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Isolation and Characterization of Endophytic Fungi from Medicinal Plant *Warburgia ugandensis*

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Abstract

The aim of this study was to isolate fungal endophytes from medicinal plant *Warburgia ugandensis* and determine antimicrobial activity of their metabolites on three human pathogens; (*Candida albicans* 90018, *E coli* 25922 and *Staphylococcus aureus* 29213). Seventeen (17) endophytic fungi were isolated and identified as; *Nigrospora oryzae*, *Aspergillus flavus*, *Cladosporium sp.* (2), *Fusarium Oxysporum*, *Phomopsis sp.*(2), *Colletotrichum acutatum*, *Altanaria sp.* (2), *Cochliobolus sativus*, *Bionectria ochroleuca*, *Phyllosticta gardeniicola*, *Guignardia mangiferae*, *Tricharina gilva*, *Diaporthe amygdali* and *Trichoderma harzianum*. Phytochemical screening of their metabolites showed absence of phenols and alkaloids; presence of saponins, tannins, alkaloids, flavonoids, sterols and glycosides in most of the extracts. Most of the fungal endophytes didn't seem to have active metabolites after screening for presence of antimicrobial activities. Extracts from *Phomopsis mali*, *Alternaria alternata* and *Fusarium oxysporum* had minimum antimicrobial activity. The study showed that fungal endophytes can be a potential source of metabolites which can be useful in pharmaceutical industry.

Key words: Fungal endophytes, *Warburgia ugandensis*, phytochemicals, active metabolites, antimicrobial activity

1. Introduction

Endophytes are microorganisms living in the tissues of plants without causing any harm, both the endophytes and the plants experience symbiotic relationship (Xin *et al*., 2017). The endophytes produce bioactive compounds which help the host plant improve the nutritional status, pest and disease resistance and physical stress tolerance (Ladoh *et al*., 2015, Jeffrey *et al*., 2008). Some of the bioactive compounds produced by endophytes inhabiting various plant species can be used in agricultural, pharmaceutical and food industries since they have been reported to have antimicrobial, antimalarial activities and can also act as enzymes (Mahsunah *et al*., 2013). Such compounds include alkaloids, terpenoids, steroids, quinones, flavanoids, phenols, tannins, anthraquinones, phenolic acids, and peptides. *Taxomyces andreanae*, an endophytic fungi of the class hyphomycete, has been reported to produce taxol which is an anti – cancer agent (Adeleye *et al*., 2015).

Warburgia ugandensis, also referred to as Kenyan green heart tree, is a spreading evergreen tree which is 4.5-30 m tall and 70 cm in diameter. The bark is smooth or scaly, pale green or brown, and it's clear of branches for about 3 m height (http://www.worldagroforestry.org, 2009). It is commonly found in tropical Africa. The extracts have been used over years as an alternative medicine for treatment of bronchial infections, parasitic infections, stomachache, cough, toothache, common cold, fever, malaria, oral thrush, muscle pain, constipation,

weak joints, cystitis, measles and diarrhoea among other diseases. Pharmacological studies have shown that *Warburgia ugandensis* extracts have antimicrobial activities (Abuto *et al*., 2016). However, it is still not clear if the symbiotic relationship between *Warburgia ugandensis* and the endophytes in it contribute to its medicinal value. This study aimed at isolating fungal endophytes from *Warburgia ugandensis*, extracting their secondary metabolites and testing their activity against *E. coli*, *Staphylococcus aureus* and *Candida albicans*.

2. Materials and Methods

2.1 Plant Material Collection

Plant material was collected from Mt Kenya region forest. Purposive sampling method was applied where by two zones which had healthy *Warburgia ugandensis* tree were chosen with the help of local guides. The leaves, stem, bark and the roots were selected for this study. The plant material was put in well labeled sterile plastic paper bags and transported to the laboratory in ice box where it was stored in a refrigerator at 4ºC awaiting processing. The plant material was processed after 48 hours of collection (Nalini *et al*., 2014).

2.2 Isolation of Endophytic Fungi

Isolation of endophytic fungi was done according to (Petrini & Fisher, 1986) with slight modifications. The plant material was thoroughly rinsed with running tap water to remove dust, soil particles and debris (Sardul, 2014). Surface sterilization was done by immersion of the plant material in 75% ethanol for 1 minute followed by 12% Sodium hypochlorite for 1 minute then rinsed twice in sterile distilled water. The plant material was allowed to dry on a sterile filter paper after which it was cut in to pieces of 3 - 3.5cm with a sterile scalpel. Four pieces of each part were placed on tap water agar plate using a sterile forceps and incubated for 6 days at 26 - 27^{0}C. Fungal hyphae tips growing from the plant tissues were sub cultured on Potato Dextrose Agar (PDA) supplemented with 250mg/L streptomycin to prevent growth of bacteria followed by incubation at 26 - 27^{0}C for 4 days (Gond *et al*., 2007). Colony purification was done by further sub culturing the fungal colonies in PDA until pure isolates were acquired.

2.3 Identification of Endophytic Fungi

Isolated fungal endophytes were identified using phenotypic and microscopic characterization up to genus level (Ellis *et al*., 2007; Hunter *et al*., 1998; www.hayesmicrobial.com/library.php). Further characterization was done using molecular methods to identify the isolates up to species level. Pure cultures of fungal isolates were sent to 'Macrogen sequencing company' Amstelveen - Netherlands, for DNA extraction, PCR, purification of PCR products and sequencing. The primers used were NS1 5' (GTA GTC ATA TGC TTG TCT C) 3' and NS24 5' (AAA CCT TGT TAC GAC TTT TA) 3'. The region of target during sequencing was 18s rRNA gene region. BLAST analysis was done on edited sequences to get the identity of the isolates from NCBI (National Center for Biotechnology Information) website depending on the maximum score, total score, query Cover and the percentage identity. Assembled sequences were submitted to NCBI for accession numbers. The same sequences were used to construct a phylogenetic tree using MEGA7 tool to establish isolate relationship (Kumar *et al*., 2016). The evolutionary history was inferred using UPGMA (Unweighted Pair Group Method with Arithmetic Mean) method. The tree was drawn to scale with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2004). All positions containing gaps and missing data were eliminated.

2.4 Extraction of Metabolites

Mass cultivation of endophytes was done according to (Karunai *et al*., 2014) with slight modifications. Agar blocks of actively growing pure fungi (3-4mm diameter) were inoculated in a 200ml universal bottle containing 100ml of sterile nutrient broth followed by incubation at 26 - 27^{0}C in a shaker for 14 days. The cultures were filtered after the incubation period by use of whatman filter papers to remove the mycelia. Part of the media sample was sterilized through microfiltration to remove the spores. This formed the crude extract which was preserved at -20^{0}C for further analysis.

Metabolites from endophytic fungi were extracted using ethyl acetate where, equal volumes of ethyl acetate were added to the filtrates then shaken well for 10 minutes to mix the contents. The solutions were transferred to separating funnel, allowed to settle for 5 minutes such that two layers (media layer and ethyl acetate layer) were formed which were collected separately. The ethyl acetate was evaporated to dryness using a vacuum rotary evaporator at 35 – 40^{0}C. The extracts were reconstituted using DMSO then sterilized through microfiltration and preserved for further analysis (Karunai and Balagengatharathilagam, 2014). This formed the ethyl acetate extract.

2.5 Screening Endophytic Fungal Extracts for Phytochemicals

Primary phytochemical screening of the crude extracts for tannins, saponins, sterols, glycosides, alkaloids, phenols, flavonoids and anthraquinones was done according to the standard protocols described by (Lemino and Bag, 2013, Mohammed *et al*., 2014).

2.6 Screening the Endophytic Fungi for Antimicrobial Activities

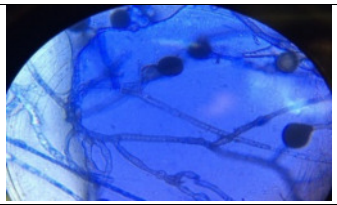
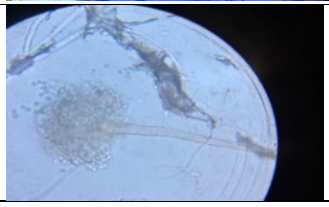
Screening for antimicrobial activities was done both for the crude extract and the ethyl acetate extract using disc diffusion technique. Three pure test organisms were obtained from Center for Microbiology Research - Kenya Medical Research Institute (CMR – KEMRI) laboratory; *Staphylococcus aureus* (ATCC 25923), *E. coli* (ATCC 25922) and *Candida albicans* (ATCC 90018). The test organisms were sub cultured in nutrient agar and after 24 hours incubation at 37^0C, 0.5 McFaland solution of the test organisms was prepared (Jennifer, 2001) to make the test inoculum. The test organisms were inoculated on well labeled nutrient agar plates by spreading the inoculum uniformly on the agar using sterile cotton sticks. Six mm (6mm) diameter sterile discs were impregnated with 20 - 25µl of the fungal extracts then transferred to the plates inoculated with the test organisms (Son and Cheah, 2002). Streptomycin and fluconazole were used as standard controls for *Staphylococcus aureus*, *E coli* and *Candida albicans* respectively. The negative control used was 0.1% DMSO. The plates were incubated for 24 hours at 37^0C after which the diameters of the zones of inhibition were measured to the nearest millimeter (mm).

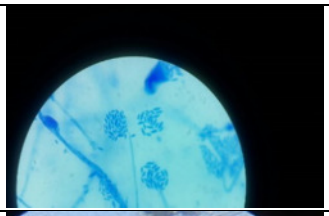
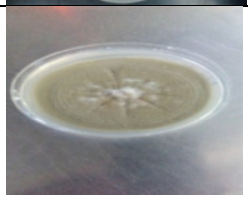
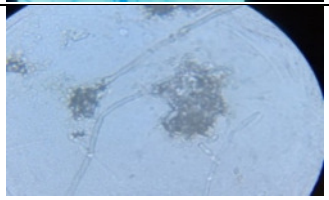
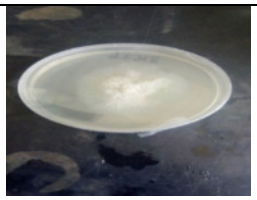
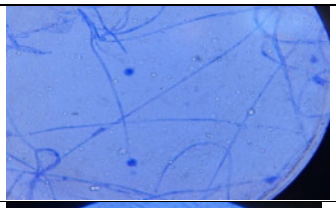
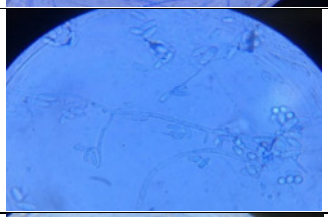
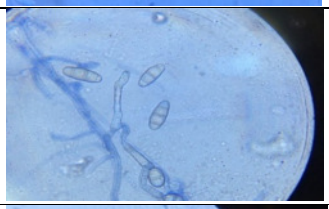
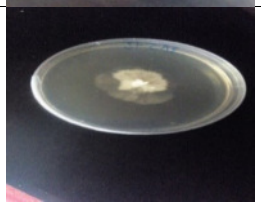
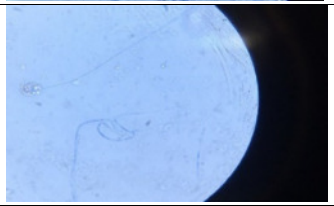
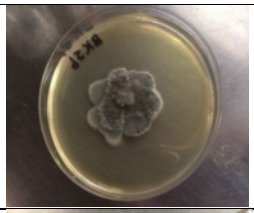
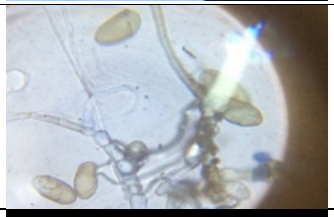
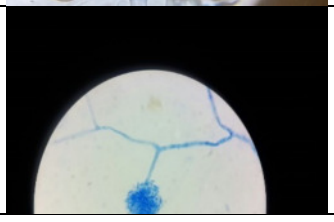
3. Results and Discussion

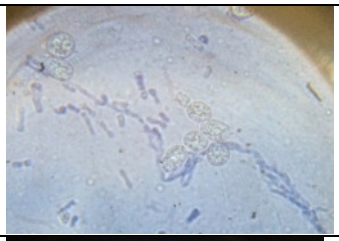
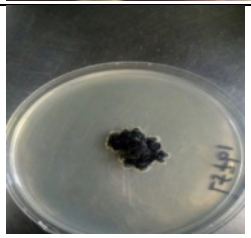
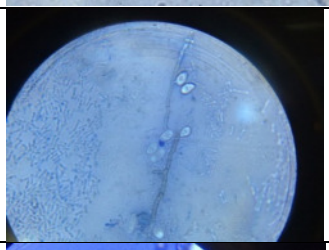
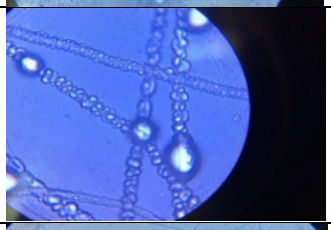
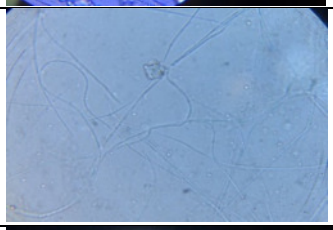
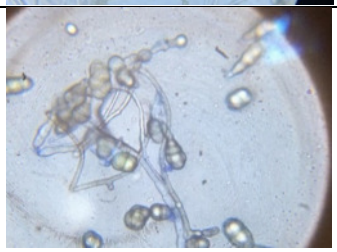
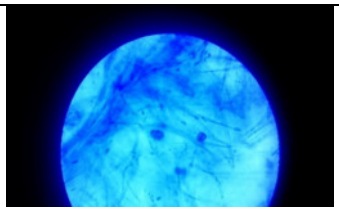
3.1 Isolation and Identification of Endophytic Fungi

A total of sixty (60) fungal isolates were isolated from *Warburgia ugandensis* collected from Mount Kenya forest in the year 2017. Twenty (20) isolates were isolated from the leaves, seventeen (17) from stem, fourteen (14) from bark and nine (9) from the root. All the isolates were characterized using phenotypic and microscopic methods. They were preliminary placed in fourteen (14) different genera namely *Nigrospora*, *Aspergillus*, *Cladosporium*, *Fusarium*, *Phomopsis*, *Colletotrichum*, *Alternaria*, *Cochliobolus*, *Bionectria*, *Phyllosticta*, *Guignardia*, *Tricharina*, *Diaporthe* and *Trichoderma*. A further characterization using sequencing methods of identification targeting 18S rRNA gene region classified the isolates in to species level (Table 1).

Table 1: Macro, micro, morphological and molecular identities of the endophytes isolated from *W. ugandensis*

Isolate Code	Fungi in culture plate	Microscopic (magnification ×100)	Identity	Accession No.
Lf3b			*Nigrospora oryzae strain: IFO 32860* max score: 2212, total score: 2212, query cover: 98%, Ident: 98%	MH014997
Lf3a2			*Aspergillus flavus strain Ya1* max score: 2179, total score: 2179, query cover: 99%, Ident: 98%	MH014996

LfZA2			*Fusarium oxysporum strain M1-EGY* max score: 2174, total score: 2174, query cover: 99%, Ident: 98%	MH015007
LfZAb			*Cladosporium bruhnei strain USN 11* max score: 2094, total score: 2094, query cover: 98%, Ident: 98%	MH015009
Bk2b			*Phomopsis sp. M-32 strain* max score: 2013, total score: 2013, query cover: 99%, Ident: 96%	MH013432
LfB7			*Colletotrichum acutatum strain BBA 68396* max score: 2174, total score: 2174, query cover: 99%, Ident: 99%	MH015004
Bkba2			*Alternaria sp. isolate KSA-SGY-12* max score: 2058, total score: 2058, query cover: 99%, Ident: 99%	MH025761
StZA8			*Phomopsis mali strain IFO 31031* max score: 2048, total score: 2048, query cover: 99%, Ident: 99%	MH016188
Bk5b			*Cochliobolus sativus strain NBRC 100205* max score: 2069, total score: 2069, query cover: 99%, Ident: 98%	MH014993
BkZA3			*Bionectria ochroleuca strain WY-1,* max score: 2192, total score: 2192, query cover: 99%, Ident: 99%	MH014995

St2b			*Phyllosticta gardeniicola isolate: MUCC0117* max Score: 2003, total Score: 2003, query cover: 99%, Ident: 99%	MH020175
Lf7b1			*Guignardia mangiferae isolate: MUCC0215* max score: 2109, total score: 2109, query cover: 96%, Ident: 98%	MH015001
Bk3a			*Tricharina gilva voucher HMAS61180* max score: 2093, total score: 2093, query cover: 99%, Ident: 99%	MH013964
St1a			*Diaporthe amygdali isolate MUCC0101* max score: 2008, total score: 2008, query cover: 99%, Ident: 99%	MH015011
Lf12b			*Cladosporium sp. strain ALEF-C1* max score: 2102, total score: 2102, query cover: 99%, Ident: 99%	MH015002
Lf6a2			*Alternaria alternata strain S-f6* max score: 2105, total score: 2105, query cover: 98, Indent: 98%	MH014998
RtZB3			*Trichoderma harzianum isolate BCS8A* max score: 2217, total score: 2217, query cover: 99%, Ident: 99%	MH015010

Alternaria sp. was isolated from both the bark and the leaf, *Nigrospora oryzae* and *Bionectria ochroleuca* from leaf, bark and stem, *Aspergillus flavus* from leaf and stem, *Colletotrichum acutatum* from leaf and root, *Fusarium oxysporum* and *Cladosporium sp.*, from leaf, root and stem, *Phomopsis sp.* from all the four parts. From the available literature, *Alternaria*, *Colletotrichum*, *Phomopsis*, *Guignardia*, *Aspergillus* and *Fusarium*

spp. are the most often isolated endophytes in variety of host plant tissues (Prabukumar *et al*., 2015; Suryanarayanan *et al*., 2009; De Siqueira *et al*., 2011). *Colletotrichum gloeosporioides complex*, *Colletotrichum coffeanum*, *Diaporthe liquidambaris*, *Guignardia mangiferae* and *Phyllosticta sp.* have been found to be *coffee Arabica* endophytes in a related previous study (Oliveira and Souza, 2014). Bogner *et al*. (2016) isolated similar isolates to the ones in the present study from tomato roots in a study done in Kenya though different species namely; *Trichoderma asperellum*, *Fusarium nygamai*, *Fusarium spp.*, *Aspergillus sclerotiorum*, *Altenaria solani* and *Cochliobolus spp.* which were reported to have nematode bio control potential. The present study also corresponds with Velma *et al*. (2017) who also isolated *Fusarium*, *Colletotrichum*, *Phomopsis*, *Cladosporium* and *Aspergillus* from selected medicinal plants in Kenya. Various *Fusarium* species (*Fusarium verticillioides*, *Fusarium boothii* and *Fusarium poae*) have been previously identified as mycotoxigenic fungi contaminating maize samples. In addition to *Fusarium*, *Lasiodiplodia theobromae*, *Mucor nidicola*, and *Nigrospora oryzae* were found in small counts (Kibe, 2015). Majority of fungal endophytes are environmental fungi. For instance, *Aspergillus flavus* and *Trichoderma harzianum* have been among the soil fungi isolated from rice growing regions in Kenya (Mwashasha *et al*., 2014).

3.2 Evolutionary Relationship of the Isolates

An optimal tree with sum of branch length = 22.59917800 was given using UPGMA method (figure 1). The evolutionary distances were in the units of the number of base substitutions per site. The analysis involved 17 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 1135 positions in the final dataset. The evolutionary relationship showed that *Aspergillus flavus* strain is the evolutionary ancestor since its nucleotide sequence has not changed over time hence there is a possibility that the rest of the strains have evolved from this specific strain over time. *Fusarium oxysporum* and *Colletotrichum acutatum* were more related to the evolutionary ancestor compared to the rest of the strains. The strains in the same clade were closely related genetically.

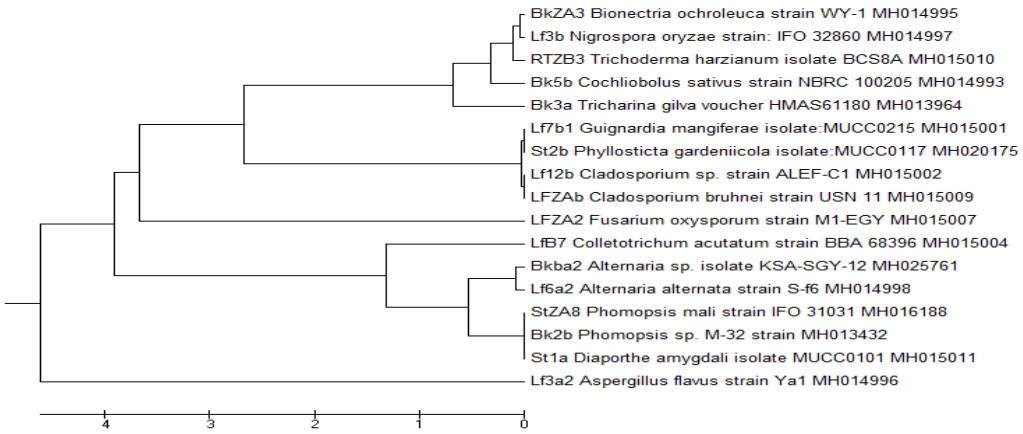


Figure 1: Evolutionary Relationship of the Endophytes isolated from *W. ugandensis*

3.3 Screening for phytochemicals

Preliminary phytochemical analysis on the extracts gave positive results for saponins, tannins, alkaloids, flavonoids, sterols and glycosides (table 2). However, the extracts showed negative results for phenols and anthraquinones (table 2). This study corresponds with Ladoh-Yemeda *et al*. (2015) who reported the presence of flavonoids, anthraquinones, tannins, phenols, steroids, coumarins and terpenoids, absence of alkaloids and saponins in ethyl acetate extracts of the endophytes *Aspergillus*, *Penicillium*, *Fusarium* and *Trichoderma*. Various fungal endophytes have been reported to have similar phytochemicals as the above mentioned isolates (Sowparthani, 2016; Kalyanaraman *et al*., 2015; Devi *et al*., 2012; Senthilmurugan *et al*., 2017).

Table 2: Phytochemical analysis of the Extracts. (-ve: absence, +ve: presence, Sap: Saponins, Tan: Tannins, Alka: Alkaloids, Flav: Flavonoids, Ster: Steroids, Glyco: Glycosides, Anthra: Anthraquinones, Phen: Phenols)

Code	Fungal Extract	Sap	Tan	Alka	Flav	Ster	Glyco	Anthra	Phen
BkZA3	*Bionectria ochroleuca*	-ve	+ve	-ve	+ve	-ve	+ve	-ve	-ve
StZA8	*Phomopsis mali*	-ve	+ve	+ve	+ve	-ve	+ve	-ve	-ve
LfZA2	*Fusarium oxysporum*	-ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve
LfB7	*Colletotrichum acutatum*	+ve	+ve	-ve	+ve	+ve	+ve	-ve	-ve
Lf12b	*Cladosporium sp.*	-ve	+ve	-ve	+ve	+ve	+ve	-ve	-ve
Bk3a	*Tricharina gilva*	+ve	+ve	+ve	-ve	+ve	+ve	-ve	-ve
Lf3b	*Nigrospora oryzae*	-ve	+ve	-ve	+ve	+ve	+ve	-ve	-ve
LfZAB	*Cladosporium bruhnei*	-ve	-ve	-ve	+ve	+ve	+ve	-ve	-ve
Bk2b	*Phomopsis sp.*	+ve	+ve	+ve	-ve	+ve	-ve	-ve	-ve
Lf7b1	*Guignardia mangiferae*	-ve	-ve	+ve	+ve	+ve	+ve	-ve	-ve
Bkba2	*Alternaria sp.*	-ve	+ve	-ve	+ve	+ve	+ve	-ve	-ve
Bk5b	*Cochliobolus sativus*	+ve	-ve	+ve	+ve	+ve	-ve	-ve	-ve
St2b	*Phyllosticta gardeniicola*	-ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve
Lf3a2	*Aspergillus flavus*	+ve	+ve	+ve	+ve	-ve	+ve	-ve	-ve
St1a	*Diaporthe amygdali*	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve
RtZB3	*Trichoderma harzianum*	+ve	+ve	-ve	-ve	+ve	+ve	-ve	-ve
Lf6a2	*Alternaria alternata*	+ve	+ve	-ve	+ve	-ve	+ve	-ve	-ve

3.4 Screening of Endophytic Fungi for Antimicrobial Activities

Screening of the endophytic fungi for antimicrobial activities was done using disc diffusion method against *Candida albicans* (90018), *E. coli* (25922) and *Staphylococcus aureus* (25923). Diameters of the zones of inhibition were measured to the nearest mm inclusive of the diameter of the disc (diameter of the disc was 6mm). Three isolates; *Phomopsis mali* (StZA8), *Alternaria alternata* (Lf6a2) and *Fusarium oxysporum* (LfZA2) showed minimum activity after 24 hours' incubation under 37^0C temperature (figures 2, 3 and 4).

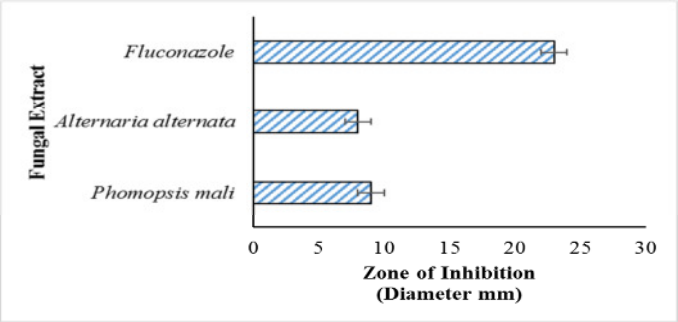


Figure 2: Antimicrobial activity against *Candida albicans*

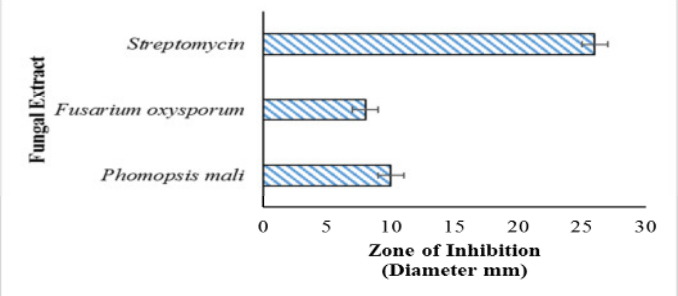


Figure 3: Antimicrobial Activity against *E coli*

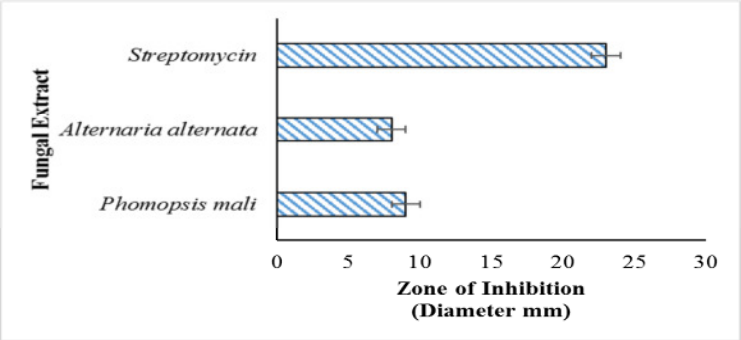


Figure 4: Antimicrobial Activity against *Staphylococcus aureus*

Phomopsis mali crude extract showed minimum activity against both bacteria (*E. coli* and *Staphylococcus aureus*) and fungi (*Candida albicans*) meaning its metabolites have both antibacterial and antifungal activity. According to the work of Tong *et al*. (2014), extracts of *Phomopsis* have antimicrobial activities against both Gram-positive and Gram-negative bacteria hence can be a good source of broad spectrum antibiotic. *Alternaria alternata* crude extract also showed similar properties with minimum activity against both *Staphylococcus aureus* and *Candida albicans*. This study corresponds with (Sabreen *et al*., 2015; Kumar *et al*., 2015) who reported that extracts of *Alternaria sp.* activity against *K. pneumoniae*, *P. aeruginosa*, *E. coli* and *S. aureus*. Both *Phomopsis sp.* and *Alternaria sp.* lie under one cluster in the evolutionary relationship meaning they could be having a similar genetic makeup. The present study reports that *Fusarium oxysporum* extract had minimum activity only against *E. coli* while Sabreen *et al*. (2015) reported that extracts of the same endophyte isolated from leaf of *Nothapodytes foetida* exhibited activity against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans*.

4. Conclusion

According to the present study, *Phomopsis mali*, *Fusarium oxysporum* and *Alternaria alternata* have metabolites with antimicrobial activities. However, there is need to study on the stability of these metabolites since improved stability may increase their activity. More different test organisms may also be used for screening the *Phomopsis mali* extract since it has indicated that it has both antibacterial and antifungal activities. It will be important also to know the active component and the section of the gene that codes for that component which may contribute to large scale production of the active component for use in the pharmaceutical industry.

5. Conflict of Interests

The authors have no any conflict of interest.

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