group $[M\text{-}COOH]^+$. This suggested likely odd number molecular weight given therefore as 175 g/mole. This implied the presence of nitrogen atom in the compound.

The ^{1}H-NMR spectrum shows four aromatic proton signals at δ 7.58 (*d*, J=1.03, 1H), 7.05 (*m*, J=0.89, 1H), 7.15 (*m*, J=0.99, 1H), 7.38 (*d*, J=1.01, 1H). The observed multiplicity pattern is typical of ortho-disubstituted benzene ring and the signals are assigned to H-4, H-6, H-5 and H-7 respectively. The deshielded proton signal at δ 7.2 (s) was assigned to H-2. The deshielding being contributed by both the inductive effect of the nitrogen atom and the anisotropic effect of the

Table 1: DNA sequence of fungal endophyte isolated from *Picralima nitida* identified as *Curvularia sp*

Source	Fungal DNA Sequence (FASTA format)	Fungus Name
Picralima nitida	TTCAAACCGGCTGGATTATTTTTCTTCACCCTTGTCTTTTGCGCACTTGTTGTTTCCTGGGCGGGTTCGC TCGCCACCAGGACCACACCATAAACCTTTTTGTTAATGCAATCAGCGTCAGTAAAAAGTAATAATTATT TTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATACGTA GTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATTCCAAAG GGCATGCCTGTTCGAGCGTCATTTGTACCCTCAAGCTTTGCTTGGTGTTGGGCGTTGTTTGTCTTTGGCC TTGCCCAAAGACTCGCCTTAAAACAATTGGCAGCCGGCCTACTGGTTTCGCAGCGCAGCACATTTTTGC GCTTGCAATCAGCAAAAGAGGTTGGCCATCCATCAAGACTACATTTATACGTTTGACCTCGGATCAGG TAGGGATACCCGCTGAACTTAAGCATATCAAT	*Curvularia sp*

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

olefenic system. The methylene proton signal observed at δ 3.69 (s) was assigned to H-1[1]A/B. Compound **3** was elucidated as the previously reported indole-3-acetic acid and the data corresponds to that reported in the literature.[23]

The extract, VLC fractions and compounds were screened against *P. aeruginosa, E. coli, B. subtilis, S. aureus, C. albicans* and *A. niger*. The results showed that none of the screened sample (extract, fractions and compounds) had any activity at 1 mg/mL concentration which is even above the maximum test concentration of 250 µg/mL stated by CLSI. It could be said that this particular strain of *Curvularia sp* from *P. nitida* does not produce antibiotic agents unlike other strains such as *Curvularia sp*. from *Argyrosomus argentatus* which was reported to exhibit strong antibacterial properties.[24] It has been reported that the genetic background, age and the environmental requirement of the plant host are some of the factors that can influence the type of secondary metabolites produced by endophytes.[25] This means that the array of secondary metabolite producible by a certain endophyte may vary according to host, health condition and geographical location of the host. Consequently, the metabolic pathways in a given strain of microorganism will vary according to these factors as well as the bioactivity of their extracts. The extract also showed no anti-tubercular property after antitubercular test and did not exhibit any observable cytotoxic property in MTT assay.

Sometimes the metabolites of different fungi may be similar. For instance, some of the metabolites reported by Eze et al. (2018) from endophytic fungi from the leaves of *Citrus jambhiri*(Rutaceae) are the same with those reported in the article. This may happen for many reasons. Though these fungi may be different based on philogeny, they are basically fungi and most have at least one metabolic pathway in common.

When they are fermented on the same medium, the substrates available to them are the same and they can easily produce similar metabolites. One of our ongoing projects (not yet published) involved large-scale fermentation of 4 different soil fungi. Two were from the same family whereas 2 were of different families but all of them produced ergosterol copiously on malt extract agar.

Conclusion

The analysis of *Curvularia sp* extract yielded three known compounds which we are reporting for the first time from both the plant and the fungal species. These compounds satisfy the Lipinski rule of five which means that they are all druggable. They should, therefore, be subjected to further bioactivity testing to determine how best they can be applied pharmaceutically for the benefit of man. They could also be modified to achieve more effective compounds.

Table 2: Comparison of the [1]H NMR data of Acropyrone

Carbon No.	δ H[a] (*J* in Hz)	δ H[b] (*J* in Hz)
1	-	-
2	-	-
3	-	-
4	-	-
5	6.90 (s)	6.85 (s)
6	-	-
7	-	-
8	6.50 (d, *J* =1.3)	6.70 (s, *J* = 1.20)
9	-	-
10	1.83 (s)	1.95 (s, *J* = 2.89)
11	2.36 (d, *J* = 1.3)	2.43 (d, *J* = 2.9)
12	3.98 (s)	4.04 s)

a. at 600 MHz in $CDCl_3$ (Reference Compound)[27]
b. Derived from [1]H spectrum at 500 MHz in MeOD from this study (Isolated Compound).

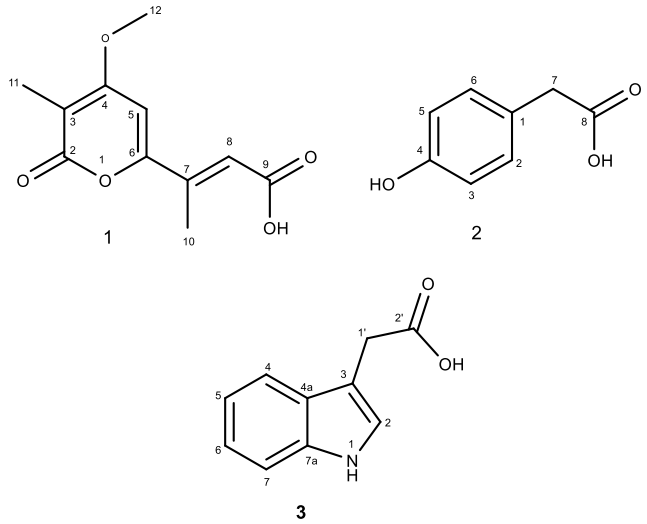

Figure 1. The chemical structures of the compounds isolated from the fermentation extract of *Curvularia sp*

Table 3: Comparison of the [1]H-NMR Data of 4-Hydroxyphenylacetic acid

Carbon No.	δ H[a] (*J* in Hz)	δ H[b] (*J* in Hz)
1	-	-
2	7.09 (m)	7.11 (m)
3	6.72 (m)	6.75 (m)
4	-	-
5	6.72 (m)	6.75 (m)
6	7.09 (m)	7.11 (m)
7	3.47 (m)	3.50 (s)
8	-	-

a. at 300 MHz in MeOD. (Reference Compound)[26]
b. Derived from [1]H NMR at 500 MHz in MeOD from this study. (Isolated Compound)

Table 4: Comparison of [1]H-NMR Data of Indole-3-Acetic Acid

Carbon No.	δ H[a] (*J* in Hz)	δ H[b] (*J* in Hz)
1	-	-
2	7.12 (s)	7.2 (s)
3	-	-
4	7.54 (d)	7.58 (d)
5	7.00 (ddd)	7.15 (m)
6	7.07 (ddd)	7.05 (m)
7	7.34 (d)	7.38 (d)
8	3.69 (s)	3.69 (s)
9	-	-
10	-	-

a. at 300 MHz in $CDCl_3$. (Reference Compound)[23]
b. Derived from [1]H spectrum at 500 MHz in MeOD from this study. (Isolated Compound)

Conflict of interest

The authors declare no conflict of interest.

Authors' 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.

Acknowledgements

The authors are grateful to Prof. Dr. Peter Proksch of Institute of Pharmaceutical Biology and Biotechnology Universitat Dusseldorf, Germany for the provision of research facilities in his laboratory for the spectral analysis. Dr. FBC Okoye wishes to acknowledge the Return Fellowship Grant from Alexander von Humboldt Foundation which enabled the completion of this work.

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