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Antimicrobial Activities of Endophytic Fungi Secondary Metabolites from *Moringa oleifera* (Lam)

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Abstract

This study presents the antimicrobial activities of secondary metabolites of endophytic fungi isolated from a medicinal plant *Moringa oleifera* growing at the University of Dar es Salaam, Tanzania. Two endophytic fungi were identified, *Nigrospora* sp. from leaves, and *Fusarium* sp. from roots and seeds. The disc method was used in testing the antimicrobial activities of the crude extracts of endophytic fungi secondary metabolites against four human pathogens, namely, *Bacillus substilis, Staphylococcus aureus, Escherichia coli,* and *Candida albicans.* The results revealed that the crude extracts of endophytes secondary metabolites from leaves showed strong antimicrobial activities against two Gram positive bacteria and fungi with Minimum Inhibitory Concentrations (MICs) ranging from 0.016 mg/mL to 0.5 mg/mL, while from roots and seeds showed moderate antimicrobial activities with MIC ranging from 0.6 mg/mL to 1 mg/mL. Qualitative phytochemical analysis of the same extracts also revealed the presence of vital constituents potential for medicinal applications such as alkaloids, phenols, flavonoids, tannins, terpenoids, and saponins. This study demonstrated that secondary metabolites of endophytic fungi from *Moringa oleifera* possess important phytochemical and exhibit antimicrobial potential against the tested human pathogens which could contribute to the endeavours for new therapeutic inventions.

Keywords: Antimicrobial activity, Endophytic fungi, Medicinal plants, *Moringa oleifera*.

Introduction

Endophytes are microbes that live in plant tissues for the whole or part of their life cycles without causing harm to the host (Zhao et al. 2010). Endophytic fungi are proven to exist in both vascular and nonvascular plants by entering plant tissues through natural openings (stomata and lenticels), and wounds caused by air current, rain waters or insects which live or breed in plants (Gennaro et al. 2003). Endophytic fungi harbour bioactive compounds such as alkaloids, steroids and triterpenoids, tannins, anthracenosides, reducing sugars, flavones, saponins which perform multiple functions including preventing plants from stress conditions like drought, heavy metals, competition, herbivorous and promotion of growth fitness (Omacini et al. 2001, Waqas et al. 2012). They are also known to produce natural bioactive compounds with great potential applications in agriculture, medicine and food industry (Stierle 1993, Verma 2009). It is further known that endophytic fungi harboured in medicinal plants produce secondary metabolites with bioactive compounds similar to those of their hosts. They are thus very important in pharmaceuticals for the discovery of new drugs (Strobel 1999, Zhao et al. 2010).

Many plants found in various places in tropical eco-region have medicinal properties, thus provide raw materials for addressing a

range of medical disorders and pharmaceutical requirements (Kubmarawa et al. 2008, Tibuhwa 2016). The World health organization (WHO) reported that about 80% of individuals from developed countries use traditional medicines derived from medicinal plants (Rajeswara et al. 2012, Tibuhwa 2017). Moreover, many Tanzanians who reside in rural and peri-urban areas rely on plants for their primary health care needs.

Moringa oleifera Lam (synonym: *Moringa pterygosperma* Gaertner) is a well-known medicinal plant that belongs to a monogeneric family Moringaceae. The tree is native to India but it is widely grown in other parts of the world (Fahey 2005). It is drought-tolerant, fast-growing, multi-purpose and one of the most useful trees in the world due to its medicinal and nutritional properties, and is therefore described as a 'miracle tree' or "tree of life" (Daba 2016). Africans have used it a long time ago in curing various diseases and sometimes it is referred to as a panacea due to its numerous applications of curing more than 300 diseases (Daba 2016). In Tanzania, Moringa tree (in Swahili, Mlonge), has been traditionally used in different ways as summarised in Table 1. It is mostly used for healing various diseases such as malaria, gastric ulcer, and controlling blood sugar and to raise the immune system and as a source of income (Sebastian 2009).

Moringa oleifera is very well studied in different parts of the world (Kumar 2017) with numerous reports on its medicinal properties as antimicrobial (Mangale et al. 2012), antipyretic agent (Chandra et al. 2010), anticancer (Purwal et al. 2010) and anti-inflammatory (Ezeamuzie et al. 1996). Various studies have also reported on the antimicrobial properties of endophytic fungi from this plant (Zhao et al. 2012, Mahdi et al. 2014), However, little is known on the characteristics of endophytic fungi found in *M. oleifera* from Tanzania. The present study thus presents the first report on the isolation and characterization of these endophytes and the antimicrobial activities of the crude extracts of their secondary metabolites against microbes of medical importance.

Table 1: Overview of uses of *M. oleifera* as traditional herbs

Plant part used	Tradition uses
Leaves	The ground powder is used to treat skin disease, headache, gastric ulcer, and aphrodisiac. Leaves juice is used to control blood sugar and diabetes and treat glandular swelling, anxiety. Also, when the leave's juice is mixed with carrot juice is used to increase urine flow - used in kidney treatment (Mbwambo et al. 2007). -Pulverized leaves are used as nutrition supplements by adding it in porridge especially for children and immune compromised people (Andrew 2010, Augustino and Gillah 2005, Olugmemi et al. 2010.)
Roots	Boiled and its syrup used indifferent treatments including antifertility, antilithic, stomach ache, lower back, and kidney pain, constipation as well as direct chewing in raw form to release cold symptoms (Choudhary et al. 2013).
Bark	Pulverized barks are used for detoxification, and treatment of rheumatism, ant inflammation (Monera and Maponga 2012).
Seeds	The ground powder is used for water treatments, and seed oil is used to treat skin disease, hysteria, and scurvy (Mbwambo et al. 2007).

Materials and Methods

Collection of plant materials and preservation

Samples were collected during March 2017 from the remnants of the natural forest behind Botany building at the University of Dar es Salaam, Mwalimu JK Nyerere campus located at S 06°78'122" E 039° 20'574". Health matured leaves, seeds, barks and roots with no visual diseases symptoms were randomly collected and stored into sterile polyethylene bags in the cool box at 4 °C then brought to the Department of Molecular Biology and Biotechnology laboratory at the University of Dar es Salaam for further processing within 72 hours as detailed in Powthong et al. (2013).

Isolation of endophytic fungi

Isolation of endophytic fungi was done from visually healthy plant parts using methods described by Suryanarayanan et al. (2003). The collected parts were carefully washed using tap water to remove all debris and soil contaminants and then dried in the air. Samples were then sterilized by immersing them sequentially in 70% ethanol for 3 min, 0.4% NaOCl for 1 min, and 70% ethanol for 2 min. They were then washed three times with sterile distilled water for 1 min each and blotted using sterile blotting paper. The processed sterile and dry plant parts were chopped into 0.5-1 cm segments. Thereafter, 4-5 segments were placed in Petri dishes containing Potato Dextrose Agar (PDA) supplemented with streptomycin (100 mg/ml). The plates with samples and without samples (control) were incubated at 30 °C for 4-6 days in the dark. The plates were observed frequently to check for the growth of fungal colonies. Purification of the fungi was done through the hyphae tip method by cutting hypha tip of growing fungi using a sterile blade and transfer to separate PDA petri dishes supplemented with 100 mg/mL streptomycin. The plates were sealed with parafilm and incubated at room temperature for 5 days. Pure cultures were obtained by serial sub culturing. The obtained endophytic fungi cultures were maintained in PDA slant at 4 °C for further screening.

Determination of colonizing frequency

The frequency of colonization was assessed on each plate daily. In the end, the colonizing frequency was calculated using the method described by Kumar and Hyde (2004) to determine endophytic fungi richness of each plant species as:

$$CF\% = \left(\frac{No\ of species}{No\ of\ segments\ screened}\right) \times 100$$

Where: CF = Colonization frequency

Morphological characterization

Both macro and micro-morphological characteristics were used to characterize the isolates. From a pure culture colony, parameters such as colony appearance, mycelia types, and colony colour were examined. Microscopic features were examined under the compound microscope (Leica 2500, Leica Microsystems CMS GmbH, Wetzler, Germany), after staining with lactophenol cotton blue stain as described in Kameshwari et al. (2015). The observed fungi were identified to the genus level using standard identification keys as described by Masumi et al. (2015).

Molecular characterization

Genomic DNA was extracted according to Pamphile and Azevedo (2002). Briefly, the mycelia were transferred from Potato Dextrose Agar (PDA) without shaking to 250 mL Erlenmeyer's flasks containing Potato Dextrose Broth (PDB). The flask with mycelia was kept for five days for massive growth at 28 ± 2 °C. From the incubated flasks, about 100 mg of mycelia were harvested for genomic DNA extraction. The isolation of DNA was carried out using DNA easy Mini kit (Qiagen). The DNA concentration was determined using the Nanodrop spectrophotometer (Thermo Scientific Nanodrop 2000) at the absorbance of 260/280 nm. The ribosomal DNA amplification of ITS1-5.8S-ITS2 region was

carried out by using PCR cycles method as described by Ramesh et al. (2017) with some modifications using primer pair ITS1 (Sequence: TCCGTAGGTGAACCTGCGG) and ITS4 (Sequence: TCCTCCGCTTATTGATATGC) for the amplification of the fungal rDNA internal transcribed spacers (ITS) regions of all isolates (White et al. 1990, Rhoden et al. 2012). The PCR products were analyzed using gel electrophoresis and photo documentation as described by Hussein et al. (2014). The amplified DNA was then sequenced for ascertaining the fungal isolates. Percentages of sequence identity and coverage were determined by comparative similarity analysis with available sequences in the GenBank at http://www.ncbi.nlm.nih.gov using Blast as detailed in Zhang et al. (2000).

Extraction of secondary metabolites from endophytic fungi for antimicrobial tests

The secondary metabolites of the endophytic fungi were extracted from separate parafilm wrapped PDA plates containing the pure endophyte isolate culture from different parts of the *M. oleifera* plant. Screening for antimicrobial activities was done following the method described by Subbulakshmi (2012). Briefly, about 4 mm mycelia agar of each endophytic fungi were aseptically cut from the actively grown pure culture using sterile cork borer and inoculated into 500 mL Erlenmeyer Flasks containing 250 mL of the autoclaved potato dextrose broth (PDB) media. It was then stationarily incubated at room temperature for three weeks as described by Sandhu et al. (2014) and Campos et al. (2015). Secondary metabolites produced from each PDB fungal culture were obtained by separating the mycelia from broth through filtering using a muslin cloth. The crude extracts were extracted twice with an equal volume of ethyl acetate (250 mL) then evaporated using the rotary vacuum evaporator under reduced pressure at 35 °C. The crude extract residuals obtained were weighed and dissolved in dimethyl sulfoxide (DMSO), stored at 4 °C as a stock solution for antimicrobial tests.

Inoculum preparation

The three bacteria strain Gram positive bacteria *Bacillus subtilis* (DSM 347), *Staphylococcus aureus* and Gram negative bacteria *Escherichia coli* (ATCC 25922) together with fungi *Candida albicans* (ATCC 90028) were obtained from the Microbiology laboratory of the Department of Molecular Biology and Biotechnology, University of Dar es Salaam. The old pure culture of bacteria and fungi were subcultured into nutrient agar and potato dextrose agar (PDA) media, respectively and incubated at 37 °C for 24 hours. The grown pure culture of bacteria and fungi colonies were then pre-cultured in nutrient broth and potato dextrose broth medium, respectively and kept overnight at 37 °C, followed by centrifuge at 10, 000 rpm for 5 min. The pellets were re-suspended and cell turbidity was assessed spectroscopically along with 0.5 McFarland standards (approximately 1.5 × 108 CFU/mL). The inocula were then used for the antibacterial assay (Jahiri 2013).

Screening for antimicrobial activity

The assay for antimicrobial activities of each extract was determined using Kirby Bauer's disc diffusion method with some modification as detailed in Sivaramakrishnan et al. (2015). The prepared bacteria and fungi inocula (size 1.5 x 108 CFU/mL) were spread aseptically using a cotton swab on Müller -Hinton agar and Sabouraud dextrose agar media, respectively. About 25 µL/disc solvent extract with concentrations of 50 mg/mL was loaded to each sterile Whatman disc 6 mm and allowed to dry and then placed on the inoculated media. Ciprofloxacin and fluconazole were used as positive controls for the bacteria and fungi evaluation, respectively while dimethyl sulfoxide (DMSO) was used as a negative control. The plates were incubated at 37 °C for 24 hours. The zones of inhibition produced were measured in millimetres (mm), while those with no zone of inhibition were

regarded as negative. The experiments were carried out in triplicates and the results are given as mean ± SE. The crude extracts of endophytic fungi with a shown high zone of inhibition against tested microorganisms were tested more for Minimum Inhibitory Concentration (MIC).

Minimum Inhibition Concentration (MIC) test

The MIC was determined on fungal crude extract which showed positive results against Gram positive and Gram negative bacteria. The tested bacteria were *Staphylococcus aureus and E. coli* grown overnight at 36 °C in Müller -Hinton agar. Inoculum for the assays was prepared by diluting in prepared 0.5 McFarland standards (approximately 1.5 × 108 CFU/mL). MIC tests were carried out according to Ellof (1998) where Muller-Hinton Broth was used on a tissue culture test plate (96 wells). The stock solutions of the fungal extracts were diluted with DMSO to the concentration of 2 mg/mL and transferred into the first well. The serial dilutions were then performed to fungal crude extract and ciprofloxacin (antibacterial standard) to make concentrations that ranged from 0.0152 to 2 mg/mL and 64 µg/mL, respectively, while DMSO was used as a negative control. The plates were sealed and incubated at 36 °C for 24 hours. Antimicrobial activity was detected by adding 20 µL of 0.5% triphenyl tetrazolium chloride to microplates well following incubation at 36 °C for 40 min. The presence of bacteria growth was detected by red/pink colour and the clear well indicated the inhibition of bacterial growth by the fungal crude extract. MIC was defined as the lowest concentration of fungal crude extract that inhibited visible growth.

Preliminary qualitative phytochemical screening of fungal metabolites produced by endophytic fungi

Phytochemical screening of alkaloids and phenols produced by the studied endophytic fungal secondary metabolites were determined following procedure as determined by (Sahu et al. 2014):

***Phenols*:** About 1 mL of crude extract of the secondary metabolites of fungal endophytes kept in a test tube was added with 2-3 drops of 20% NaOH solution. The colour of the solution changed to yellow then the solution turned colourless after the addition of acid, this test showed the presence of flavonoids.

***Alkaloids*:** The 1 mL crude extract of the fungi secondary metabolites was dissolved in an equal amount of 2 N HCl solutions. Then the mixture was treated with a few drops of Mayer's reagent (a mixture of 3 mL of potassium iodide solution with 2 mL mercuric chloride solution). The presence of the cream precipitate indicated the presence of alkaloids.

***Tannins*:** About 2 mL of fungal extracts were treated by adding an equal amount of alcoholic $FeCl_3$ reagent which turned the solution into bluish-black colour. Two drops of dilute H_2SO_4 were added which turned the colour to yellowish-brown precipitate that indicated the presence of tannins.

***Saponins*:** Determination of the presence of saponins was performed by the frothing test. About 1 mL of the fungal extract was vigorously shaken with distilled water then allowed to settle for 10 minutes. Then the formation of a fairly stable emulsion indicated the presence of saponins.

***Terpenoids*:** About 1 mL of fungal crude extract was added with 2 mL of chloroform followed by the addition of 3 mL of concentrated H_2SO_4. The formation of reddish-brown precipitate coloration at the interface indicated the presence of terpenoids.

***Steroids*:** Libermann-Burchard reaction was performed to determine the presence of steroids. The fungal extract was added in 2 mL of chloroform solution. Then the mixture was treated with acetic anhydride following the addition of a few drops of concentrated H_2SO_4. The presence of steroids was confirmed by the formation of a blue-green ring in the solution.

Secondary quantitative phytochemical screening of fungal crudes metabolite produced by endophytic fungi

Determination total flavonoid content

Determination of the total flavonoid content of the fungal crudes secondary metabolites were analyzed spectrophotometrically according to Chang et al (2002). Briefly, about 0.5 mL of each extract was diluted with 0.5 mL of 80% aqueous ethanol containing 10% aluminium chloride ($AlCl_3$). After one incubation hour at room temperature, the absorbance was detected spectrophotometrically at 420 nm using the same UV-Vis spectrophotometer model 6305 Jenway UK. Total flavonoid concentration was calculated using a quercetin standard calibration curve. Extract samples were evaluated at a final concentration of 0.1 mg/mL. Total flavonoids content was calculated as quercetin (mg/g) using a standard calibration curve. Data were expressed as Rutin equivalent/100 g of fungal crude extracts of secondary metabolites.

Determination of total phenol content

The analysis of the total phenolic content was determined by using the Folin-Ciocalteu colorimetric method described by Cai et al. (2004). Briefly, the diluted sample of the fungal crude extract of 0.4 mL was oxidized with 0.5 N Folin-Ciocalteu reagents for 4 min at room temperature, and then, following neutralization with saturated sodium carbonate (75 g/L). The absorbance of the resulting blue colour was measured at 765 nm after incubation for 2 hours at room temperature in dark with gallic acid taken as standard.

Results

Macro-morphological characterization

In this study, five endophytic fungi were isolated from the leaves, roots, barks, and seeds of *Moringa oleifera* Lam. Based on the macro-morphological characteristics, the five isolates fall in two distinct endophytic fungal groups corresponding to two genera *Fusarium* and *Nigrospora*. However, morphological characters could not identify the isolates into the species level, thus further identification was employed through a molecular method.

Fusarium species

The culture portrayed cottony mycelia texture with the regular shape at the edge. The colour of the colonies was white to pink then dark grey darkening on the surface with age while at the reverse side of the plate it was relatively black (Figure 1a & b). The microscopic feature observed was septate-branched hyphae with ellipsoid microconidia (Figure 1c).

Figure 1: *Fusarium* species isolated from (a) root, (b) seed and (c) hyphae observed under the light microscope.

Nigrospora species

Nigrospora species were growing very fast producing cottony white mycelia at a young stage (Figure 2a), which darken to grey and black with age while the reverse side of the colony was also black. The colony edge was

typically undulated. Microscopic observations revealed the abundant of ovoid to ellipsoid black conidia (Figure 2b) and the presence of undulated and branched septate hyphae lacking clamp connections (Figure 2c).

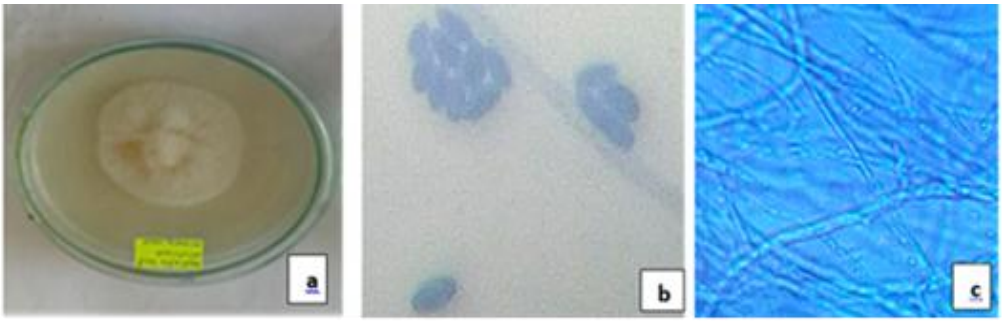

Figure 2: *Nigrospora* species from leaves (a) culture (b) conidia and (c) hyphae, microscopic observation under light microscope.

Colonization frequency

The frequency of colonization of the five endophytic fungi isolates is summarised in Table 2. *Fusarium* sp. occurred in all three parts of the plant where roots contributed 50% while the other parts (bark and seed) portrayed equal frequencies of 14.26% each. The *Nigrospora* sp. was isolated only from the roots with 50% frequency and leaves with 14.26% as shown in Table 2.

Molecular characterization of endophytic fungi

Confirmation of the conventionally identified endophytic fungi isolates from leaves, roots, barks, and seeds were done using rDNA sequences. The ITS sequences of each endophyte were searched against the NCBI database. The analysis showed that all sequences had greater than 95% identity similarity with previously reported fungal species in NCBI Genbank as shown in Table 2. The analysis, therefore, confirmed them to belong to two conventionally identified species of the genera *Nigrospora* and *Fusarium* in class Sordariomycetes of the phylum Ascomycota (Table 2).

Table 2: Identified fungi, colonizing frequency, and the percentage identity of isolated endophytic fungi from different parts of *M. oleifera* and that of taxa found in the NCBI

Plant parts	Identified fungi	Identity (%)	Colonizing frequency (%)
Leaves	*Nigrospora* sp.	98	14.6
Barks	*Fusarium* sp.	96	14.6
Roots	*Fusarium* sp.	95	50%
	Nigospora sp.	95	50%
Seeds	*Fusarium* sp.	98	14.6

Antimicrobial activities of secondary metabolites from endophytes

The study revealed that the ethyl acetate crude extracts of the metabolites of endophytic fungi isolated from leaves and roots exhibited antimicrobial activities with inhibition zones ranging from 9 to 13 mm against *B. substilis*, *S. aureus*, and *C. albicans* but had no activity against *E. coli* (Table 3). It was also noted that the crude extract of endophytic fungi from

seeds possessed the least inhibition zones ranging from 7 to 9 mm against tested *B. substilis*, *S. aureus*, *C. albicans* while no zone of inhibition against *E. Coli*. Extracts from the bark also showed antibacterial activities with inhibitory zones ranging from 8 to 9 mm against *B. substilis* and *S. aureus* while no zones of inhibitions were produced against both *E. coli* and *C. albicans* (Table 3).

Table 3: Antimicrobial activities of the crude extract of endophytic fungi isolated from *M. oleifera* against the pathogen of medical importance

Source	Endophytic fungi	Inhibition zone (mm) concentration 20 µg/disc			
		Bacillus subtilis	*Staphylococcus aureus*	*Escherichia coli*	*Candida albicans*
Leaves	*Nigrospora* sp.	10 ± 0.33	11 ± 0.33	–	9 ± 0.44
Roots	*Fusarium* sp.	13 ± 0.29	12 ± 0.44	–	10 ±0.67
Barks	*Fusarium* sp.	8 ± 0.33	9 ± 0.33	–	–
Seeds	*Fusarium* sp.	9 ± 0.33	9 ± 0.17	–	7.3±0.33
	+ve	25 ± 1.0	25 ± 0.8	21 ± 2.0	24 ± 1.9
	–ve	–	–	–	–

Note: +ve = positive control –ve = negative control (DMSO), Inhibition zones expressed as mean ± standard deviation and - = no zone of inhibition

Minimal Inhibitory Concentration (MIC)

The results showed that both Gram positive and fungi were susceptible to all three crude extracts tested. The MIC values of two tested pathogens were isolated from seeds and roots in all tested pathogen bacteria and fungi ranged from 0.6 mg/mL to 1 mg/mL. It was also observed that the fungal crude extracts from the leaf had antimicrobial activities against *Bacillus subtilis* and *Staphylococcus aureus* at MIC 0.016 mg/mL and *Candida albicans* at0.5 mg/mL, while the fungi extract from seeds and roots performed antimicrobial activity against *Candida albicans* with MIC 0.5 mg/mL (Table 4).

Table 4: Minimum Inhibitory Concentrations (mg/mL) of the three endophytic fungi crude extracts from *M. oleifera* against bacteria and fungi stains.

Source	Endophyte	Tested pathogens		
		Bacillus subtilis	*Staphylococcus aureus*	*Candida albicans*
Leaves	*Nigrospora* sp.	0.016 ± 0.03	0.016 ± 0.02	0.5 ± 0.03
Roots	*Fusarium* sp.	0.50 ± 0.02	0.5 ± 0.01	1 ± 0.01
Seeds	*Fusarium* sp.	1 ± 0.09	1 ± 0.02	1 ± 0.02
	SR	0.006 ± 0.01	0.006 ± 0.03	0.006 ± 0.01

Note: SR = Standard reference, Concentration presented as mean ± standard deviation

Preliminary qualitative phytochemical composition of fungal secondary metabolites produced by endophytic fungi

The results from the quantitative screening of the three crude extracts of endophytic fungi secondary metabolites showed the presence of alkaloids, phenols, flavonoids, tannins, terpenoids, and steroids while saponins were absent in crude extracts from secondary metabolites of endophytic fungi *Fusarium* sp. from seeds.

Secondary quantitative phytochemical composition of fungal crude metabolites produced by endophytic fungi

The secondary quantitative phytochemical screening of extracts of endophytic fungi secondary metabolites was performed to

determine the total flavonoid content and the results revealed that the three tested extracts have total flavonoid content ranging from 168.14 to 284.07 mg/g as shown in Figure 3. The highest flavonoid values 284.07 mg/g were from *Fusarium* sp isolated from root while the lowest values 168.14 mg/g was from *Fusarium* sp. isolated from seed.

Furthermore, the results revealed that the total phenolic content of all three fungal extracts ranged from 300.12 mg/g to 380.31 mg/g. The maximum value was from *Fusarium* sp. 380.31 mg/g of the root and lowest value was recorded from *Fusarium* sp. 300.12 mg/g of the seed as shown in Figure 3.

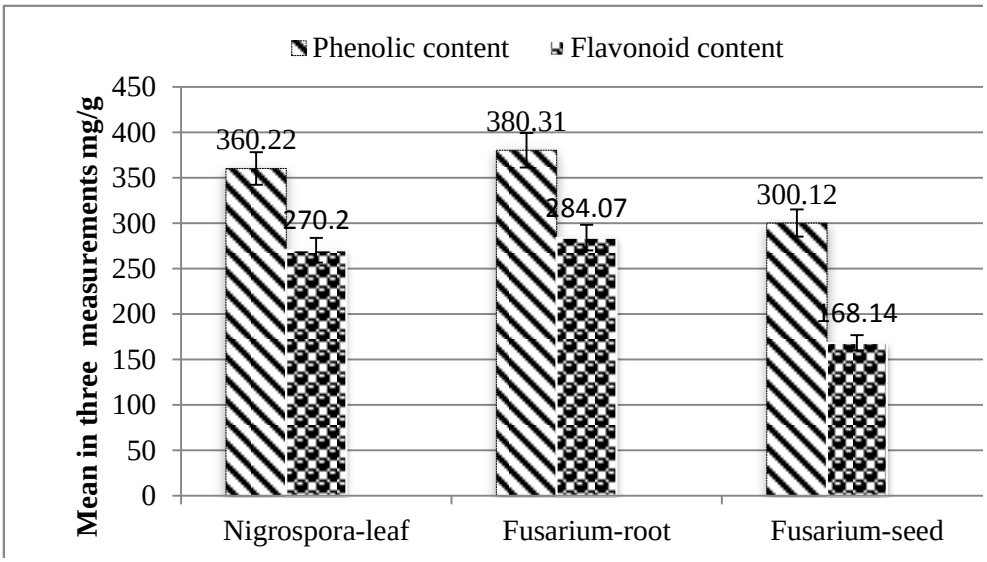

Figure 3: Total Phenolic and flavonoid content in three crude extracts of endophytic fungi secondary metabolites from *M.oleifera*

Discussion

This paper constitutes the first report of the isolation of endophytic fungi from *M. oleifera* from Tanzania, intending to characterize and screen their antimicrobial activities against pathogens of medical importance. It was observed that different parts of this plant harboured five endophytic fungi (Table 2). Basing on morphological and molecular characterization, the isolates were confirmed to belong to *Nigrospora* sp (2 species) and *Fusarium* sp (3 species). The isolate identified revealed that *Fusarium* sp. were dominant in seeds, barks, and roots while *Nigrospora* sp. was dominant only in leaves and completely missing in other parts. These findings are similar to the previous reports, for example, Dhanalakshmi et al. (2013), and Masumi et al. (2015). The former isolated the same endophytic fungi from *Moringa oleifera* of Yercaud hills in India, while the latter reported on the abundance of *Fusarium* species isolated from a different Thymus medicinal plant. This shows that the isolated endophytes have a wide host range as noted by Huang et al. (2008). On the other hand, the colonization frequency of endophytic fungi in the studied plant parts varied with roots flaunted the highest percentage (Table 2) than the rest of the plant parts. Additionally, the studied *Moringa oleifera* harboured a relatively low number of endophytic isolates compared to what has been reported in other studies (see for example Naik et al. 2014). This might be due to many factors including diversified microhabitats within the studied plant,

geographical locations and seasonal changes among others; the factors that have been well reported by Wilson and Carroll (1994), Asan et al. (2010), and Rajeswari et al. (2014), to influence the diversity and abundance of endophytes in host plants.

Screening of antimicrobial activities of the crude extracts of secondary metabolites from endophytic fungi revealed that the studied endophytic fungi metabolites exhibited varied inhibitory effects against Gram positive bacteria *Bacillus subtilis*, *Staphylococcus aureus*, and a pathogenic fungi *Candida albicans*, while they showed no activity to the Gram negative bacteria *E. coli*. According to Powthong et al. (2013), the Gram negative bacteria are resistant to most of the antibacterial activity due to the presence of lipopolysaccharide protein and addition thinner layer of peptidoglycan which makes their cells more complex as compared to Gram positive bacteria. Similar observations have been reported in other studies (e.g. Zhao et al. 2012, Souza et al. 2016).

Also, MIC results were carried out on the three crude extracts of the fungal secondary metabolites which showed the effects against three tested pathogens. The MIC results revealed that the crude extracts of fungal metabolites from leaf observed to have strong antimicrobial activity against three tested pathogens with MIC values ranging from 0.5 mg/mL to 0.016 mg/mL. It was further recorded that the extracts from fungal metabolites from root and seed showed moderate, antimicrobial activities against all tested pathogen with MIC values 0.6 mg/mL to 1 mg/mL. The results are similarly to that of Arora and Kaur (2019) who determined the antimicrobial activities of the *M. oleifera* from botanical garden of Guru Nanak Dev University, Amritsar, India. Basing in our findings, the MIC values were classified as strong and others moderate according to a proposed classification done by Aligiannis et al. (2001) who proposed that, the strong inhibitors MIC have values < 0.5 mg/ mL; moderate inhibitors have MIC values between 0.6 and 1.5 mg /mL) and weak inhibitors have MIC values above 1.6 mg /mL). Based on this classification, our study results considered MIC values below 1 mg/mL of the crude extracts of fungal secondary metabolites as having potential antimicrobial activities.

Additionally, it was recorded that quantitative phytochemical screening of the fungal crude extracts revealed the presence of phytochemical constituents such as alkaloids, phenols, flavonoids, tannins, and terpenoids and steroids while saponins were not present in the crude extracts of the secondary metabolites of endophytic fungi *Fusarium* sp. from seeds. On the other hand, the secondary screening of the crude extracts from secondary metabolites of endophytic fungi of *Fusarium* sp. from roots revealed to have the highest phenols and flavonoids content than that of *Nigrospora* sp. from leaves and seeds. According to various findings, it has been reported that endophytic fungi harboured in medicinal plants may produce secondary metabolites with bioactive compounds similar to those of their hosts (Strobel 1999, Zhao et al. 2010) which is same as our findings. For example, Kasolo et al. (2011) identified phytochemicals contained in medicinal plant *Moringa oleifera* roots which are tannins, catechol tennins, steroids and triterpenoids, flavonoids, saponins, anthraquinones, alkaloids, while Brighente et al. (2007) reported that natural compounds from medicinal plants contain taninns flavonoids, tocopherols, cathechins, phenols and flavonoids with promising antimicrobial activities against human pathogens. The results of this study revealed the ability of the studied crude extracts of secondary metabolites from endophytic fungi isolated from the studied medicinal plants to have both, antimicrobial activity against human pathogens and possess phytochemical compounds. It is also interesting in this study to note that, the secondary metabolites of endophytes isolated from *Moringa oleifera* in Tanzania exhibited similar antimicrobial activities against the pathogens of medical importance like those endophytic fungi metabolites isolates from the

same medicinal plant found in India reported by Arora and Kaur (2019). The crude extracts of the secondary metabolites of the endophytic fungi had marked inhibitory activities on pathogens of medical importance that underscore their enormous traditional uses in the country (Table 1). Results also showed antimicrobial activities against the eminent human pathogens (Table 3). These findings are in agreement with the known traditional uses of the studied medicinal plant that specify the used plant parts (Table 1). For example, the leaves and roots of *M. oleifera* are traditionally used in the treatment of headache, stomach ache, antifertility, gastric ulcers and controlling of blood sugar while the seeds are used in treating scurvy and hysteria (Mbwambo et al. 2007). The used plant parts undergo different processing including boiling that might alter the chemical compositions of the compounds in the plant. It was, however, interesting to note that the secondary metabolites of the studied endophytic fungi isolated from medicinal plant *M. oleifera* portrayed some medicinal properties (antimicrobial activities) as that of the host plant (Table 3), similar findings also reported by Strobel (1999) and Zhao et al. (2010). It is therefore of paramount importance to investigate the endophytic fungi from these medicinal plants which could be an alternative source of novel compounds, thus help in conserving the threatened medicinal plants due to overexploitation. A future study could be focused on understanding the influence of seasonality on the colonization and abundance of endophytic fungi from this noble plant and the elucidation of active compounds contributing to the activities observed.

Conclusion

This study presents the antimicrobial activities of secondary metabolites from endophytic fungi isolated from *Moringa oleifera*. Both morphological and molecular methods delineated two important endophytic fungal genera; *Fusarium* and *Nigrospora* species harboured by the plant. The secondary metabolites of the isolated endophytic fungi portrayed antimicrobial activities against human pathogens *Bacillus subtilis*, *Staphylococcus aureus*, and *Candida albicans*. It was also noted that the crude extracts of the secondary metabolites from endophytic fungi isolates contained phytochemical content, which might be a source of therapeutic potential for the studied plants. Further studies are recommended for the determination of the toxicity of the secondary metabolites of these endophytic fungi in paving a way for new therapeutic inventions.

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References

Aligiannis N, Kalpoutzakis E, Mitaku S, and Chinou IB 2001 Composition and antimicrobial activity of the essential oils of two Origanum species. *Journal of Agricultural and Food Chemistry* 49(9): 4168-4170.

Andrew A 2010 *Effect of Moringa oleifera leaf powder supplement to improve the nutritional status of severely malnourished children aged 6-24 months in Arusha region*. Ph.D. Dissertation, Sokoine University of Agriculture, Tanzania.

Arora DS and Kaur N 2019 Antimicrobial potential of fungal endophytes from *Moringa oleifera*. *Appl. Biochem. Biotechnol*. 187(2): 628-648.

Asan A, Okten SS and Sen B 2010 Airborne and soilborne microfungi in the vicinity Hamitabat Thermic Power Plant in Kirklareli City (Turkey), their seasonal distributions and relations with climatological factors. *Environ. Monitor. Assess*. 164(1-4): 221-231.

Augustino S and Gillah PR 2005 Medical plants in urban districts of Tanzania: plants,

gender roles, and sustainable use. *Int. Forest. Rev*. 7 (1): 44-58.

Brighente IMC, Dias M, Verdi LG, and Pizzolatti MG 2007 Antioxidant activity and total phenolic content of some Brazilian species. *Pharmaceut. Biol.* 45(2): 156-161.

Campos FF, Junior S, Policarpo A, Romanha AJ, Araújo MS, Siqueira EP, Resende JM, Alves T, Martins-Filho OA, Santos VLD, and Rosa CA 2015 Bioactive endophytic fungi isolated from *Caesalpinia echinata* Lam. (Brazilwood) and identification of beauvericin as a trypanocidal metabolite from *Fusarium* sp. *Memórias do Instituto Oswaldo Cruz* 110(1): 65-74.

Cai Y, Luo Q, Sun M, Corke H 2004 Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. *Life Sci*. 74: 2157-2184.

Chandra R, Kumarappan CT, Kumar J, and Mandal SC 2010 Antipyretic activity of JURU-01-a polyherbal formulation. *Global J. Pharmacol.* 4(1): 45-47.

Chang C, Yang M, Wen H, Chern J 2002 Estimation of total flavonoid content in propolis by two complementary colorimetric methods. *J. Food Drug Analysis* 10: 178-182.

Choudhary MK, Bodakhe SH, and Gupta SK 2013 Assessment of the antiulcer potential of *Moringa oleifera* root-bark extract in rats. *J. Acupunct. Meridian Stud*. 6: 214-220.

Daba M 2016 Miracle tree: A review on multi-purposes of *Moringa oleifera* and its implication for climate change mitigation. *J. Earth Sci. Clim. Change* 7(4).

Dhanalakshmi R, Umamaheswari S, Sugandhi P and Prasanth DA 2013 Biodiversity of the endophytic fungi isolated from *Moringa oleifera* of Yercaud hills. *Int. J. Pharm. Sci. Res*. 4(3): 1064-1068.

Ellof JN 1998 A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. *Planta Medica* 64: 711-713.

Ezeamuzie IC, Ambakederemo AW, Shode FO and Ekwebelem SC 1996 Anti-inflammatory effects of Moringa oleifera root extract. *Int. J. Pharmacog.* 34: 207-212.

Fahey JW 2005 *Moringa oleifera*: A review of the medical evidence for its nutritional, therapeutic and prophylactic properties. Part 1. *Trees for Life J.* 1(5).

Gennaro M, Gonthier P, and Nicolotti G 2003 Fungal endophytic communities in healthy and declining Quercus robur L. and Q. cerris L. trees in northern Italy. *J. Phytopathol.* 151: 529-534.

Huang WY, Cai YZ, Hyde KD, Corke H, Sun M 2008 Biodiversity of endophytic fungi associated with 29 traditional Chinese medicinal plants. *Fungal Diversity* 33: 61-75.

Hussein J, Tibuhwa DD, Mshandete A, and Kivaisi AK 2014 Molecular phylogeny of saprophytic wild edible mushroom species from Tanzania based on ITS and nLSU rDNA sequences. *Curr. Res. Environ. Appl. Mycol.* 4: 250-260.

Jahiri X 2013 *Isolation of fungal endophytes from grasses by laser micro dissection and pressure catapulting*. Master Thesis The Artic University Norway.

Kameshwari S, Mohana B, and Thara Saraswathi KJ 2015 Isolation and identification of endophytic fungi from Urginea indica, a medicinal plant from diverse regions of south India. *Int. J. Latest Res. Sci. Technol*. 4(1): 75-80.

Kasolo JN, Bimenya GS, Ojok L, and Ogwal-Okeng JW 2011 Phytochemicals and acute toxicity of Moringa oleifera roots in mice. *J. Pharmacog. Phytother.* 3(3): 38-42.

Kubmarawa D, Khan ME, Punah AM and Hassan M 2008 Phytochemical screening and antimicrobial efficacy of extracts from *Khaya senegalensis* against human pathogenic bacteria. *Afr. J. Biot*. 7: 4563-4566.

Kumar DS and Hyde KD 2004 Biodiversity and tissue recurrence of endophytic fungi in Tripterygium wilfordii. *Fungal Diversity* 17: 69-90.

Kumar S 2017 Medicinal importance of *Moringa oleifera*: drumstick plant. *Indian J. Sci. Res*. 16(1): 129-132.

Mahdi T, Mohamed I and Yagi S 2014 Endophytic fungal communities associated with ethnomedicinal plants from Sudan and their antimicrobial and antioxidant prospective. *J. Forest Prod. Indust.* 3(6): 248-256.

Mangale SM, Chonde SG and Raut PD 2012 Use of *Moringa oleifera* (drumstick) seed as natural absorbent and an antimicrobial agent for groundwater treatment. *Res. J. Recent Sci*. 1(3): 31-40.

Masumi S, Mirzaei S, Zafari D and Kalvandi R 2015 Isolation identification and biodiversity of endophytic fungi of Thymus. *Progress in Biological Sciences* 5(1): 43-50.

Mbwambo Z, Mainen J, Moshi, Edmund J, Kayombo MC, Kapingu LM, George T, Ndunguru J and Aroldia M 2007 Promotion of community-based cultivation of *Hibiscus sabdariffa*, *Moringa oleifera*, *Adansonia digitata* and *Aloe vera* for use as herbal nutritional supplements for people living with HIV/AIDS. The MUHAS repository Project report.

Monera TG and Maponga CC 2012 Prevalence and patterns of *Moringa oleifera* use among HIV positive patients in Zimbabwe: a cross-sectional survey. *J Public Health Africa* 3: 6-8.

Naik BS, Krishnappa M and Krishnamurthy YL 2014 Biodiversity of endophytic fungi from seven herbaceous medicinal plants of Malnad region, Western Ghats, southern India. *J. Forestry Res.* 25: 707-711.

Olugmemi TS, Mutayoba SK and Lekule FP 2010 Effect of Moringa (*Moringa oleifera)* inclusion in a cassava based diets to Broiler chickens. *Int. J. Poultry Sci*. 9(4): 363-367.

Omacini M, Chaneton EJ, Ghersa CM, and Müller CB 2001 Symbiotic fungal endophytes control insect host-parasite interaction webs. *Nature* 409: 78-81.

Pamphile JA and Azevedo JL 2002 Molecular characterization of endophytic strains of *Fusarium verticillioides* (*Fusarium moniliforme*) from maize (*Zea mays* L). *World J. Microbiol. Biotechnol*. 18: 391-396.

Powthong P, Jantrapanukorn B, Thongmee A and Suntornthiticharoen P 2013 Screening of antimicrobial activities of the endophytic fungi isolated from *Sesbania grandiflora* (L). Pers. *J. Agr. Sci. Tech*. 15: 1513-1522.

Purwal L, Pathak AK and Jain UK 2010 In vivo anticancer activity of the leaves and fruits of *Moringa oleifera* on mouse melanoma. *Pharmacologyonline* 1: 655-665.

Rajeswari S, Umamaheswari S, Prasanth D and Rajamanikandan KP 2014 Study of the endophytic fungal community of *Moringa oleifera* from Omalur region Salem. *Int. J. Pharmaceut. Sci. Res.* 5(11): 4887-4892.

Ramesh V, Arivudainambi USE, Rajendran A 2017 The molecular phylogeny and taxonomy of endophytic fungal species from the leaves of Vitex negundo L. *Studies in Fungi* 2(1): 26-38.

Rajeswara RBR, Syamasundar KV, Rajput DK, Nagaraju G and Adinarayana G 2012 Biodiversity, conservation and cultivation of medicinal plants. *J. Pharmacogn* 3: 59-62.

Rhoden SA, Garcia A, Rubin Filho CJ, Azevedo JL and Pamphile JA 2012 Phylogenetic diversity of endophytic leaf fungus isolates from the medicinal tree *Trichilia elegans* (Meliaceae). *Genet. Mol. Res.* 11: 2513-2522.

Sahu M, Vermaand D and Harris KK 2014 Phytochemicalanalysis of the leaf, stem and seed extracts of Cajanus cajan L (Dicotyledoneae: Fabaceae). *World J. Pharm. Pharmaceut. Sci*. 3(8): 694-733.

Sandhu SS, Kumar S and Aharwal RP 2014 Isolation and identification of endophytic fungi from *Ricinus communis* Linn. and

their antibacterial activity. *Int. J. Res. Pharm. Chem.* 4: 611-618.

Sebastian M 2009 RAVI "Impact Stories" Arusha MAC and the Moringa Network, Tanzania. www.linkinglearners.net

Sivaramakrishnan S, Prabukumar S, Rajkuberan C, and Ravindran K 2015. isolation and characterization of endophytic fungi from medicinal plant *Crescentia cujete* L. and their antibacterial, antioxidant and anticancer properties. *Int. J. Pharm Sci*. 7: 316-321.

Souza IF, Napoleão TH, de Sena KX, Paiva PM, de Araújo JM and Coelho LC 2016 Endophytic microorganisms in leaves of *Moringa oleifera* collected in three localities at Pernambuco State, Northeastern Brazil. *Brit. Microbiol. Res. J.* 13(5): 1-7.

Stierle A, Strobel GA and Stierle D 1993 Taxol and taxane production by *Taxomyces andreanae*, an endophytic fungus of Pacific yew. *Science* 260 (5105): 214-216.

Strobel GA, Miller RV, Martinez-Miller C, Condron MM, Teplow DB and Hess W M 1999 Cryptocandin, a potent antimycotic from the endophytic fungus Cryptosporiopsis cf. quercina. *Microbiol*. 145: 1919-1926.

Subbulakshmi GK, Thalavaipandian A and Ramesh V 2012 Bioactive endophytic fungal isolates of *Biota orientalis* (L) Endl., *Pinus excelsa* Wall. and *Thuja occidentalis* L. *Int. J. Adv. Lif. Sci*. 4: 9-15.

Suryanarayanan TS, Venkatesan G and Murali TS 2003 Endophytic fungal communities in leaves of tropical forest trees: diversity and distribution patterns. *Curr. Sci.* 85: 489–493.

Tibuhwa DD 2016 Oxalis corniculata L. in Tanzania: traditional use, cytotoxicity and antimicrobial activities. *J. Appl. Biosci*. 105: 10055-10063.

Tibuhwa DD 2017 Antioxidant potentialities and antiradical activities of *Oxalis Corniculata* Linn from Tanzania. *J. Appl. Biosci*. 116: 11590-11600.

Verma VC, Kharwar RN and Strobel GA 2009 Chemical and functional diversity of natural products from plant-associated endophytic fungi. *Nat. Product. Commun*. 4: 1511-1532.

Waqas M, Khan AL, Kamran M, Hamayun M, Kang SM, Kim YH and Lee IJ 2012 Endophytic fungi produce gibberellins and indoleacetic acid and promote host-plant growth during stress. *Molecules* 17: 10754-10773.

White TJ, Bruns TD, Lee S and Taylor JW 1990 Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics. In: PCR Protocols: A Guide to Methods and Applications (Innis MA, Gelfand DH, Sninsky JJ and White TJ, eds.). Academic Press, San Diego, 315-322.

Wilson D and Carroll GC 1994 Infection studies of *Discula quercina* and endophyte of *Quercus garryana*. *Mycologia* 86: 635-647.

Zhang Z, Schwartz S, Wagner L and Miller W 2000 A greedy algorithm for aligning DNA sequences. *J. Comput. Biol*. 7: 203-214.

Zhao JH, Zhang YL, Wang LW, Wang JY and Zhang CL 2012 Bioactive secondary metabolites from Nigrospora sp. LLGLM003, an endophytic fungus of the medicinal plant *Moringa oleifera* Lam. *World J. Microbiol. Biotechnol*. 28: 2107-2112.

Zhao J, Zhou L, Wang J, Shan T, Zhong L, Liu X and Gao X 2010 Endophytic fungi for producing bioactive compounds originally from their host plants. *Curr. Res. Technol. Educ. Trop. Appl. Microbiol. Microbial Biotechnol.* 1: 567-576.