Identification and bioactive potential of endophytic fungi isolated from *Calotropis procera* (Ait.) R. Br.

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Abstract: *Calotropis procera* (Ait.) R. Br., was investigated for endophytic fungi as a possible source of bioactive secondary metabolites *in vitro*. Leaf and stem sections from naturally grown *Calotropis procera* plants were processed for the presence of endophytic fungi. A total of four endophytic fungi isolated from *Calotropis procera* were cultivated and identified as *Myrothecium verrucaria, Alternaria alternata, Epicoccum nigrum* and *Aspergillus terreus.* The endophytic fungal crude extracts were screened for their antimicrobial activity against a panel of thirteen important pathogenic microorganisms. The most effective extract was *Myrothecium verrucaria* extract, which demonstrated significant activity against most of the tested microorganisms under investigation. By screening the antitumor activity, non of the fungal crude extracts had antitumor activity, except for *Myrothecium verrucaria* extract, which possessed comparable cytotoxic activities to vinblastine sulphate as reference drug against colon cancer cell line (HCT-116) and liver cancer cell line (HepG2), while in the case of breast cancer cell line (MCF-7) its IC_{50} was better than the reference drug. All fungal extracts exhibited antioxidant activity at 5 µg/ml, but with variation in % exploitation of scavenge DPPH radicals. *Myrothecium verrucaria* extract revealed the most promising antioxidant activity. These results indicate that *Myrothecium verrucaria* extract could be a promising source of bioactive compounds and warrant further study. The antimicrobial, antitumor and antioxidant fraction of *Myrothecium verrucaria* extract was isolated and characterized using elemental analysis, IR, Mass spectrum and ^{13}C-NMR spectrum.

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Key Words: *Calotropis procera*, endophytic fungi, cytotoxicity, antimicrobial, antioxidant activity.

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; **DPPH**, 2,2-diphenyl-1-picrylhydrazyl; **DMSO**, dimethyl sulphoxide; **ITS**, internal transcribed spacer; **PCR**, polymerase chain reaction; **PDA**, potato dextrose agar; **NMR**, nuclear magnetic resonance; **IR**, infra red

1. Introduction

Calotropis procera (Ait.) R. Br., commonly known as calotrope, rubber tree, akando, milk-weed or swallow wart, is a widely used medicinal plant. It has long botanical history and extensive uses in traditional medicine. *C. procera* belongs to the family Asclepidaceae. It is a shrub or small tree, drought-resistant, salt tolerant to a relatively high degree. The plant contains cardiotoxic, emetocathartic and digitalic properties. It also possesses bactericidal and vermicidal activities (Sing *et al*., 2002; Khan *et al.*, 2007). The plant is very effective in treating leprosy, elephantiasis, chronic rheumatism, ulcer and skin diseases (Akinloye *et al.*, 2002; Kumar and Roy, 2007). *C. procera* is grown naturally in Egypt, in the Red Sea coastal region, Sinai, oases of the Western Desert and Gebel Elba (Boulos, 2000).

Endophytes are micro-organisms that reside in the internal tissues of living plants without causing any apparent negative effects. Generally, the relationship between the plant and its endophytes is one of a symbiotic nature where the endophytes colonise the internal tissues of the plant (Zhang *et al*., 2006). ‘‘Endophytism’’ is, thus, a unique cost-benefit plant-microbe association defined by ‘‘location’’ (not ‘‘function’’) that is transiently symptomless, unobtrusive, and established entirely inside the living host plant tissues (Kusari and Spiteller, 2012).

Plant endophytic fungi have been recognized as an important and novel resource of natural bioactive products with potential applications in agriculture, medicine and food industry (Strobel *et al*., 2004; Verma *et al*., 2009). As reported by Kusari *et al.* (2012), the production of bioactive compounds by endophytes, especially those exclusive to their host plants, is not only important from an ecological perspective but also from a biochemical and molecular standpoint. Exciting possibilities exist for exploiting endophytic fungi for the production of known and novel biologically active secondary metabolites. This could lead to a cost-effective environmentally friendly, continuous, and reproducible yield compliant to commercial scale-up. In the case of endophytes capable of producing host plant compounds, would then be independent of the variable quantities

produced by plants influenced by environmental conditions, and have advantages over the *in vitro* production of these active compounds through plant callus cultures. Therefore, it is beneficial to study the relations between the endophytes and their host plants, and to develop a substitutable approach for efficient production of these scarce and valuable bioactive compounds. Thus, if a microbial source of the drug would be available, it would eliminate the need to harvest and extract the slow growing and relatively rare trees for the drug. The price for the drug would then be reduced (Gunatilaka, 2006; Zhou, *et al*., 2009).

In the past two decades, many valuable bioactive compounds, both known and novel (Bok *et al*., 2006; Bergmann *et al*., 2007; Knappe *et al*., 2008; Schroeckh *et al*., 2009; Riyaz-Hassan *et al*., 2012), with antimicrobial (e.g. cryptocandin from *Cryptosporiopsis quercina*), insecticidal and anticancer activities (e.g. taxol from *Taxomyces andreanae*) have been successfully discovered from the endophytic fungi. These bioactive compounds could be classified as alkaloids, terpenoids, steroids, quinones, lignans, phenols and lactones (Strobel *et al*., 2004; Zhang *et al*., 2006; Xu *et al*., 2008). According to Li *et al*. (2005), at least one active endophytic fungal isolate should be obtained from each plant species, but the percentage of active isolates may differ accordingly. So, the aim of this study was the *in vitro* isolation of endophytic fungi from *Calotropis procera* and the identification of them, also to determine the probability of the production of bioactive compounds from endophytic fungal extracts by testing their antimicrobial, antitumor and antioxidant activities.

2. Materials and Methods

2.1. Samples collection and endophytic fungi isolation

Stems and leaves of *C. procera* were randomly collected from healthy and mature naturally grown plants for the investigation of endophytic fungi. Samples were brought to the laboratory in wet papers and processed within a few hours to reduce the chances of contamination. Stem and leaf explants were washed thoroughly under running tap water to remove dust and debris. After proper washing, explants were then surface sterilized by dipping them in 70% ethanol for 30 seconds, then submerged in 40 and 20% (v/v) commercial bleach solution (Clorox) (2 and 1% sodium hypochlorite solution) for 20 minutes, for stem and leaf sections, respectively, followed by three rinses with sterile distilled water. Stem explants were cut into 0.5-1 cm long sections, and leaves were cut into 0.5 cm x 0.5-1 cm sections with and without midrib under aseptic conditions. Explants were then placed in Petri dishes containing potato dextrose agar (PDA) supplemented with chloramphenicol (50 mg/litre) to suppress bacterial growth. All the dishes were sealed with parafilm and incubated at 25±2 °C for up to 21 days in dark, and emerging fungi were transferred to fresh PDA plates and incubated for 7 days with periodical check for purity (Son *et al*., 2003).

2.2. Identification of isolated endophytic fungi

Three of total four fungal isolates were identified at the Regional Center for Mycology and Biotechnology (RCMB) using image analysis system (Leica CTR 5000, 280 DFC). The fourth isolate was difficult to identify through the regular method without the presence of conidia in culture, therefore sequence similarities and phylogenetic analysis were used to identify this fungus, i.e. endophytic fungus was transferred to malt extract medium and incubated with shaking (180 rpm) at 25 °C for 7 days. Fungal identification method was based on their internal transcribed spacer ribosomal DNA (ITS-rDNA) sequences. Mycelia were collected by centrifugation and DNA was extracted. Purified DNA was subjected to PCR amplification using a pair of ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) primers for ITS-rDNA amplification (White *et al*., 1990), which produces an amplicon of approximately 550 bp of the ITS region. Sequence data was analyzed in the Gene Bank database by using the BLAST program available on the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov). The unknown sequence was compared to all of the sequences in the database to assess the DNA similarities (Altschul *et al*., 1997). Alignment and molecular phylogeny were evaluated using Bio Edit software. Purification and sequencing of PCR products for the isolate under study were performed in the Sigma company of scientific service. By reviewing the previous researches about this fungus, it was found that the production of conidia is independent of light and dense conidia production is self-inhibitory though the secretion of growth substances, but this inhibition could be removed by the addition of biotin to the culture medium according to Domsch *et al.* (1980). Therefore, biotin at a concentration of 10 mg/100 ml was added to the culture medium of the unknown fungus to promote the production of its conidia.

2.3. Extraction of the secondary metabolites from isolated endophytic fungi

Concerning the production of the secondary metabolites using static flask culture, spores from ten to fourteen days old culture were transferred into 500 ml Erlenmeyer flask containing 250 ml of malt extract medium. The seeded flasks were incubated at 25 ^{o}C for 21 days under static conditions. The cultures were harvested and filtered through Whatman no. 1 filter paper to give

clear filtrate that was exposed to the extraction process.

Five liters of each fungal broth filtrate were extracted with chloroform and methanol (2:1 v/v) in a separating funnel. The mixture was shaken vigorously and left to settle down forming a dense lower aqueous layer containing the secondary metabolites. The lower layer was separated, then the solvent was evaporated to dryness and evaluated for its antimicrobial, antitumor and antioxidant activities.

2.4. Screening for antimicrobial activity

The extracts prepared from the endophytes were evaluated for their antimicrobial activity at a concentration of 1 mg/ml DMSO (dimethyl sulphoxide) against human pathogenic microorganisms obtained from Regional Center for Mycology and Biotechnology (RCMB) at Antimicrobial Unit to test microorganisms, namely; *Aspergillus fumigatus* (RCMB 02564), *Candida albicans* (RCMB 05035), *Absidia corymbifera* (RCMB 09584) and *Trichophyton mentagrophytes* (RCMB 0925) as pathogenic fungi, *Escherichia coli* (RCMB 010056), *Pseudomonas aeruginosa* (RCMB 010043), *Shigella flexneri* (RCMB 0100542), *Klebsiella pneumoniae* (RCMB 0010093), *Proteous vulgaris* (RCMB 010085) and *Neisseria gonorrhoeae* (RCMB 010076) as Gram negative bacteria, and *Streptococcus pyogenes* (RCMB 010015), *Staphylococcus epidermidis* (RCMB 010024) and *Staphylococcus aureus* (RCMB 010027) as Gram positive bacteria. Antimicrobial activity was determined using the agar well diffusion assay method as described by Holder and Boyce (1994). The tested organisms were subcultured on nutrient agar medium (Oxoid laboratories, UK) for bacteria and Saboroud dextrose agar (Oxoid laboratories, UK) for fungi. Ampicillin and gentamycin (Sigma Chemical Co., St. Louis, Mo, USA) were used as a positive control against Gram positive and Gram negative bacteria, respectively. Amphotericin B (Sigma Chemical Co., St. Louis, Mo, USA) was used as a positive control for fungi. The plates were done in triplicate. Bacterial cultures were incubated at 37°C for 24 hours, while the fungal cultures were incubated at 37°C for 2-7 days. Antimicrobial activity was determined by measurement zone of inhibition as described by (Agwa *et al*., 2000).

2.5. Cytotoxicity assay

2.5.1. Cell culture

Mammalian cell lines: MCF-7 cells (breast cancer cell line), HCT-116 (colon cancer cell line) and HepG2 (liver cancer cell line) were obtained from VACSERA Tissue Culture Unit. The cells were propagated in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (Sigma Chemical Co., St. Louis, Mo, USA), 1% L-glutamine, HEPES buffer and 50 µg/ml gentamycin (Sigma Chemical Co., St. Louis, Mo, USA). All cells were maintained at 37°C in a humidified atmosphere with 5% CO_2 and were sub-cultured two times a week.

2.5.2. Evaluation of cellular cytotoxicity

The cytotoxic activity of extracts was evaluated by the crystal violet staining (CVS) method described by Saotome *et al.* (1989) and modified by Itagaki *et al*. (1991). Briefly, in a 96-well tissue culture microplate, the cells were seeded at a cell concentration of 1×10^4 cells per well in 100 µl of growth medium. Fresh medium containing 50 µl concentrations of endophytic fungal extract were added after 24 hours of seeding at 37°C. Serial two fold dilutions of the tested extracts were added to confluent cell monolayers dispensed into 96-well, flat-bottomed microtiter plates using a multichannel pipette. The microtiter plates were incubated at 37°C in a humidified incubator with 5% CO_2 for a period of 48 hours. Three wells were used for each concentration of the test sample. Control cells were incubated without test sample and with or without DMSO. The little percentage of DMSO present in the wells was found not to affect the experiment. After the 48 hours incubation period, the viable cells yield was determined by a colorimetric method. After the end of the incubation period, media were aspirated and the crystal violet solution (1%) was added to each well for at least 30 minutes. The stain was removed and the plates were rinsed using distilled water. Glacial acetic acid (30%) was then added to all wells and mixed thoroughly. The quantitative analysis (colorimetric evaluation of fixed cells) was performed by measuring the absorbance in an automatic microplate reader (TECAN, Inc.) at 595 nm. All results were corrected for background absorbance detected in wells without added stain. Treated samples were compared with the cell control in the absence of the tested extracts. All experiments were carried out in triplicate. The effect on cell growth was calculated as the difference in absorbance percentage in presence and absence of the tested extracts and illustrated in a dose-response curve. The concentration at which the growth of cells was inhibited to 50% of the control (IC_{50}) was obtained from this dose-response curve. The standard antitumor drug used was vinblastine sulphate.

2.6. Antioxidant Assay

The antioxidant activity of extracts was determined by the DPPH free radical scavenging assay. Methanol solution (0.004% w/v) of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was freshly prepared and stored at 10ºC in the dark. A methanol solution of the test compound was also prepared. A 40 ul of the methanol solution was added to 3 ml of DPPH solution. Absorbance measurements were recorded immediately with a Milton Roy Spectronic 201 UV-visible

spectrophotometer. The decrease in absorbance at 515 nm was determined continuously, with data being recorded at 1 minute intervals until the absorbance stabilized (16 minutes). Ascorbic acid was used as a reference standard. The absorbance of the DPPH radical without antioxidant was also measured as control and 95% methanol was used as blank. All the determinations were performed in three replicates and averaged. Percentage of scavenging of the DPPH free radical was measured using the following equation (Yen and Duh, 1994): % DPPH radical scavenging = [(Absorbance of control - Absorbance of test sample) / (Absorbance of control)] x 100

2.7. Fractionation and isolation of bioactive compounds from Myrothecium verrucaria extract

The crude extract was subjected to silica gel (Merck, 0.02-0.04 mm) column chromatography. The elution was conducted with a step gradient CH_2Cl_2 / MeOH (100:0, 95:5, 90:10, 80:20, and 50:50) (Anastasiadi *et al.,* 2009). The different fractions were collected. Those fractions were pooled and concentrated using lyophilisation.

2.8. Physicochemical properties and spectroscopic analysis of the most active fraction

Physical and chemical properties of the purified active substance were studied. The elemental analysis, IR, ^{13}C-NMR spectrum and mass spectrum were determined at Regional Center for Mycology and Biotechnology (RCMB) at Al-Azhar University.

2.9. Cytotoxicty of the most active fraction against normal Vero cell line

Cytotoxic effect of the isolated pure compound at concentration of 10 µg/ml against normal Vero cells was evaluated by following the same steps mentioned in the cytotoxicity assay.

3. Results and Discussion

3.1. Description of isolated endophytic fungi

Leaf and stem explants of *C. procera* were processed for the isolation of endophytic fungi. A total of 4 fungal species were isolated (Figures 1-4).

Aspergillus terreus

Colonies yellowish-brown to cinnamon-brown, spreading rapidly consisting of a dense felt of conidiophores (Figure 1a). Conidial heads densely columnar, condiophore stipes smooth walled, hyaline. Vesicles subspherical, 10-20 µm in diameter. Conidiogenous cells biseriate. Metulea as long as the phialides. Conidia smooth walled, hyaline, spherical to proadly ellipsoidal, 1.5-2.5 µm in diameter (Figure 1b).

Alternaria alternata

Colonies expanding grey to olivaceous, powdery or felty (Figure 2a). Conidiophores unbrached, with one or few conidial scars, up to 50 µm long, 3-6 µm wide. Conidia obclavate to ellipsoidal, with short, cylindrical beak, 23-56 µm x 8-17 µm, medium brown, regulose with muriform septation with a single scar at the tip (Figure 2b).

Epicoccum nigrum

Colonies grow fairly slowly and progress from cottony yellow to orange. The reverse is red, diffusible yellow and orange pigment is present (Figure 3a). Hyphae branch repeatedly in a focal pattern known as sporodochia, Short conidiophores arise from sporodochia. Muriform macroconidia are spherical to club shaped (Figure 3b).

Myrothecium verrucaria

Colonies are white reaching 3.0-4.0 cm in diameter in 14 days at 25 on PDA, mycelium white, forming black sporodochia, reverse rosy buff (Figure 4a). Phialides 3-6 in whorl, 10.5-14.5 x1.5-2.0 µm (Figure 4b). Conidia broadly fusiform, the epical end pointed and the basal truncate bearing an apical. Funnel-shaped appendage (these characteristic have been appeard after addition of biotin) (Figure 4c). The culture had been identified by using ITS-rDNA sequence similarities and phylogenetic analysis to identify this fungus (Table 1, Figure 5).

3.2. Antimicrobial activity

The results of screening isolated endophytic fungal extracts for their antimicrobial activity against a panel of thirteen important pathogenic microorganisms were summarized in Table 2. Only three of four isolated endophytic fungi namely; *Alternaria alternata, Epicoccum nigrum* and *Myrothecium verrucaria* extracts were effective against tested pathogenic microorganisms, but with variation in the exploitation of antimicrobial property and that is may be due to the findings of Oh *et al*. (2003) who demonstrated that secondary metabolites are generally produced following active growth, and many have an unusual chemical structure. Some metabolites are found in a range of related fungi, while others are only found in one or a few species. The restricted distribution implies a lack of general function of secondary metabolites in fungi. *Aspergillus terreus* extract was totally ineffective against all tested microorganisms at 1 mg/ml. These findings are in contrary to the results of Awaad *et al*. (2012) who demonstrated that extracellular metabolites of *Aspergillus terreus* have antimicrobial activity against some human pathogenic microbes, but this may be due to tested microorganisms.

Figure 1a. Colony morphology of *A. terreus*

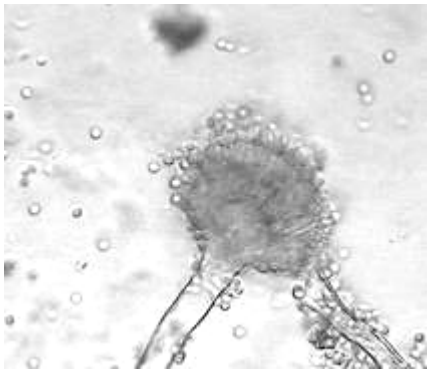

Figure 1b. *A. terreus* under microscope

Figure 2a. Colony morphology of *A. alternata*.

Figure 2b. *A. alternata* under microscope.

Figure 3a. Colony morphology of *E. nigrum*.

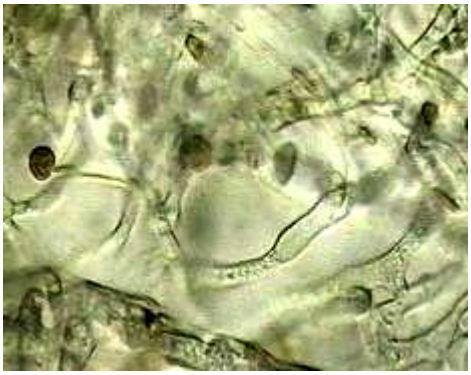

Figure 3b. *E. nigrum* under microscope detecting muriform macroconidia.

Figure 4a. Colony morphology of *M. verrucaria.*

Figure 4b. Mycellium and phialides in whorl of *M. verrucaria*.

Figure 4c. Conidia of *M. verrucaria* after addition of biotin to the media.

Table 1. Sequence of ITS-rDNA producing significant alignment for *Myrothecium verrucaria* isolate.

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
HQ389476.1	Uncultured Hypocreales clone 9A4S48N internal transcribed spacer	981	981	98%	0.0	99%
GU183129.1	Myrothecium verrucaria strain NRRL 52420 internal transcribed spa	981	981	98%	0.0	99%
FJ235085.1	Myrothecium verrucaria strain CGMCC 3.2190 18S ribosomal RNA	981	981	98%	0.0	99%
EU750696.1	Myrothecium sp. 14016 18S ribosomal RNA gene, partial sequence;	981	981	98%	0.0	99%
AF455507.1	Myrothecium atroviride isolate wb256 small subunit ribosomal RNA	981	981	98%	0.0	99%
AJ302002.1	Myrothecium atroviride 18S rRNA gene, 5.8S rRNA gene, 28S rRNA	981	981	98%	0.0	99%
AJ302003.1	Myrothecium verrucaria 18S rRNA gene, 5.8S rRNA gene, 28S rRNA	981	981	98%	0.0	99%
JQ356542.1	Myrothecium verrucaria isolate E16 internal transcribed spacer 1, p	979	979	98%	0.0	99%
JN650593.1	Myrothecium verrucaria strain DCF88 18S ribosomal RNA gene, par	977	977	97%	0.0	99%
AJ301999.1	Myrothecium verrucaria 18S rRNA gene, 5.8S rRNA gene, 28S rRNA	976	976	98%	0.0	99%
EF017211.1	Myrothecium verrucaria internal transcribed spacer 1, partial sequer	974	974	98%	0.0	99%
JX501292.1	Myrothecium verrucaria strain MV01 18S ribosomal RNA gene, part	970	970	96%	0.0	99%
GQ131886.1	Myrothecium verrucaria isolate MYRver2 18S ribosomal RNA gene,	968	968	95%	0.0	99%
EU520634.1	Uncultured ascomycete clone W2 internal transcribed spacer 1, part	968	968	98%	0.0	99%
FJ235932.1	Myrothecium roridum strain CGMCC 3.3682 18S ribosomal RNA ge	966	966	97%	0.0	99%
HQ607996.1	Myrothecium verrucaria isolate CY157 18S ribosomal RNA gene, pa	963	963	98%	0.0	99%
HM162053.1	Uncultured Ascomycota clone 278 18S ribosomal RNA gene, partial	963	963	98%	0.0	99%
HM162010.1	Uncultured Ascomycota clone 219 18S ribosomal RNA gene, partial	963	963	98%	0.0	99%
HM162013.1	Uncultured Ascomycota clone 222 18S ribosomal RNA gene, partial	963	963	98%	0.0	99%
HM162002.1	Uncultured Ascomycota clone 205 18S ribosomal RNA gene, partial	963	963	98%	0.0	99%
HM043804.1	Myrothecium verrucaria 18S ribosomal RNA gene, partial sequence;	963	963	98%	0.0	99%
HQ608048.1	Myrothecium verrucaria isolate CY235 18S ribosomal RNA gene, pa	957	957	98%	0.0	99%
JN859394.1	Myrothecium sp. REF174 18S ribosomal RNA gene, partial sequenc	953	953	94%	0.0	99%
FR682255.1	Uncultured Ascomycota genomic DNA containing 18S rRNA gene, I	953	953	94%	0.0	99%
AY303603.1	Myrothecium verrucaria 18S ribosomal RNA gene, partial sequence;	946	946	94%	0.0	99%
HQ596904.1	Myrothecium verrucaria internal transcribed spacer 1, partial sequer	942	942	93%	0.0	99%
EF458487.1	Myrothecium sp. IMER1 internal transcribed spacer 1, partial seque	942	942	93%	0.0	99%
HQ625520.1	Myrothecium verrucaria strain Hmp-F73 internal transcribed spacer	941	941	93%	0.0	99%
JN545776.1	Myrothecium sp. E-000535633 18S ribosomal RNA gene, partial se	937	937	98%	0.0	98%

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Query  9    AACTCCC-AACCCTTTGTGAACCTTACCATATTGTTGCTTCGGCGGGACCGCCCCGGCGC  67
            ||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    AACTCCCAAACCCTTTGTGAACCTTACCATATTGTTGCTTCGGCGGGACCGCCCCGGCGC  60
Query  68   CTTCGGGCCCGGAACCAGGCGCCCGCCGGAGGCCCCAAACTCTTATGTCTTTAGTGGTTT  127
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   CTTCGGGCCCGGAACCAGGCGCCCGCCGGAGGCCCCAAACTCTTATGTCTTTAGTGGTTT  120
Query  128  TCTCCTCTGAGTGACACATAAACAAATAAATAAAAACTTTCAACAACGGATCTCTTGGTT  187
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  TCTCCTCTGAGTGACACATAAACAAATAAATAAAAACTTTCAACAACGGATCTCTTGGTT  180
Query  188  CTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTG  247
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  CTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTG  240
Query  248  AATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTC  307
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  AATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTC  300
Query  308  GAGCGTCATTTCAACCCTCAGGCCCCCAGTGCCTGGTGTTGGGGATCGGCCCAGCCTTCT  367
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  GAGCGTCATTTCAACCCTCAGGCCCCCAGTGCCTGGTGTTGGGGATCGGCCCAGCCTTCT  360
Query  368  CGCAAGGCCGCCGGCCCCGAAATCTAGTGGCGGTCTCGCTGTAGTCCTCCTCTGCGTAGT  427
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  CGCAAGGCCGCCGGCCCCGAAATCTAGTGGCGGTCTCGCTGTAGTCCTCCTCTGCGTAGT  420
Query  428  AGCACAACCTCGCAGTTGGAACGCGGCGGTGGCCATGCCGTTAAACACCCCACTTCTGAA  487
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421  AGCACAACCTCGCAGTTGGAACGCGGCGGTGGCCATGCCGTTAAACACCCCACTTCTGAA  480
Query  488  AGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAA-AAGCGGGAGG  546
            |||||||||||||||||||||||||||||||||||||| ||||| |||
Sbjct  481  AGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAATAAGCGG-AGG  539
Query  547  AA  548
            ||
Sbjct  540  AA  541
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Figure 5. *Myrothecium verrucaria* strain NRRL 52420 ITS1, partial sequence, 5.8S ribosomal RNA gene and ITS2, complete sequence and 28S ribosomal RNA gene, partial sequence length=1079.

Table 2. Evaluation of antimicrobial activity of endophytic fungal extracts against a range of clinically pathogenic microorganisms.

Samples / Tested microorganisms	*A. terreus*	*A. alternata*	*E. nigrum*	*M. verrucaria*	Standard
	Mean zone of inhibition in mm ± Standard deviation				
Fungi					**Amphotericin B**
***Aspergillus fumigatus* (RCMB 02564)**	NA	NA	NA	22.3±0.37	23.7±0.10
***Candida albicans* (RCMB 05035)**	NA	NA	NA	14.00.25	21.9±0.12
***Absidia corymbifera* (RCMB 09584)**	NA	NA	NA	19.3±0.58	26.4±0.20
***Trichophyton mentagrophytes* (RCMB 0925)**	NA	NA	NA	17.8± 0.63	25.4±0.16
Gram Positive Bacteria					**Ampicillin**
***Staphylococcus aureus* (RCMB 010027)**	NA	12.6± 0.63	14.6± 0.63	20.9± 0.63	28.9±0.14
***Staphylococcus epidermidis* (RCMB 010024)**	NA	10.2± 0.58	15.2± 0.58	19.3± 0.58	25.4±0.18
***Streptococcus pyogenes* (RCMB 010015)**	NA	NA	12.6± 0.44	22.6 ± 0.44	26.4±0.34
Gram negative Bacteria					**Gentamycin**
***Neisseria gonorrhoeae* (RCMB 010076)**	NA	NA	NA	NA	19.9±0.18
***Proteous vulgaris* (RCMB 010085)**	NA	NA	NA	18.9 ± 0.44	23.4±0.30
***Klebsiella pneumoniae* (RCMB 0010093)**	NA	NA	13.4 ± 0.25	21.6± 0.58	26.3±0.15
***Shigella flexneri* (RCMB 0100542)**	NA	NA	NA	18.7± 0.63	24.8±0.24
***Pseudomonas aeruginosa* (RCMB 010043)**	NA	NA	NA	NA	17.3±0.12
***Escherichia coli* (RCMB 010056)**	NA	NA	12.8±0.58	20.3 ± 0.25	25.3±0.18

*NA: No activity

Alternaria alternata extract revealed moderate activity against some Gram positive bacteria, but no activity was observed in the rest of tested microorganisms. These results were consistent with the result of Vieira- Fernandes *et al*. (2009), who demonstrated that *Alternaria alternata* extract had antibacterial activity against some pathogenic bacteria such as *Staphylococcus aureus*. *Epicoccum nigrum* extract showed activity against all Gram positive and some of Gram negative bacteria with diameters of inhibition zone ranged from 12.6-15.2 mm, while no antifungal activity was detected. On the other hand, *Epicoccum nigrum* P16, a sugarcane endophyte, produces antifungal compounds as demonstrated by Fávaro *et al.* (2012). This discrepancy may be due that the antifungal activity was tested against plant pathogenic fungi not human pathogenic fungi like the tested fungi in this study. *Myrothecium verrucaria* extract demonstrated significant activity with diameters of inhibition zone ranged from 14.0-22.6 mm against most of tested microorganism. These results are in the same line with Pervez *et al.* (2013) who demonstrated that isolated *Myrothecium* spp. exhibited good antimicrobial potential against human pathogenic microorganisms *in vitro*. Amphotericin B, Ampicillin and Gentamycin demonstrated the highest activities against fungi, Gram positive bacteria and Gram negative bacteria, respectively.

3.3. Cytotoxic activity

The cytotoxic activity of isolated endophytic fungal extracts against MCF-7, HepG2 and HCT-116 carcinoma cell lines was determined by CVS method using vinblastine sulphate as a reference drug. The response parameter (IC_{50}) was calculated for each cell line. Three fungal extracts namely; *Aspergillus terreus, Alternaria alternata* and *Epicoccum nigrum* have not any cytotoxic activity against any of tumor cell lines under investigation (data not shown), they may have cytotoxic activity against other cell lines such as in the case of *Alternaria alternata* extract, which displayed a cytotoxic activity towards HeLa cells *in vitro* as reported by Vieira-Fernandes *et al.* (2009).

From the results revealed in Table 3, it could be observed that *Myrothecium verrucaria* extract possessed a dose dependent cytotoxic effect against all three tumor cell lines. However, it exhibited more selective cytotoxic activity against MCF-7 (IC_{50} = 107 ng/ml) followed by HCT-116 cell lines (IC_{50} = 380 ng/ml), then HepG2cell line (IC_{50} = 711 ng/ml), compared to vinblastine sulphate as reference drug with IC_{50} = 460, 260 and 460 ng/ml against MCF-7, HCT-116 and HepG2 carcinoma cell lines, respectively.

Table 3. *In vitro* cytotoxic activitiy of *Myrothecium verrucaria* extract on different cell lines.

Samples / Conc. (µg/ml)	*M. verrucaria* extract against MCF-7	Vinblastine sulphate (reference drug) against MCF-7	*M. verrucaria* extract against HCT-116	Vinblastine sulphate (reference drug) against HCT-116	*M. verrucaria* extract against HepG2	Vinblastine sulphate (reference drug) against HepG2
	Percent of cell viability % ± [a]S.D					
50	6.48 ± 0.48	7.82 ± 0.98	7.56±0.29	16.27± 1.12	8.64±0.82	14.38 ± 1.41
25	8.64 ± 0.84	15.18 ± 1.23	11.04±0.87	21.68± 2.38	12.36±0.64	16.13 ± 2.24
12.5	10.87± 0.61	29.26 ± 2.74	14.49±0.92	28.2 ± 4.56	18.58±1.26	24.25 ± 2.96
6.25	15.00 ± 1.54	42.35 ± 2.21	15.41±0.72	38.06± 5.32	21.27±1.18	45.13 ± 2.04
3.125	19.78 ± 0.92	56.54 ± 1.96	16.53±1.20	47.54± 4.04	29.40±1.62	55.00 ± 2.33
1.56	23.15 ± 1.38	67.24 ± 2.94	19.08±0.67	53.42± 3.96	37.86±1.44	72.13 ± 3.06
0.78	26.52 ± 0.61	73.28 ± 1.25	34.86±1.44	63.47 ±1.34	48.67±2.26	86.72±1.95
0.39	29.57 ± 1.23	84.66. ± 2.24	49.8±2.60	71.62±2.13	56.21±3.03	94.58±2.63
0.195	38.76 ± 1.44	97.44 ± 1.44	61.67±2.32	80.36±5.65	66.18±4.12	98.58±3.58
0.098	51.12 ± 3.03	100.00±0.98	78.21±3.20	62.72±2.86	80.39±3.20	100.00±2.16
0.049	65.14 ± 4.71	100.00±1.22	87.40±5.65	98.42±1.34	89.64±1.38	100.00±1.58
0.024	78.21 ± 3.20	100.00±1.44	94.17±2.86	100.00±2.24	96.34±2.64	100.00±2.63
0.012	85.36 ± 2.19	100.00±2.32	100.00±1.97	100.00±1.42	100.00±2.16	100.00±2.34
0	100.00 ± 0.92	100.00±1.55	100.00±2.24	100.00±1.67	100.00±1.66	100.00±1.84
[b]IC $_{50}$	107 ng/ml	460 ng/ml	380 ng/ml	260 ng/ml	711 ng/ml	460 ng/ml

[a] Mean of three assays % ± standard deviation, [b] IC_{50} sample concentration required to inhibit tumor cell proliferation by 50%.

Myrothecium verrucaria extract possessed comparable cytotoxic activities to vinblastine sulphate as reference drug against HCT-116 and HepG2, while in the case of breast cancer cell line (MCF-7) its IC_{50} was more effective than the reference drug (vinblastine sulphate) by at least six two fold serial dilution (Figure 6). These findings are compatible with that of Abbas *et al.* (2002) who demonstrated that *Myrothecium* extract had excellent antitumor effect against four mammalian cell lines H4TG, MDCK, NIH3T3 and KA31T *in vitro* with low IC_{50}.

3.4. Antioxidant activity

The crude extracts of *Aspergillus terreus, Alternaria alternata, Epicoccum nigrum* and *Myrothecium verrucaria* were evaluated for their antioxidant ability to scavenge DPPH radicals. The results are shown in Figure 7. The fungal extracts and the standard (ascorbic acid) were assessed at a concentration of 0 – 40 µg/ml. At concentration of 5 µg/ml (the least concentration used), a significant difference between the sequestering capability of DPPH radicals by the crude extracts of the four fungi was observed, but *Myrothecium verrucaria*

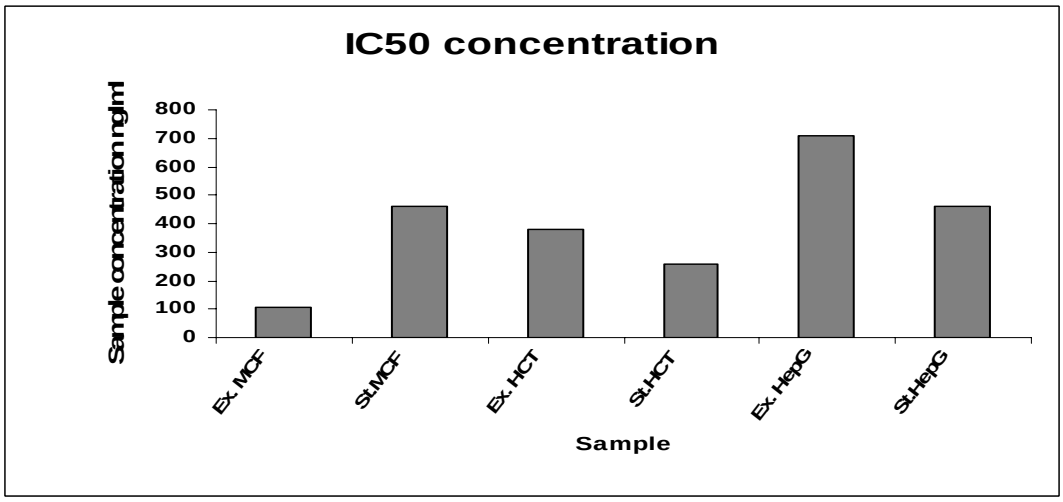

Figure 6. IC_{50} values of different fungal extracts and vinblastine sulphate as reference drug against different cell lines.

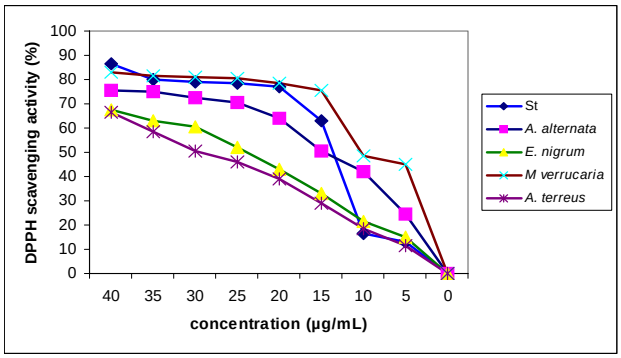

Figure 7. DPPH scavenging activity (%) of fungal crude extracts at different concentrations comparing to the standard ascorbic acid.

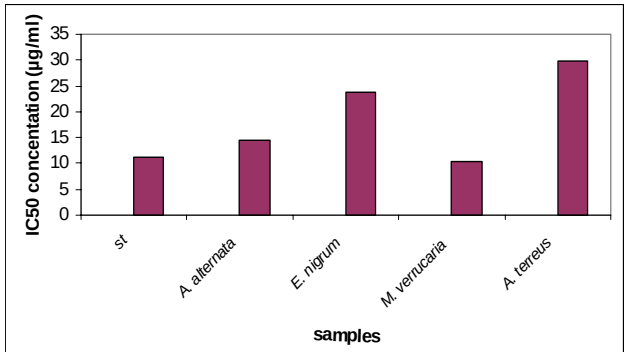

Figure 8. IC_{50} concentrations of DPPH scavenging activity (%) of fungal crude extracts.

extract revealed promising antioxidant activity with the highest DPPH scavenging activity of 44.76%, which indicates the presence of at least one promising antioxidant compound in the crude extract of *Myrothecium verrucaria*. Unfortunately, the evaluation of the antioxidant activity of *Myrothecium verrucaria* extract in previous researches was rare. To conclude, all fungal crud extracts under investigation had antioxidant activity. These results are in the same line with the results of Dewi *et al.* (2012) who demonstrated that *Aspergillus terreus* possessed significant antioxidative activity with an IC_{50} of 19.91 µg/ml. Also, Vieira–Fernandes *et al.* (2009) found that the endophytic fungus *Alternaria alternata* isolated from *Coffea arabica* L. exhibited good ability to scavenge DPPH radicals and antioxidant activity by β-carotene/linoleic acid system oxidation, in addition to Hai-Hong *et al.* (2011) who detected that two compounds (ENP2 and ENP1) isolated from extracelluler metabolites of *Epicoccum nigrum* JJY-40 had good antioxidant activities. The IC_{50} values (sample concentration that gives 50 % scavenging capacity) are represented in Figure 8.

From the obtained results, the IC_{50} values were 11.2, 14.6, 23.7, 10.3 and 29.7 µg/ml for ascorbic acid (standard), *Alternaria alternata, Epicoccum nigrum*, *Myrothecium verrucaria* and *Aspergillus terreus,* respectively.

From the above results it was declared that the endophytic fungus *Myrothecium verrucaria* could be a promising source of bioactive compounds and warrant further study, so its extract was fractioned for isolating active compounds, subsequently it was found that there were eighteen fractions (1-18); fractions no. 5, 9 and 13 had antimicrobial activity; while fractions no. 8, 13 and 16 had antitumor activity and finally fractions no. 10, 13 and 17 had antioxidant activity. So, fraction no. 13 was selected to be characterized for its triple activity at concentration of 10 µg/ml with no cytotoxicity against normal Vero cells at the same concentration.

3.5. Spectroscopic characteristics

Mass spectrometry analysis of the active substance (mass spectrum) showed the following fragments: 59.7 (100), 55.97 (88.43), 124.10 (66.99), 60.51 (50.60), 94.5 (35.87), 72.6 (39.49),

65 (26.58) and 581 (8.80), and gave molecular weight of 581 atomic mass unit (Figure 9). IR spectroscopy (Figure 10) showed the following absorption peaks: abroad peak at 3606 cm-1 for (OH), 1639 for (C=O), 2931 for (CH_3 asy.), 2873 for (CH_2 sym.), 1523 for (CH_2 bend) and 1053 for (C-O). C^{13}-NMR (400 MHZ, DMSO, TMS) of this spectra compound showed δ (ppm) at range 38-40 for –CH_2 (alkyl chain), δ (111-129) for =C=C= and δ (151-159) for C=O (Figure 11). The elemental analysis data showed the following: C= 63.91%, H= 5.88% and O= 30.21% from which the empirical formula is $C_{31}H_{34}O_{11}$. From the fragmentation pattern of the MS, C^{13}-NMR, IR and elemental analysis it was found that the active purified metabolite from *Myrothecium verrucaria* extract may be related to Macrocyclic antibiotic.

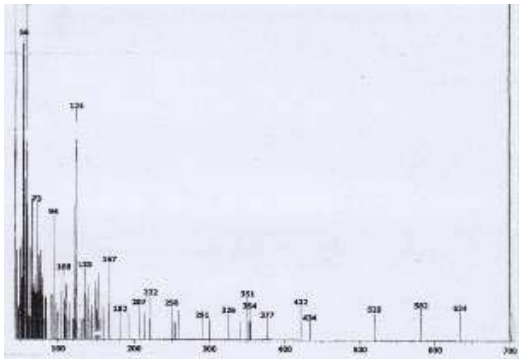

Figure 9. Mass spectrum of isolated compound.

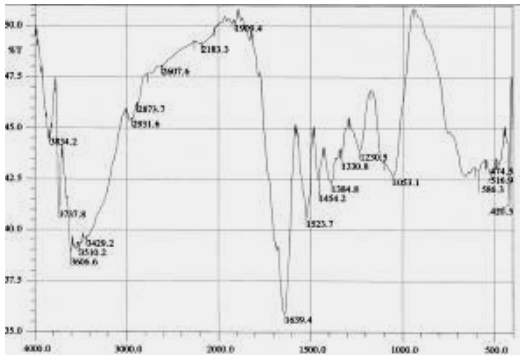

Figure 10. IR spectrum of isolated compound.

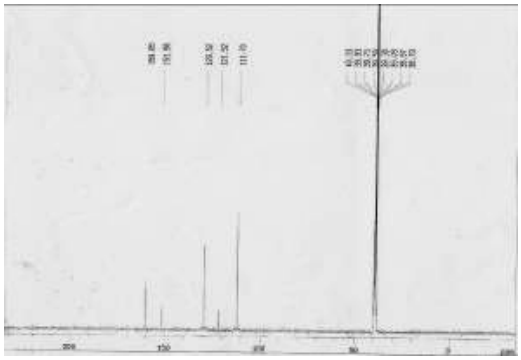

Figure 11. C^{13}-NMR spectrum of isolated compound.

In conclusion, endophytic fungi play an important role in the search for bioactive compounds and might also represent an alternative source for the production of antimicrobial, antitumor and antioxidant agents that are not easily obtained by chemical synthesis. The potential of these fungi is of great interest and warrants further investigations.

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