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Secondary metabolites from endophytic fungi of *moringa oleifera*: antimicrobial and antioxidant properties

Abstract

The exponential increase in antimicrobial resistance among several microbes, now more than ever, poses a tremendous challenge to the health system. This has created an urgency to look outside the "norm" for antimicrobial agents that would be useful in tackling the various disease-causing microorganisms. Endophytes are microorganisms that not only have a niche within the plant tissues but also produce bioactive compounds which could be a significant breakthrough in the pharmaceutical industry, especially with the manufacture of novel drugs having an impactful effect on these disease-causing microbes. The study examined the secondary metabolites produced by endophytic fungi associated with *Moringa oleifera* as well as the antioxidant and antimicrobial activities of the crude extracts of these secondary metabolites against microbes of medical importance. To achieve this, the fresh leaves of moringa (*Moringa oleifera*) were collected from Ngwo in Enugu's north local government area, Enugu State. Isolation of endophytic fungi was done using the protocol described by.[13] The fungus was cultured by placing agar blocks of actively growing pure culture (3 mm in diameter) in a 500 ml Erlenmeyer flask containing 100 g of rice medium. Incubation was done at room temperature for 3 weeks. Afterward, the fermentation was stopped by introducing 500 ml of ethyl acetate into the flask. Whatman No. 1 filter paper was used to separate the fermentation mixture. Cultures of the bacteria and fungi were maintained on nutrient and Sabouraud dextrose agar respectively at 4 °C. After the extraction had been done, antimicrobial activity was determined by agar well diffusion assay by Kirby-Bauer's method. The result of the DPPH antioxidant assay of the endophytic fungal extract revealed that at a concentration of 100 µg/ml, Two Endophytic fungi, MA and MB showed antioxidant activity with % Inhibition of 55.1 and 48.3% respectively. The result of HPLC analysis of the fungal endophytic fungal metabolites revealed the presence of active compounds in the extracts. It was concluded that *Moringa oleifera* leaves harbor endophytic fungi. These endophytes could be exploited in medicine as novel metabolites.

Keywords: *Moringa oleifera,* secondary metabolites, endophytic fungi, antimicrobial, antioxidant

Volume 10 Issue 5 - 2022

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Received: August 30, 2022 | **Published:** September 27, 2022

Introduction

The exponential increase in antimicrobial resistance among several microbes, now more than ever, poses a tremendous challenge to the health system. Antimicrobial resistance has not only challenged the structure of the public health system but has also become a global burden. This has created an urgency to look outside the "norm" for antimicrobial agents that would be useful in tackling the various disease-causing microorganisms.

The moringa plant (*Moringa oleifera* Lam.) has been known over the years as a "miracle plant" importantly because all of its parts have been used either nutritionally or for medicinal purposes.

Antioxidants, antibacterial, antifungal, anti-cholesterol, anti-inflammatory, anti-ulcer, pain relief, immunomodulatory, and wound healing properties are attributed to this plant.[1,2] *M. oleifera* has also been found to form associations with various microbes that act as symbionts in multiple tissues of the plant. These symbiont microbes which can either be bacteria or fungi are generally referred to as endophytes.[3,4]

Endophyte is a general term used to describe microorganisms that not only have a niche within the plant tissues but also undertake either a part or the whole of their life cycle within this host plant without causing any damage.[5] They enter and habit the tissues of the host plant via natural openings such as stomata and lenticels, and also by injuries inflicted by air currents, rain waters, or insects associated with the plant.[6] Endophytic fungi have been discovered to be the most predominant of endophytes found in plant tissues. Various bioactive compounds have been associated with these endophytic fungi ranging from alkaloids, steroids, triterpenoids, tannins, and anthracene sides, to reducing sugars and even compounds with tremendous applications in agriculture, medicine, and the food industry.[7-9]

These bioactive compounds produced by these endophytic fungi would be a significant breakthrough in the pharmaceutical industry, especially with the production of novel drugs having an impactful effect on these disease-causing microbes. Various researchers in Nigeria have probed the antimicrobial and antioxidant properties of endophytic fungi isolated from medicinal plants.[10-12]

The present study thus aimed to investigate the secondary metabolites produced by endophytic fungi associated with *M. oleifera* and the antioxidant and antimicrobial activities of the crude extracts of these secondary metabolites against microbes of medical importance.

Materials and methods

Collection of plant materials

The fresh leaves of moringa (*M. oleifera*) were collected from ngwo in Enugu north local government area, Enugu state. The samples were taken from a healthy plant leaf in September 2021.

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Isolation of endophytic fungi

Isolation of endophytic fungi was done using the protocol described by Suryanarayanan.[13] Running tap water was used to wash the leaf sample, to remove dust and debris after which the sample was air-dried before further processing. Sterilization of the leaf material was done by immersion into 70 % ethanol for 3 min and 0.5 % Sodium hypochlorite (NaOCl) for 1 min. The leaf material was again dipped into 70 % ethanol and rinsed thoroughly with sterile distilled water. After air-drying the samples, a sterile scalpel was used to cut the leaves (3-4 mm in diameter and 1 cm in length) while the midribs were cut separately. In each Petri dish, 5-6 segments of both leaf blade and midrib were placed on the Sabouraud Dextrose agar (SDA) medium. The media were supplemented with chloramphenicol (250 mg) to repress any bacterial growth. The plates were sealed and incubated at room temperature and observed daily for the growth of endophytic fungi. The hyphal tips growing from the plant materials were transferred to fresh Potato Dextrose Agar (PDA) plate without the addition of antibiotics to obtain pure cultures for investigations.

Cultivation of endophytic fungi and metabolite extraction

The fungus was cultured by placing agar blocks of actively growing pure culture (3 mm in diameter) in a 500 ml Erlenmeyer flask containing 100 g of rice medium. Incubation was done at room temperature for 3 weeks. Afterward, the fermentation was brought to a halt by the introduction of 500 ml of ethyl acetate into the flask. Whatman No. 1 filter paper was used to separate the fermentation mixture.

The metabolite from the fungus was extracted using ethyl acetate as an organic solvent. Ethyl acetate was evaporated using a rotary evaporator at 50 ºC and the resultant compound was dried in $CaCl_2$ desiccators to yield the crude metabolite. The crude extract was then kept in an Eppendorf tube at 25 ºC for bioassay.

Determination of antimicrobial activity

Inoculum preparation

Cultures of the bacteria and fungi were stored on nutrient and Sabouraud dextrose agar respectively at 4 ºC. Before the test, bacterial and fungal cultures were prepared as follows: the bacterial cultures were subcultured in nutrient broth at 37 ºC for 24 h while the cultures of fungi were subcultured in liquid Sabouraud dextrose medium for 48 h at 25 ºC. The turbidity of the broth culture was then equilibrated to 0.5 McFarland standards using normal saline as diluents.

Antimicrobial assay of fungal metabolites

Antimicrobial activity was determined by agar well diffusion assay by Kirby-Bauer's method.[14] The crude extract was dissolved in dimethyl sulphoxide (DMSO) giving a concentration of 1 mg ml^{-1} for antimicrobial bioassay. A loopful of bacterial and fungal cultures from broth cultures was evenly distributed on the surface of nutrient agar. The plates were kept for some time to solidify. Wells were made in the media using a 6 mm diameter sterile metallic borer. Then, 50 µl of the sample (1 mg/ml concentration) was added to each of the wells. Others were supplemented with DMSO and reference antimicrobial drugs as negative and positive controls respectively. The plates were incubated at 37 ºC overnight. At the end of the incubation period, the diameter of inhibition zones formed in all three replicates was measured in mm using a calibrated measuring ruler and their mean values were calculated.

Antioxidant Activity

Procedure

To obtain 0.1 mM of DPPH, 2 mg of 1, 1-diphenyl-2-picrylhydrazyl (DPPH) was dissolved in 50 ml of methanol. The endophytic fungal metabolites were prepared by dissolving in methanol to yield a concentration of 100 µg/ml. Ascorbic acid was used as standard. The negative control used was a mixture of methanol and DPPH solution, which was also used as a blank. Reaction mixtures containing 25 µl of the test sample and 25 µl of DPPH were added to the wells of microtiter plates and made up to 200 µl by adding 150 µl of methanol. Plates were incubated at 27.7 ºC for 30 min.

A spectrophotometer was used to ascertain the absorbance at 490 nm. Percentage inhibition by sample treatment also referred to as the DPPH free radical scavenging effect of the samples, was determined by comparing a methanol-treated control group using the formula below:

$$\%\ Inhibition\ = \frac{Absorbance\ of\ control - Absorbance\ of\ sample}{Absorbance\ of\ control} \times 100$$

High-performace liquid chromatography analysis of fungal metabolites

Procedure

To perform this experiment, 2 mg of dried fungal metabolites was reconstituted with 2 ml of HPLC grade methanol. Sonication was done for 10 mins followed by centrifugation at 13000 rpm for 5 min. The supernatant was pipetted, filtered using cotton wool, and introduced into the HPLC vials. 100 µl of the dissolved samples were each transferred into HPLC vials containing 500 µl of HPLC grade methanol. Each sample was allowed to run for an hour. The analysis was carried out on the samples with a Dionex P580 HPLC system coupled to a photodiode array detector (UVD 340S, DIONEX Softron Gmb H, Germering, Germany). Detection was at wavelengths of 235, 254 and 340 nm. The separation column (125 x 4 mm; length x 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 as eluent.

Results

Two Endophytic fungi, MA and MB were isolated from the leaves of *M. oleifera.* The metabolite yield from these endophytic fungi was 48.5 mg and 54.2 mg for MA and MB respectively after cultivation and extraction.

Antimicrobial assay of fungal metabolites

A 1.0 mg/ml concentration of the extract from MA showed a spectrum of antimicrobial activity against *S. aureus* (IZD = 6.7 mm), *B. subtilis* (IZD = 6.9 mm), *S. typhi* (IZD = 5.7 mm), *Candida albican* (IZD = 5.0 mm) and *A. fumigatus* (IZD = 4.7 mm). However, the 1.0 mg/ml concentration of the extract from MB only showed antimicrobial activity against *S. aureus* (IZD = 8.7 mm), *B. subtilis* (IZD = 7.4 mm), and *E. coli* (IZD = 4.7 mm). The extract from MB showed no antifungal activity against *A. fumigatus* and *C. albicans* Table 1.

Gentamicin (10 µg/ml) was used as a positive control for the Bacteria samples while Fluconazole (50 µg/ml) was used as a positive control for the fungal samples (I. e. *A. fumigatus and C. albicans*).

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Table 1 Antimicrobial evaluation of the Secondary metabolites showing Mean Inhibition Zone diameter (IZD) produced against test organisms (mm)

Test organisms	Endophytic fungal metabolites (1.0mg/ml)		Positive Control	Negative Control (DMSO)
	MA	MB		
S. areus	6.7	8.7	16	0
B. subtilis	6.9	7.4	17	0
S. typhi	5.7	0	22	0
E. coli	0	4.7	21	0
A fumigatus	4.7	0	17	0
C. albicans	5	0	4	0

Antioxidant Activity

The result of the DPPH antioxidant assay of the endophytic fungal extract revealed that at a concentration of 100 µg/ml, MA and MB showed antioxidant activity with % Inhibition of 55.1 and 48.3% respectively. These values are comparable with the 64.7% inhibition recorded for the positive control (Ascorbic Acid) at the same concentration Figure 1.

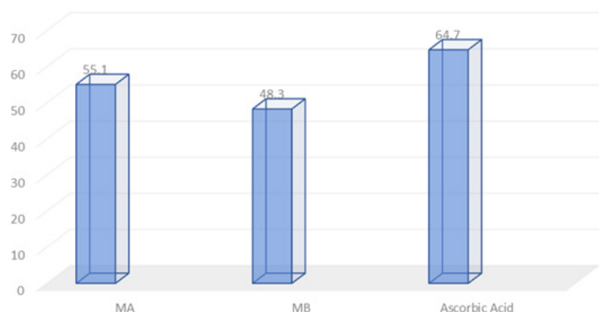


Figure 1 DPPH antioxidant assay.

Data presented as mean, values are statistically significant from standard control with a p-value <0.05.

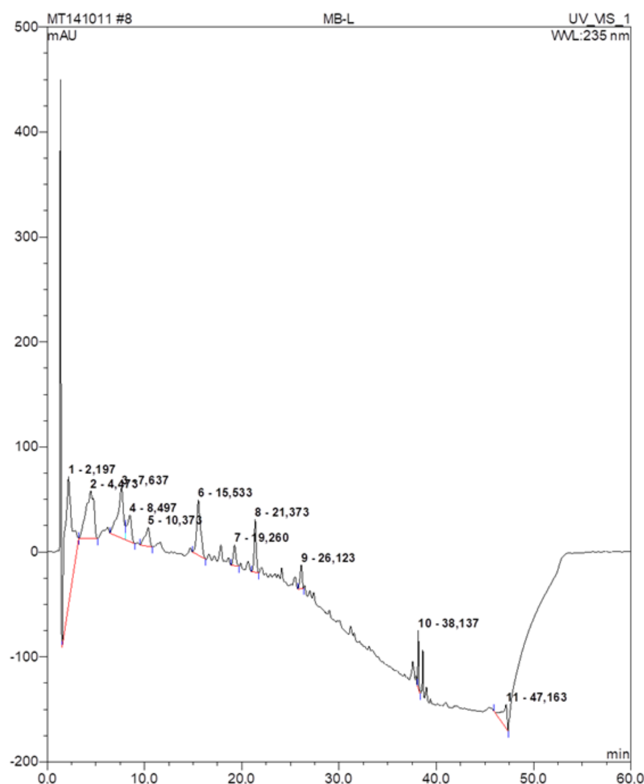
High-performace liquid chromatography analysis of fungal metabolites

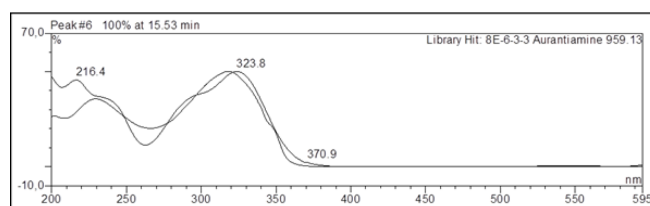
The result of HPLC analysis of the fungal endophytic fungal metabolites revealed the presence of active compounds in the extracts. Benzylpyridine B, Aurantiamine were present in the crude extract of MB, while Pavetanin A1Ac was found in the crude extract of MA. Figures 2 and 3 show the UV spectra and chemical structures of the detected compounds present in the fungal extracts.

Discussion

This paper entails a report on the isolation of endophytic fungi from *M. oleifera* from Nigeria, intending to screen the antimicrobial and antioxidant characteristics of the active metabolites against pathogens of medical importance. Results gotten from this study have shown that the leaf tissues of *M. oleifera* serve as a niche for endophytic fungi of medical importance. This finding is consistent with literature by various researchers that endophytic fungi are found in the leaf tissues of *M. oleifera*.[4,15,16]

Analysis of the potential of the crude extracts of secondary metabolites from endophytic fungi to serve as antimicrobial agents showed that the endophytic fungi metabolites exhibited varied inhibitory effects against Gram-positive bacteria *Bacillus subtilis, Staphylococcus aureus*, and pathogenic fungi *Candida albicans* and *A. fumigatus*. From the obtained result, it was noted that the Gram-positive bacteria (*S. aureus* and *B. subtilis*) were the most susceptible among the test organisms to endophytic fungal metabolites (8.7 mm and 6.11 mm respectively), while fungi were the least susceptible (*A. fumigatus* and *C. albicans*) 4.7 mm and 5.0 mm respectively. However, these crude metabolite extracts from the endophytic fungi showed no activity in the Gram-negative bacteria E. coli. Powthong.[17] revealed that Gram-negative bacteria are more resistant to most of the antibacterial activity due to the possession of lipopolysaccharide protein and an additional tiny layer of peptidoglycan resulting in a more complex cellular structure as opposed to Gram-positive bacteria. Similar scientific findings were reported in various other literature.[18,19]





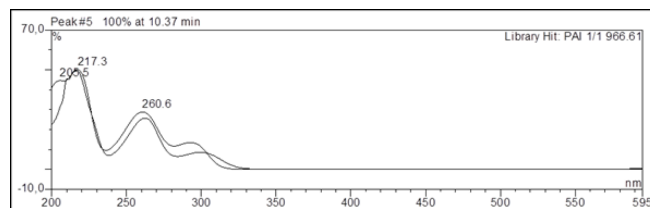


Figure 2 HPLC chromatogram of the endophytic fungal extract (MB) and the UV Spectrum of the detected compounds -Benzylpridine B and Aurantiamine.

Oxidative stress is the result of the imbalance that exists between the production of free radicals and the antioxidant defense system. This oxidative stress poses potential damage to cells.[20,21] Oxidative stress conditions have been linked to several diseases such as cancer, diabetes, and premature aging including, generation disorders.[22]

Citation: Enyi EO, Ekpunobi NF. Secondary metabolites from endophytic fungi of *moringa oleifera*: antimicrobial and antioxidant properties. *J Microbiol Exp.* 2022;10(5):150–154. DOI: 10.15406/jmen.2022.10.00367

Several plants have been found to contain several antioxidant constituents that have various modes of action and are employed in traditional medicine.[23]

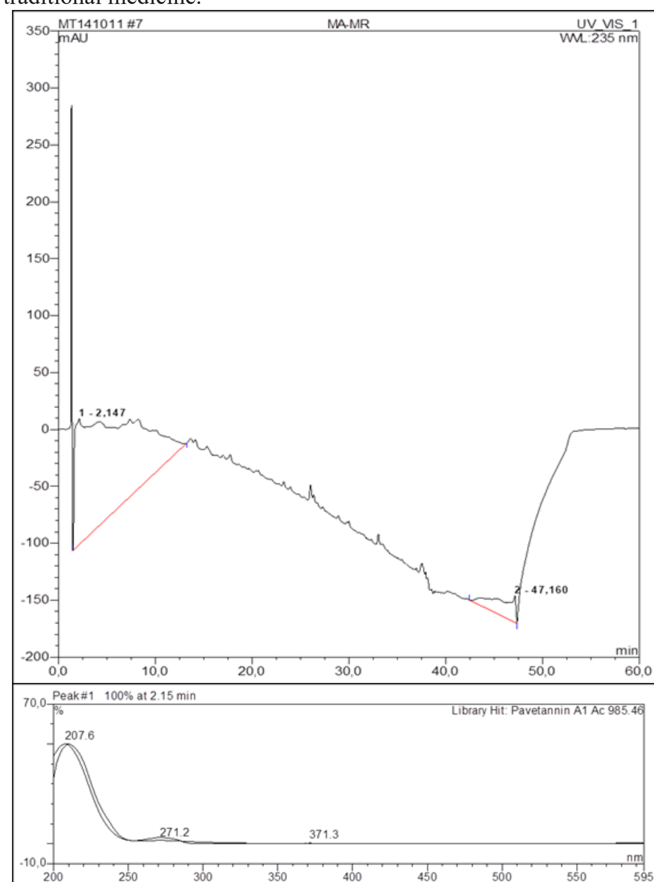


Figure 3 HPLC chromatogram of the endophytic fungal extract (MB) and the UV Spectrum of the detected compound - Pavetanin A1Ac.

The crude extracts of secondary metabolites from endophytic fungi isolated from *Moringa oleifera* were found to show strong antioxidant activity (free radical scavenging effect of DPPH) in comparison to the standard (ascorbic acid). These secondary metabolites could be a reliable and novel way of combatting oxidative stress conditions as they have been shown to possess the proton-donating ability and could serve as free radical inhibitors, possibly playing the role of primary antioxidants.

Aurantiamine, Benzylpyridine B, and Pavetanin are secondary metabolites that are known to exhibit medical and physiological activities. Aurantiamine is a blue fluorescence metabolite that belongs to diketopiperazines that exhibit important biological activities such as anticancer or neurotoxic effects.[24]

Conclusion

Based on our research, it could be concluded that *Moringa oleifera* leaves harbor endophytic fungi. These endophytic fungi produce secondary metabolites that possess antimicrobial and antioxidant activities and thus, endophytic fungi of *Moringa oleifera* may be a good source of bioactive compounds when compared with available data in the literature. These endophytes could be exploited in medicine as novel metabolites.

Acknowledgements

None.

Conflicts of interest

Author declares that there is no conflict of interest.

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Citation: Enyi EO, Ekpunobi NF. Secondary metabolites from endophytic fungi of *moringa oleifera*: antimicrobial and antioxidant properties. *J Microbiol Exp.* 2022;10(5):150–154. DOI: 10.15406/jmen.2022.10.00367