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ORIGINAL ARTICLE

Bioactive potential of endophytic *Myrothecium* sp. isolate M1-CA-102, associated with *Calophyllum apetalum*

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Abstract

Context: Endophytes colonizing medicinal plants are diverse, constituting a rich bioresource for novel natural products.

Objective: *Myrothecium* sp. isolate M1-CA-102 was the most promising among the 16 *Myrothecium* isolates screened. The bioactive potential of the crude extract from the *Calophyllum apetalum* Willd. endophytic *Myrothecium* sp. (Alb. & Schwein.) Ditmar (Incertae sedis) isolate M1-CA-102 and its thin layer chromatography (TLC) fractions were screened based on antioxidant, anti-inflammatory, antimicrobial activities, and cytotoxicity.

Materials and methods: The antioxidant activity was measured by 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) radical scavenging capacities. Further, 15-lipoxygenase (15-LOX) and human cyclooxygenase-2 (COX-2) inhibition were assessed at different concentrations (25, 50, and 100 μg/mL for the crude extract, 5, 25, and 50 μg/mL for the TLC fractions). DNA-nicking assay as an indicator of the capacity of extracts to scavenge hydroxyl radical was recorded at a concentration of 50 μg/mL. Cell cytotoxicity was recorded by colorimetric 3-(4,5-dimethylthylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Antibacterial (*Bacillus subtilis*) and anti-*Candida* (*Candida albicans*) assays were performed by the microdilution method.

Results: The DPPH and ABTS IC_{50} values of M1-CA-102 extract were 10 and 6 μg/mL compared with 6.1 and 7.03 μg/mL for the positive control quercetin. The cytotoxicity IC_{50} value of M1-CA-102 extract was 37 μg/mL, while the M-I TLC fraction was 21 μg/mL. The M1-CA-102 extract gave an IC_{50} value of 58 and 8 μg/mL for 15-LOX and COX-2, respectively. The MIC values for antimicrobial activity for M1-CA-102 extract ranged from 35 to 54 μg/mL, while for the TLC fractions, it ranged from 91 to 515 μg/mL.

Conclusion: The results indicate that *Myrothecium* M1-CA-102 isolated from *C. apetalum* is a potential source of natural metabolites of pharmaceutical importance.

Keywords

15-LOX, Antimicrobial, antioxidant, COX-2, cytotoxicity

History

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Introduction

Calophyllum apetalum Willd. (Clusiaceae) is an evergreen, endemic tree species of Western Ghats, India. The genus *Calophyllum* is composed of about 130 species confined to the warm humid tropics of the world. Leaf decoction is traditionally used as an eye remedy in Asian medicine (Peres & Nagem, 1997). Different parts of the tree are a source of secondary metabolites such as triterpenes, steroids, benzopyrans, xanthones, coumarins, and neoflavonoids with biological properties (Laure et al., 2008). The bioactive constituents of this genus exhibit a variety of biological activities including antibacterial, hypotensive, antiviral, antiretroviral, phagocyte stimulation, piscicidal (phenyl coumarins), and molluscicidal activity (Ha et al., 2009). Anti-HIV activity was exhibited by some dipyranocoumarins isolated from the genus *Calophyllum* (Ishikawa, 2000). Calophyllolide, a complex 5-Ph-coumarin, isolated from the nuts of *C. inolphyllum* L., showed antiarrhythmic, anti-inflammatory, antiarthritic activity, bradycardiac coronary dilator, and anticoagulant properties (Khare, 2007). Phongpaichit et al. (2006) reported that some of the medicinal properties of host plants have also been exhibited by endophytic fungi. The fungal endophytes exist in the intercellular space of plant tissues, which are capable of producing a large number of natural products (Kesting et al., 2011).

Endophytes could synthesize similar secondary metabolites as the host plant, presumably due to horizontal gene transfer from the host plant to the residing endophytes (Staniek et al., 2008). One ubiquitous example is paclitaxel (Taxol®) isolated from a fungal endophyte, *Taxomyces andreanae* A. (Stierle et al., 1993; Strobel et al., 1993). Several researchers have isolated this natural bioactive molecule from different endophytes of yews (Staniek et al.,

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2008; Stierle et al., 1993). Earlier studies on endophytes as potential sources of bioactive agents have led to the postulation that many unknown potent bioactive compounds are yet to be discovered from endophytes.

The endophytic fungus *Myrothecium* sp. (Alb. & Schwein.) Ditmar (Incertae sedis) colonizes a wide range of hosts (Wang et al., 2005). Bioherbicidal, antimalarial, and antifungal activities are exhibited by several isolates of the genus *Myrothecium*, but this genus also produces mammalian-sensitive mycotoxins (Hoagland et al., 2007; Isaka et al., 1999; Liu et al., 2006). In the present study, *Myrothecium* species was isolated from the endemic tree *C. apetalum*, and it is the first report on the endophytes and their bioactive potential from *C. apetalum*.

Materials and methods

Chemicals, reagents, cell lines, and reference cultures

Linoleic acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 15-LOX (soybean), 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), quercetin, trypsin, and 3-(4,5-dimethylthylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO). COX-2 inhibition kit was obtained from Cayman Chemicals (Ann Arbor, MI). Ascorbic acid (AA), butylated hydroxyl toluene (BHT), chloramphenicol, 10% fetal bovine serum, L-glutamine, potato dextrose agar (PDA), sodium biocarbonate, non-essential amino acids, and minimum essential medium Eagle were purchased from HiMedia (Mumbai, Maharashtra, India). Plasmid pBR322 was purchased from Merck Biosciences (Bangalore, Karnataka, India). HeLa (human cervix) cell lines were obtained from National Centre for Cell Science (Pune, Maharashtra, India). All the microbial strains of human pathogens used in the antimicrobial bioassay were procured from the Institute of Microbial Technology (IMTECH) (Chandigarh, India).

Isolation of endophytes

Fungal endophytes were isolated from five endemic trees of Western Ghats, India *viz*., *C. apetalum* Willd.; *Garcinia gummigutta* (L.) N. Robson, *G. indica* Choisy, *Garcinia morella* (Gaertn.) Desr., and *Garcinia xanthochymus* Hook.f.ex. T. of the Clusiaceae family as described earlier by Ruma et al. (2012). Sixteen isolates of *Myrothecium* sp. were isolated from *C. apetalum* and *G. morella* during three seasons, such as summer, rainy, and winter, which were used for the present study.

Preparation of crude extract of endophyte culture broth

Endophytic fungal isolates were grown on potato dextrose agar at 27 °C for 5 days. Three pieces (0.5–0.5 cm^2) of mycelial agar plugs were inoculated into 1000 mL Erlenmeyer flasks containing 500 mL potato dextrose broth and incubated at room temperature for 28 days under stationary condition. The broth and mycelia were blended together and extracted with equal volumes of ethyl acetate and mycelia were removed by filtration through four layers of muslin cloth. The filtrates were evaporated to dryness using a rotary evaporator. The dried extracts of the 16 isolates of *Myrothecium* sp. were dissolved in methanol and used further for the assays.

Purification of bioactive metabolites

Among the 16 isolates of *Myrothecium*, the isolate M1-CA-102 showed potent bioactivity, so it was used for further studies.

Thin layer chromatography (TLC) analysis of the extracts

The extract of *Myrothecium* (M1-CA-102; 20 µL, 10 mg/mL) was spotted on silica gel-coated TLC plates (20 cm × 10 cm with a 250-µm thickness, E. Merck, Darmstadt, Germany). The solvent system used was chloroform:methanol::10:0.5 (v/v). The optimized chamber saturation time for mobile phase was 30 min at room temperature [25 °C ± 2] and the length of chromatogram run was 8 cm. The plates were visualized under UV as well as in iodine chamber. The clearly visualized bands, such as M-I, M-flu and M-II with R_f values of 0.79, 0.75, and 0.6, respectively, were eluted and pooled separately from prep-TLC. They were then extracted from silica gel using methanol and concentrated in a vacuum evaporator.

The TLC fractions were analyzed for purity through analytical HPLC. The fractions were further analyzed for antioxidant (DPPH, ABTS), antimicrobial (antibacterial, antifungal), 15-LOX, COX-2, and cytotoxicity activities.

Determination of antioxidant activity

The antioxidant activity was determined for the crude extracts of 16 isolates of *Myrothecium* sp. and also for the TLC fractions of the isolate M1-CA-102.

DPPH scavenging assay

The free radical scavenging capacity of the extracts was determined by the DPPH method described by Brand-Williams et al. (1995) with minor modifications. The DPPH radical solution (300 µM) was prepared in ethanol and 95 µL of DPPH was added to each well of a microtitre plate. Different concentrations of test samples (5 µL) were added to the respective wells. The plate was incubated for 30 min at room temperature and the absorbance was recorded at 517 nm using Spectra max 340PC (Molecular Devices, Sunnyvale, CA). Ascorbic acid (AA), BHT, and quercetin (Q) were used as positive controls. The results were expressed as total antioxidant capacity (TAC) and a dose-dependent curve was plotted to calculate the IC_{50} value. The values are mean ± SD of three independent experiments. The activity is represented as % radical scavenging.

ABTS radical cation decolorization assay

The antioxidant activity was analyzed by the 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) method described by Re et al. (1999). ABTS was diluted in water to a final concentration of 7 mM. ABTS radical cations are generated by mixing 7 mM ABTS and 2.45 mM potassium persulfate and incubating the mixture at room temperature for

12–16 h in dark. This solution was diluted in methanol to obtain an absorbance of 0.70 at 734 nm. In this study, 10 µL of different concentrations (10, 25, 50, and 100 µg) of endophytic extracts were mixed with 990 µL of ABTS solution and the absorbance was measured using a UV/visible spectrophotometer (Beckman Coulter, DU 730 Life Sciences, Webster, TX) exactly 5 min after initial mixing. Ascorbic acid (AA), BHT, and quercetin (Q) were used as positive controls. A dose-dependent curve was plotted to calculate the IC_{50} value.

Total phenolic content

The total phenol content of the 16 isolates of *Myrothecium* extracts was determined by the Folin–Ciocalteu method as described by Singleton et al. (1999). The crude extracts (100 µL) were mixed with 500 µL of Folin–Ciocalteu reagent and incubated for 5 min at room temperature. Aqueous Na_2CO_3 (7.5%; 1.5 mL) was added to solution and it was mixed by vortexing. This solution was incubated at 37 °C in the dark for 2 h. The absorbance was measured at 734 nm using a UV/visible light spectrophotometer (Beckman Coulter, DU 730 Life Sciences). The results were analyzed in gallic acid equivalents using a (0–0.1 mg/mL) standard curve.

Antimicrobial activity

Antibacterial activity

Antibacterial activity was performed using Gram negative bacteria such as *Escherichia coli* (MTCC 724), *Klebsiella pneumoniae* (MTCC 661), *Salmonella typhi* (MTCC 733), *Shigella flexneri* (MTCC 1457); and Gram positive bacteria such as *Staphylococcus aureus* (MTCC 96) and *Bacillus subtilis* (MTCC 441). These cultures were maintained on nutrient agar slants and stored at 4 °C. The strains were sub-cultured on a fresh agar plate 24 h prior to antimicrobial testing. A disc-diffusion method was followed as described in Clinical and Laboratory Standards Institute (CLSI, 2012). The crude *Myrothecium* extract and its TLC fractions (10 µL; 10 mg/mL) were loaded onto 10 mm discs placed on these plates. They were incubated at 37 °C for 16–18 h. The diameter of inhibition zones as well as the minimum inhibitory concentration (MIC) was recorded and compared against the reference compound chloramphenicol under similar conditions of experiment.

Antifungal activity

Antifungal activity was tested using *Candida albicans* (MTCC 183). This culture was maintained on Sabouraud agar slants and stored at 4 °C. This strain was sub-cultured on a fresh agar plate 24 h prior to antimicrobial testing. The disc-diffusion method was followed as described by NCCLS (2003). The Sabouraud agar plates were inoculated with the fungal strain. The crude *Myrothecium* extract and its TLC fractions (10 µL; 10 mg/mL) were loaded onto 10 mm discs placed on these plates and were incubated at 25 °C for 48–72 h. The diameter of inhibition zones was recorded and compared against the reference compound, nystatin under similar experimental conditions. Zones of inhibition (mm) were measured after 72 h.

Determination of minimal inhibitory concentration

The MIC was determined by the broth microdilution method as described by Klančnik et al. (2010) for antibacterial assay. The bacterial strains were diluted with Muller Hinton Broth (MHB) to obtain the optimum cell density of 10^5–10^6 CFU/mL. Whereas the fungal strain was diluted with Sabouraud dextrose broth to obtain the optimum cell density 2×10^4 CFU/mL (CLSI, 2008). Crude *Myrothecium* extract and its TLC fractions were diluted in methanol with a range of concentrations which were 10, 5, 2.5, 1.25, 0.625, 0.3125, 0.156, 0.078, 0.039, and 0.019 mg/mL. These extracts (50 µL each) were added to the wells of a sterile 96-well microtitre plate. Each microbial suspension (50 µL) was added to these wells making the final volume 100 µL in each well. The plates were incubated for 24 h at 37 °C and 48–72 h at 25 °C for determining antibacterial and antifungal MIC, respectively. The minimum inhibitory concentration is considered to be the lowest concentration that inhibits the viability of microorganisms. This activity was measured by addition of 10 µL of MTT (2 mg/mL) to each well. The plates were incubated at 25 °C for 30 min in the dark. All the MIC values were based on three replicates. Chloramphenicol was used as a positive control for determining antibacterial MIC whereas; Nystatin was used as a positive control for determining antifungal MIC. The development of formazan was measured at an absorbance of 460 nm by using microtitre plate reader Varioskan Flash with SkanIt Software 2.4.3 RE (Thermo. Fisher Scientific Inc., Waltham, MA)

Kinetics of inactivation using broth macrodilution method

The kinetics of antimicrobial assay was performed using the cultures of *B. subtilis* and *C. albicans* with slight modifications as described by Burt (2004) and Klančnik et al. (2010). As per the results obtained by the disc diffusion and broth microdilution methods, the crude *Myrothecium* (M1-CA-102) extract was added to 5 mL of growth media. The diluted *B. subtilis* (10^5–10^6 CFU/mL) and *C. albicans* (2×10^4 CFU/mL) cultures were inoculated in their specific growth media as described earlier, containing the endophyte extract and were incubated for 24 h. The microbial growth samples were taken at 0, 3, 6, 9, 18, and 24 h. The microorganism count was calculated after incubation of the plates and also by colony counting. Positive control experiments were done without the addition of the endophyte extract. The mean log CFU/mL was calculated along with the standard deviation.

Anti-inflammatory assay

In vitro *15-lipoxygenase (LOX) inhibition assay*

The inhibition of 15-LOX was determined as described by Kemal et al. (1987). The substrate 0.2 µM linoleic acid was prepared in 0.2 M borate buffer (pH 9). Different concentrations of endophyte extracts were mixed with 15-LOX enzyme and incubated for 5 min at room temperature. The crude extract was used in the concentrations of 25, 50, and 100 µg/mL, and for the TLC fractions, the concentrations used were 5, 10, and 25 µg/mL for the inhibition studies. The substrate was added to the mixture and the absorbance was

measured at 243 nm using a UV–Vis spectrophotometer (Beckman Coulter, DU 730 Life Sciences). Quercetin was used as a positive control and methanol as a negative control. A dose-dependent curve was plotted to calculate the IC_{50} value.

Human cyclooxygenase-2 (COX-2) inhibition assay

Cyclooxygenase (COX) inhibition was measured using a Colorimetric Human COX-2 inhibitor screening assay kit (Cayman, Ann Arbor, MI). The crude endophytic extracts dissolved in methanol to a concentration of 25, 50, and 100 µg were used for inhibition studies as per manufacturer's protocol. The absorbance at 415 nm was measured by using a microtitre plate reader (Varioskan Flash with SkanIt Software 2.4.3 RE).

DNA protection studies

A DNA nicking assay was performed by using supercoiled pBR322 plasmid (Lee et al., 2002). Fenton reagent was prepared by mixing 30 mM H_2O_2, 50 µM ascorbic acid, and 80 µM $FeCl_3$. Plasmid DNA was added to 10 µL of the crude M1-CA-102 extract (5 mg/mL) and its TLC fractions (5 mg/mL) and incubated at 37 °C for 5 min. Fenton reagent was added to the solution and incubated at 37 °C for 30 min. The reaction mixture was analyzed by 1% agarose gel electrophoresis. The positive control reaction contains plasmid DNA and Fenton reagent whereas the negative control consists of only the plasmid DNA, incubated for 30 min under similar conditions. The results were documented using XR + Molecular Imager Gel documentation system (Bio Rad, Hercules, CA).

Cytotoxicity assay

HeLa cell lines were maintained in Eagle's minimum essential medium (2 mM L-glutamine and Earle's salts). The cytotoxicity was evaluated by MTT according to Mossmann (1983). HeLa cell cultures (5×10^5 cells/mL) were cultured in a 96-well flat bottomed microtitre plate and incubated for 48 h at 37 °C in a humidified 5% CO_2 incubator. Different concentrations of the crude *Myrothecium* extract and its TLC fractions were filtered through 0.11 µm filters and added to the wells. The plate was incubated for 48 h at 37 °C in a humidified incubator with 5% CO_2. The preparation of MTT (5 mg/mL) was carried out in phosphate buffered saline (PBS). To each well, 10 µL of MTT was added and incubated in dark for 4 h in CO_2 incubator. The supernatant was removed from the wells and the plate was washed three-times with Dulbecco's formula PBS (pH 7.3). To all the wells, DMSO (100 µL) and 0.1 M glycine buffer (25 µL, pH 10.5) were added. The absorbance of the samples was measured at 570 nm after 15 min. Doxorubicin was used as a positive control and methanol as a negative control. The IC_{50} values are the average of three assays. HeLa cells were treated with crude *Myrothecium* extract (M1-CA-102) and Doxorubicin. Later these cells were stained with acridine orange: ethidium bromide and observed under a fluorescent microscope.

Hemolytic activity assay

Indirect hemolytic activity of the *Myrothecium* extract was determined according to the method of Boman and Kaletta (1957) using packed human erythrocytes (blood group B) washed several times with PBS (0.15 mM, pH 7.2) and sedimented by gentle centrifugation (3000 rpm for 5 min) until a clear supernatant was obtained. For the assay, the stock was prepared by mixing packed 1 mL of erythrocytes, 1 mL of egg yolk, and 8 mL of PBS. About 1 mL of suspension from stock solution was incubated with various concentrations of the crude *Myrothecium* extract and the TLC fractions at 37 °C for 30 min. The reaction was terminated by adding 10 mL ice cold PBS and centrifuged at 4 °C at 1500 rpm for 10 min. The amount of hemoglobin released in the supernatant was measured at 540 nm. About 1 mL of stock erythrocytes with 10 mL ice cold PBS alone is considered as 0% lysis and the lysis with water was considered as 100% lysis.

HPLC analysis

Reverse phase-HPLC analysis of the crude endophyte extracts and the TLC fractions, such as *Myrothecium* (M1-CA-102), M-I, M-II, and M-flu, was performed using Waters (Milford, MA) system 515 HPLC pump equipped with UV–Vis detector (Waters, 2489, Milford, MA). The stationary phase was C18 Waters symmetry(R) column (4.6 × 250 mm, 5 µm). An isocratic mobile phase consisting of acetonitrile:water:acetic acid::18:82:2 (v/) was delivered at a flow rate of 1 mL/min. The elution profiles were read at 280 nm and 320 nm. A mixture of reference compounds such as gallic acid, quercetin, and phloroglucinol-R were used.

MS analysis

Mass spectrometry (MS) was performed using a Waters Synapy G2 with UPLC Acquity System (Waters, Milford, MA) to measure the mass of the TLC fractions M-I, M-II, and M-flu. Mass spectra data were acquired by electrospray ionization (ESI) in a negative ion/positive ion mode. The sample preparation was carried out in acetonitrile:water:formic acid::50:50:1 (v/v) (0.1%). ESI was carried out within a range of mass to charge (*m*/*z*) 100–1000.

Statistical analysis

All assays were conducted in triplicates. The reported value was calculated as the mean of three independent experiments ± standard deviation (SD). Statistical comparisons for IC_{50} values, 15-LOX and human COX-2 inhibition, were carried out using a one-way ANOVA.

Results

Fungal endophytes

A total of 169 fungal endophytes were recovered from *C. apetalum* (Ruma et al., 2012). From the consortium of endophytes, 16 isolates of the genus *Myrothecium* sp. (Table 1) were selected for this study.

Partially purified fractions

Preparative TLC

Three bands were observed for the extract of *Myrothecium* (M1-CA-102) (Figure 1). The bands visible after developing

in iodine were M-I and M-II and the band visible under UV M-flu, were eluted.

Antioxidant activity

The crude extracts of 16 isolates of *Myrothecium* reduced the radicals DPPH and ABTS significantly (Table 2). The extract of *Myrothecium* isolate M1-CA-102 showed highest DPPH and ABTS scavenging activities. The reduction capacity of DPPH radicals was determined by the decrease of the absorbance induced at 517 nm. Among the crude extracts, *Myrothecium* (M1-CA-102) extract showed the strongest DPPH activity of 77.26%. In DPPH assay, the IC_{50} for quercetin, BHT, and AA were 6.1, 14.46, and 32.30 µg/mL, respectively. The percent DPPH radical scavenging activity for the TLC fractions was highest in M-flu which was 58.64% at a concentration of 50 µg with an IC_{50} value of 45 µg/mL (Table 3).

The IC_{50} value of ABTS inhibition assay for the reference compounds was 7.03 µg/mL for quercetin, 19.5 µg/mL for BHT, and 75.2 µg/mL for ascorbic acid. In ABTS radical cation decolorization assay, *Myrothecium* isolate M1-CA-102 showed 99.45% scavenging activity. The highest percent ABTS scavenging activity of 79.65% was observed for the TLC fraction M-flu at a concentration of 50 µg with an IC_{50} value of 21 µg/mL (Table 3).

Total phenol

The results showed that the total phenol content in the 16 *Myrothecium* crude extracts varied considerably. Among the different extracts of endophytes, the highest content of total phenol 154.02 mg GAE/mg was found in *Myrothecium* isolate M1-CA-102.

Table 1. Isolates of *Myrothecium* spp. obtained from *Calophyllum apetalum* and *Garcinia Morella*.

Sl. no.	Isolate code	Source	Season	Locations	Accession number
Calophyllum apetalum					
1	M1-CA-102	Bark	Summer	Shimoga	JX862206
2	M2-CA-203	Twig	Rainy	Shimoga	JX908884
3	M3-CA-213	Twig	Rainy	Shimoga	JX908885
4	M4-CA-222	Bark	Rainy	Shimoga	JX908886
5	M5-CA-158	Bark	Summer	Mysore	JX908887
6	M6-CA-301	Twig	Winter	Kajre	JX984612
7	M7-CA-302	Twig	Winter	Kajre	JX984613
8	M8-CA-305	Twig	Winter	Kajre	JX984614
9	M9-CA-360	Twig	Winter	Mysore	JX984615
Garcinia morella					
10	M10-GM-111	Twig	Summer	Nagara Fort	JX984616
11	M11-GM-123	Twig	Summer	Nagara Fort	JX984617
12	M12-GM-130	Twig	Summer	Madikeri	JX984618
13	M13-GM-345	Bark	Winter	Nagara Fort	JX984619
14	M14-GM-367	Twig	Winter	Shimoga	JX984620
15	M15-GM-368	Twig	Winter	Madikeri	JX984621
16	M16-GM-369	Twig	Winter	Madikeri	JX984622

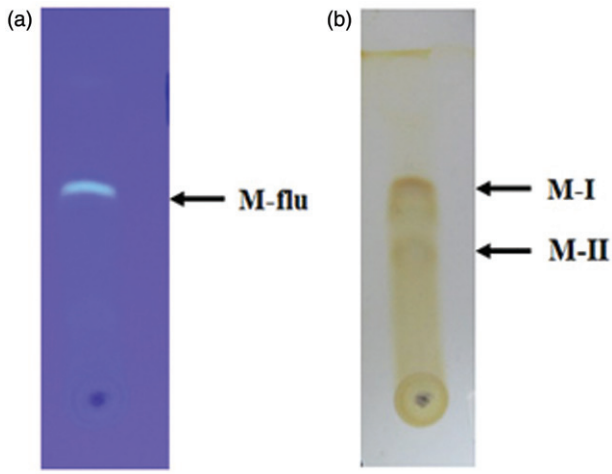

Figure 1. TLC profiles of the crude *Myrothecium* (M1-CA-102) extract. (a) TLC plate visualized under UV light and (b) TLC plate after development in iodine.

Table 2. Percent scavenging activity of DPPH and ABTS radicals by extracts of 16 *Myrothecium* isolates.

Sl. no.	Isolate code	% DPPH radical scavenging activity	% ABTS radical scavenging activity	Total phenol content (mg/mL)
1	M1-CA-102	**71.24 ± 2.02**	**99.45 ± 3.55**	**154 ± 2.45**
2	M2-CA-203	23.87 ± 1.89	39.22 ± 3.75	23 ± 3.35
3	M3-CA-213	35.9 ± 2.22	35.35 ± 2.45	28 ± 3.27
4	M4-CA-222	15.2 ± 3.09	22.3 ± 3.64	17 ± 3.49
5	M5-CA-158	52.09 ± 3.45	68 ± 2.85	55 ± 2.65
6	M6-CA-301	11.05 ± 3.23	21.89 ± 2.48	13 ± 1.99
7	M7-CA-302	40.21 ± 2.67	33.66 ± 2.53	31 ± 1.58
8	M8-CA-305	16.25 ± 1.22	18 ± 3.33	10 ± 2.66
9	M9-CA-360	10.26 ± 2.36	15.03 ± 3.85	8 ± 2.95
10	M10-GM-111	21.5 ± 2.87	28.55 ± 3.05	17 ± 3.88
11	M11-GM-123	22.36 ± 3.56	26.79 ± 3.66	20 ± 2.76
12	M12-GM-130	35.45 ± 1.65	38.75 ± 2.85	27 ± 3.22
13	M13-GM-345	21.22 ± 1.90	27.5 ± 2.58	20 ± 3.82
14	M14-GM-367	36.85 ± 2.85	41.44 ± 3.57	38 ± 3.63
15	M15-GM-368	19.32 ± 2.69	23.7 ± 3.23	17 ± 2.87
16	M16-GM-369	33.62 ± 2.55	38.29 ± 2.55	32 ± 2.90
BHT (+control)	–	85.25 ± 3.05	100 ± 2.95	–

Bold values signify that amongst the 16 isolates screened the isolate M1-CA-102 has given the highest antioxidant activity and has been taken up for further studies.
Each result is expressed as mean ± SD ($n = 3$).
Total phenolic content is expressed in mg gallic acid equivalent (GAE)/100 g dry weight (DW).

Antimicrobial activity

The crude extract of *Myrothecium* isolate M1-CA-102 and its TLC fractions were most active against Gram negative bacteria. The inhibition zones ranged from 8 to 23 mm (Table 4). The crude extract displayed the most potent antimicrobial activity in comparison to the TLC fractions. The crude extract of *Myrothecium* isolate M1-CA-102 was most effective against *K. pneumoniae*, *B. subtillis*, and S. *flexneri*, exhibiting an inhibition zone ranging from 20 to 23 mm, where as the inhibition zone for *S. aureus*, *E. coli,* and *S. typhi* ranged between 17 and 18 mm. Among the TLC fractions, M-flu displayed the most potent antimicrobial activity followed by M-1. The crude extract of *Myrothecium* isolate M1-CA-102 showed clear antifungal activity against *C. albicans*. MIC values were determined by the microdilution method ranged from 35 to 200 µg/mL for bacteria, whereas for *C. albicans*, it ranged from 54 to 225 µg/mL (Table 4). The range of sensitivity measured by the disc diffusion and broth microdilution method was comparable.

In this study, we also evaluated the growth inhibition kinetics at the concentration pre-determined as the MIC by the broth microdilution method. The effect was visible as growth inhibition and, consequently, no growth was observed in 24 h. The growth, survival, and death curves for *B. subtilis* and *C. albicans* at various concentrations of the crude *Myrothecium* extract are shown in Figure 2(a) and (b), respectively.

Table 3. Antioxidant activity of the crude extract of *Myrothecium* sp. M1-CA-102 isolated from twigs of *Calophyllum apetalum* and its TLC fractions.

Sl. no.	Test samples	DPPH (IC_{50}) (µg/mL)	ABTS (IC_{50}) (µg/mL)
1	M1-CA-102 (crude)	10 ± 1.25	6 ± 2.32
2	M-I (TLC fraction)	45 ± 2.09	43 ± 1.46
3	M-II (TLC fraction)	45 ± 2.5	40 ± 1.37
4	M-flu (TLC fraction)	40 ± 1.95	21 ± 2.22
5	Ascorbic acid (+ control)	32.30 ± 1.86	75.2 ± 2.83
6	Butylated hydroxytoluene (+ control)	14.46 ± 1.57	19.5 ± 2.51
7	Quercetin (+ control)	6.1 ± 2.08	7.03 ± 1.64

Each result is expressed as mean ± SD ($n = 3$).

15-LOX inhibition

The anti-inflammatory activity was evaluated through the percent inhibition of lipoxygenase enzyme. The crude extract of *Myrothecium* isolate M1-CA-102 at 100 mg concentration showed a 90.20% LOX inhibition. The IC_{50} value of LOX inhibition for quercetin was 1.45 µg/mL, whereas the IC_{50} of *Myrothecium* isolate M1-CA-102 was 58 µg/mL. The TLC

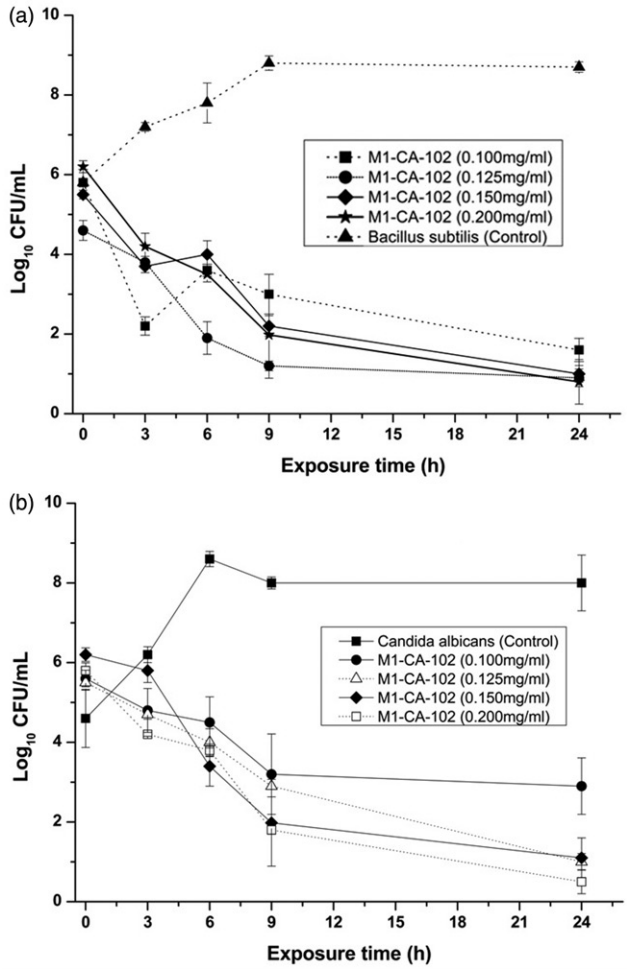

Figure 2. *Bacillus subtilis* (a) and *Candida albicans* (b) growth, survival, and death curves on exposure to the crude extract of *Myrothecium* (M1-CA-102). Each point represents the log of the mean ± SD CFU/mL.

Table 4. Antimicrobial activity of the crude extract of *Myrothecium* sp. M1-CA-102 and the TLC fractions.

Microorganisms	Zone of inhibition (mm)[a]/(minimum inhibitory concentration [µg/mL])[b]						
	Antibacterial activity						Antifungal activity
Test samples	*Klebsiella pneumoniae*	*Staphylococcus aureus*	*Escherichia coli*	*Salmonella typhi*	*Shigella flexneri*	*Bacillus subtilis*	*Candida albicans*
M1-CA-102 (crude)	23 ± 1.25 (35)	17 ± 1.22 (88)	17 ± 2.32 (82)	18 ± 1.5 (64)	23 ± 2.07 (40)	20 ± 1.5 (57)	21 ± 1.22 (54)
M-I (TLC fraction)	12 ± 2.09 (143)	11 ± 1.85 (156)	13 ± 2.01 (137)	9 ± 1.0 (185)	12 ± 2.2 (155)	11 ± 2.02 (160)	7 ± 1.5 (225)
M-II (TLC fraction)	6 ± 2.22 (186)	7 ± 2.65 (181)	10 ± 2.0 (165)	7 ± 1.25 (200)	11 ± 1.45 (152)	9 ± 2.0 (170)	0 (515)
M-flu (TLC fraction)	19 ± 1.5 (91)	15 ± 2.05 (110)	12 ± 1.07 (138)	12 ± 1.23 (145)	17 ± 1.25 (105)	18 ± 1.56 (82)	11 ± 2.04 (167)
Chloramphenicol[c]	25 ± 2.1 (13.5)	27 ± 2.08 (22)	21 ± 1.9 (21)	23 ± 2.05 (27)	25 ± 1.9 (32)	27 ± 1.25 (47)	–
Nystatin[d]	–	–	–	–	–	–	23 ± 2.12 (21.5)

[a]All determinations were done in triplicates, standard deviation for 95% confidence.
[b]The values are mean of three determinations, the ranges of which are <5% of the mean in all case.
[c]Positive control for antibacterial activity.
[d]Positive control for antifungal activity.

fraction M-flu at a concentration of 25 μg showed 81.57% LOX inhibition (Table 5).

Human COX-2 inhibiton

The inhibitory effect of the endophyte extracts on human COX-2 was measured by *in vitro* enzymatic activities. *Myrothecium* isolate M1-CA-102 crude extract exhibited 94.10% inhibition to human COX-2. The TLC fraction M-flu showed the highest percent COX inhibition of 76.99% at a concentration of 50 μg (Table 5).

DNA protection studies

Plasmid pBR322 DNA was exposed to Fenton's reagent for 30 min at 37 °C which caused a super shift from native form (Form I) to nicked DNA (Form II) displaying a differential pattern in gel (Figure 3). The plasmid DNA incubated with the crude extract and the TLC fractions of *Myrothecium* isolate M1-CA-102 at a concentration of 50 μg for 30 min at 37 °C and further exposure to Fenton's reagent protected the plasmid DNA from damage. The crude extract of *Myrothecium* and the TLC fractions M-I and M-flu provided protection to the DNA when compared to fraction M-II.

Cytotoxicity assay

The extracts and the TLC fractions were subjected to the MTT assay in order to dertermine their cytotoxicity against the HeLa cell line. The percentage of cell inhibition was determined and compared to those of untreated controls. Less than 10% cell survival indicated cytotoxicity and IC_{50} values were estimated for the corresponding TLC fractions. The crude extract of *Myrothecium* isolate M1-CA-102 showed the highest cell growth inhibition, and the IC_{50} was 37 μg/mL. Doxorubicin showed an IC_{50} value of 16 μg/mL. The results indicate that the crude extract exhibits potential cytotoxicity as evaluated by the *in vitro* screening test (Table 6). HeLa cells treated with crude *Myrothecium* (M1-CA-102) extract and doxorubicin showed clear cell disruption (Figure 4).

Hemolytic activity analysis

The extract and its TLC fractions were found to be non-toxic at the concentrations of 25, 50, and 100 μg as it did not lead to any hemolysis of the cells.

HPLC analysis

The partially purified TLC fractions of the crude extracts were analyzed by HPLC. Reference compounds used for the study were gallic acid, quercetin, and phloroglucinol-R. The crude extract of *Myrothecium* showed the presence of a peak with highest intensity at 14.362 retention time (Figure 5a). The chromatograms of the TLC fractions M-I (Figure 5b) showed a major peak at 8.532 retention time, whereas the fractions M-II and M-flu showed a major peak at 6.677 and 8.788 retention time, respectively (Figure 5c and 5d). The chromatogram of the mixture of reference compounds, gallic acid, quercetin, and phloroglucinol-R (Figure 5e) showed specific peak at retention times 3.044, 5.730, and 9.609 min, respectively, scanned at wavelength 280 nm.

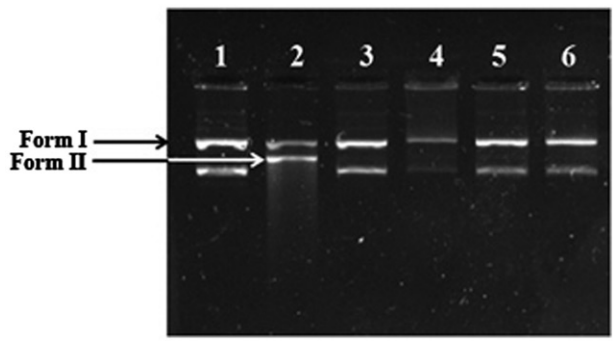

Figure 3. Endophyte extracts assessed on plasmid pBR322 DNA treated by Fenton's reagent. Lane 1: pBR322 (native plasmid DNA); Lane 2: pBR322 DNA + Fenton's reagent; Lane 3: pBR322 + *Myrothecium* extract (50 μg) + Fenton's reagent; Lane 4: pBR322 + TLC fraction M-II (50 μg) + Fenton's reagent; Lane 5: pBR322 + TLC fraction M-I (50 μg) + Fenton's reagent; Lane 6: pBR322 + TLC fraction M-flu (50 μg) + Fenton's reagent.

Table 5. Anti-inflammatory activity of *Myrothecium* extract and its TLC fractions.

	15-Lipoxygenase activity			Human cyclooxygensae-2 activity		
Test samples	Final concentration of methanolic extract (μg/mL)	% inhibition	IC_{50} (μg/mL)	Final concentration of methanolic extract (μg/mL)	% inhibition	IC_{50} (μg/mL)
M1-CA-102 (crude)	25	22.74		25	79.55	
	50	42.57	58	50	94.10	8
	100	90.20		100	100	
M-I (TLC fraction)	5	11.22		5	6.03	
	10	22.89	25	25	15.11	50
	25	45.16		50	33.45	
M-II (TLC fraction)	5	9.31		5	6.27	
	10	17.89	57	25	11.9	68
	25	26.16		50	23.42	
M-flu (TLC fraction)	5	27.98		5	22.04	
	10	49.44	10	25	58.43	21
	25	81.57		50	79.66	
Quercetin (+control)	25	35.72		25	81.29	
	50	68.55	36	50	98.65	5
	100	100		100	100	

MS analysis

The mass spectrometry technique was facilitated to analyze the mass of the TLC fractions, M-I, M-II, and M-flu. The MS spectrum of TLC fractions of *Myrothecium* (M1-CA-102) exhibited intense peak of 114.1016, 247.1340, and 345.1 *m/z* for M-I, M-II, and M-flu, respectively (Figure 6a–c).

Discussion

With the increasing awareness and knowledge regarding mutualistic microorganisms, endophytes have been revealed as a rich and consistent source for chemically active novel metabolites that may produce potential medicines. The majority of the fungal endophytes undiscovered are inhabitants of tropical trees (Arnold, 2008). *Myrothecium* species are ubiquitous, and the biochemical investigations have afforded a wide variety of natural bioactive molecules (Li et al., 2007). Although several bioactive secondary metabolites have been isolated and characterized from *Calophyllum* species, reports are not available on its endophyte profile and the secondary metabolites from those endophytes. This study reports that the endophytes of *C. apetalum* possess bioactive compounds that displayed diverse biological activity.

Table 6. Cytotoxicity of crude extract of *Myrothecium* sp. M1-CA-102 and its TLC fractions against HeLa cervix cancer cell lines.

Test samples	Concentration (μg/mL)	% inhibition of HeLa cells	IC_{50} (μg/mL)
M1-CA-102 (crude)	25	35.47	
	50	68.90	37 ± 1.25
	100	98.54	
M-I (TLC fraction)	15	25.26	
	25	70.54	21 ± 1.2
	50	92.47	
M-II (TLC fraction)	15	20.71	
	25	42.35	36 ± 1.22
	50	60.12	
M-flu (TLC fraction)	15	22.15	
	25	47.92	27 ± 1.09
	50	71.15	
Doxorubicin (+ control)	15	48.30	
	25	64.57	16 ± 1.53
	50	99.05	

Our study demonstrated that the crude extract of *Myrothecium* M1-CA-102 isolate as well as its TLC fractions has good antioxidant properties when assessed by DPPH and ABTS radical scavenging methods. The results showed the ability of these extracts to reduce the free radical initiation or retard free radical chain reaction in the oxidation mechanism. Antioxidant activities have also been reported from endophytes, which can be estimated by different methods (Harper et al., 2003; Yu et al., 2002). In the present study, the IC_{50} value for the crude extract of *Myrothecium* (M1-CA-102) for scavenging DPPH radical was 10 μg/mL, whereas for $ABTS^{+}$, the IC_{50} value was 6 μg/mL. Among the TLC fractions, the lowest IC_{50} value of 40 μg/mL for DPPH radical scavenging was recorded in M-flu. The IC_{50} values for $ABTS^{\cdot+}$ were 21 μg/mL for M-flu. The potential of these extracts with persuasive antioxidant capacity could be credited to the presence of phenols in them. Earlier reports also provided evidence that phenolic compounds are potent antioxidants, and

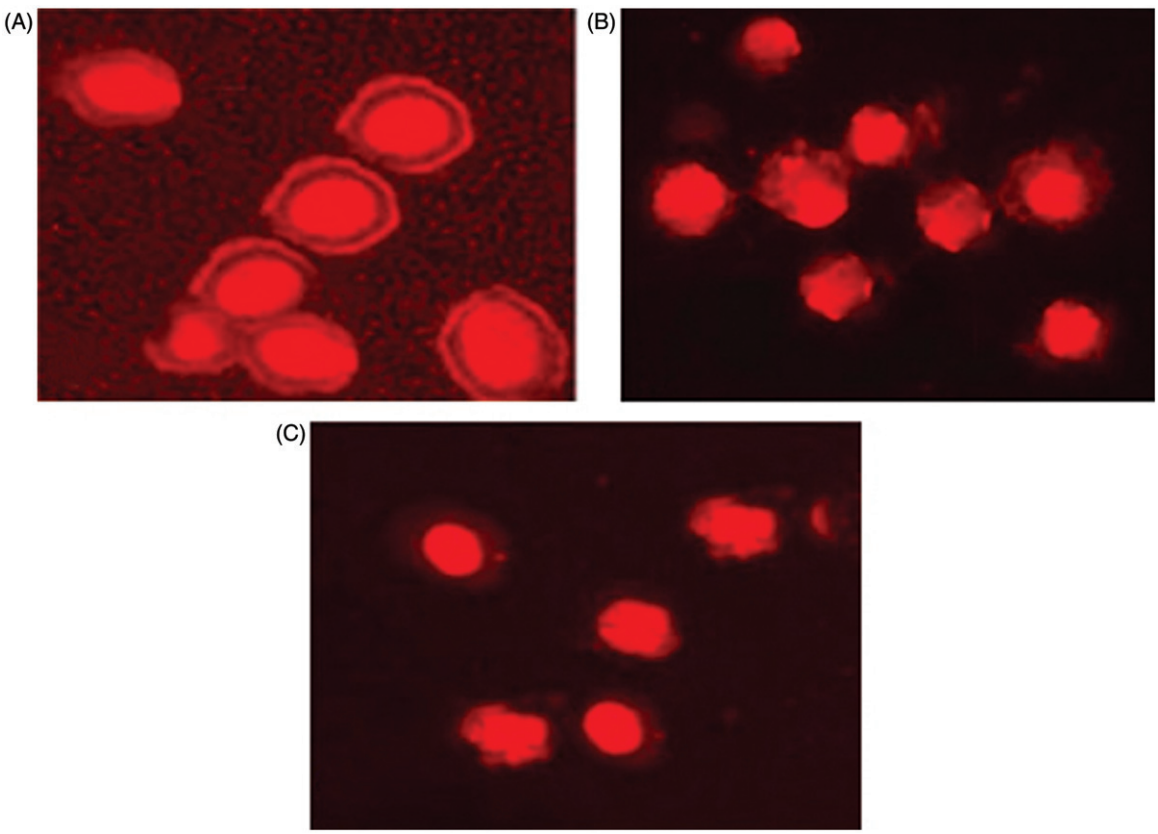

Figure 4. Acridine orange – ethidium bromide staining of HeLa cells observed under fluorescent microscope. (A) Control (untreated cells), (B) cells treated with doxorubicin, and (C) cells treated with the crude extract of *Myrothecium* (M1-CA-102).

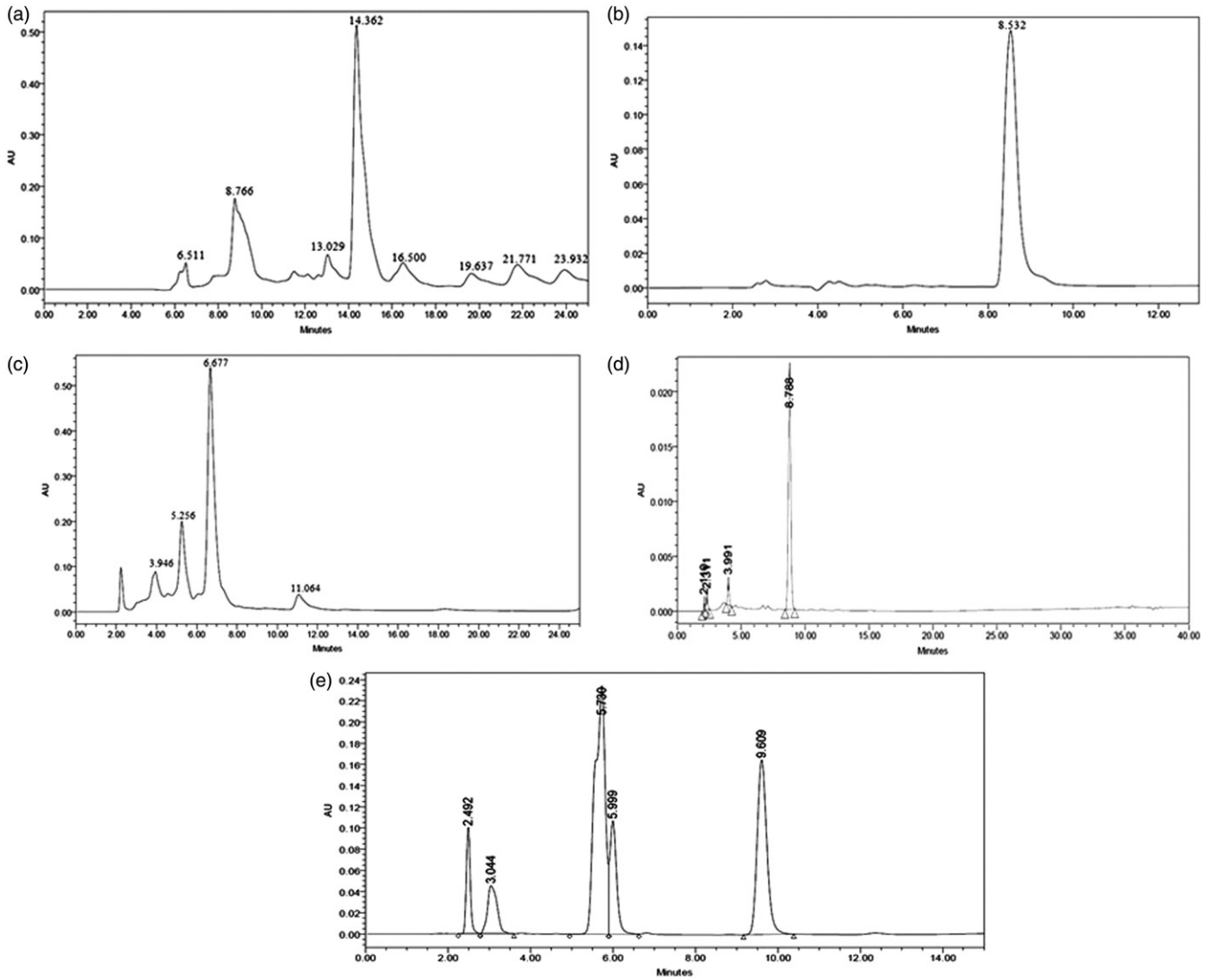

Figure 5. Analytical HPLC chromatograms of standards and crude endophyte extracts separated on a semi-preparative RP-HPLC column. (a) HPLC chromatogram of *Myrothecium* extract. (b) HPLC chromatogram of TLC fraction M-I from *Myrothecium.* (c) HPLC chromatogram of TLC fraction M-II from *Myrothecium*. (d) HPLC chromatogram of TLC fraction M-flu from *Myrothecium.* (e) HPLC chromatogram of a mixture of gallic acid, quercetin, and phloroglucinol-R showing specific peak at retention time 3.044, 5.730, and 9.609 min, respectively.

they possess other properties such as anticancer, antimicrobial, antimutagenic, immunomodulatory, antiviral, or anti-inflammatory activities (Li et al., 2005a,b; Tapiero et al., 2002; Yu et al., 2005). The presence of phenols in the extract obtained in the present study could be responsible for the antioxidant properties of these endophytes and their fractions.

The poly-unsaturated fatty acids (PUFAs) generated from the phospholipids of the cell membrane act as the substrates for the enzymes, COX and lipoxygenases (LOX), resulting in the production of metabolites, such as the eicosonoids and the prostaglandins, which are responsible for a range of allergic and inflammatory ailments (Reddy et al., 2009). Non-steroidal anti-inflammatory drugs (NSAIDs) are usually used for the treatment of inflammation by inhibiting these enzymes. The present study reports a dual inhibitor of the COX-LOX from the endophyte extracts such as *Myrothecium* (M1-CA-102) and its TLC fractions such as M-I, M-II, and M-flu. This work adds to the few studies showing the inhibition of both LOX and COX-2 by endophyte extracts. Reddy et al. (2009) reported chebulagic acid isolated from the fruits of *Terminalia chebula* as a dual inhibitor of COX-2 and 5-LOX, which also induces apoptosis in COLO-205 cells.

Secondary metabolites produced by endophytes help to surmount the invasion of pathogens as it is believed to bring out a resistance mechanism in the host (Tan & Zou, 2001). There are several reports on antimicrobial compounds isolated from endophytes, belonging to diverse classes such as alkaloids, peptides, steroids, terpenoids, phenols, and flavonoids (Pimentel et al., 2011). The antifungal bioactives were identified from the anamorphs of the endophytic genera such as *Colletotrichum*, *Alternaria*, *Ovulariopsis*, *Pestalotiopsis*. *Phomopsis*, and *Phoma* (Li et al., 2001; Lu et al., 2000; Weber et al., 2004; Xiang et al., 2003). The crude extract of *Myrothecium* showed a better zone of inhibition for *C. albicans* as well as for a majority of the Gram negative bacteria that could be endorsed to the presence of bioactive compounds like phenols and flavonoids. The present study reveals that the crude extract of *Myrothecium* (M1-CA-102) possessed a broad spectrum antibacterial property with special reference to Gram negative bacteria.

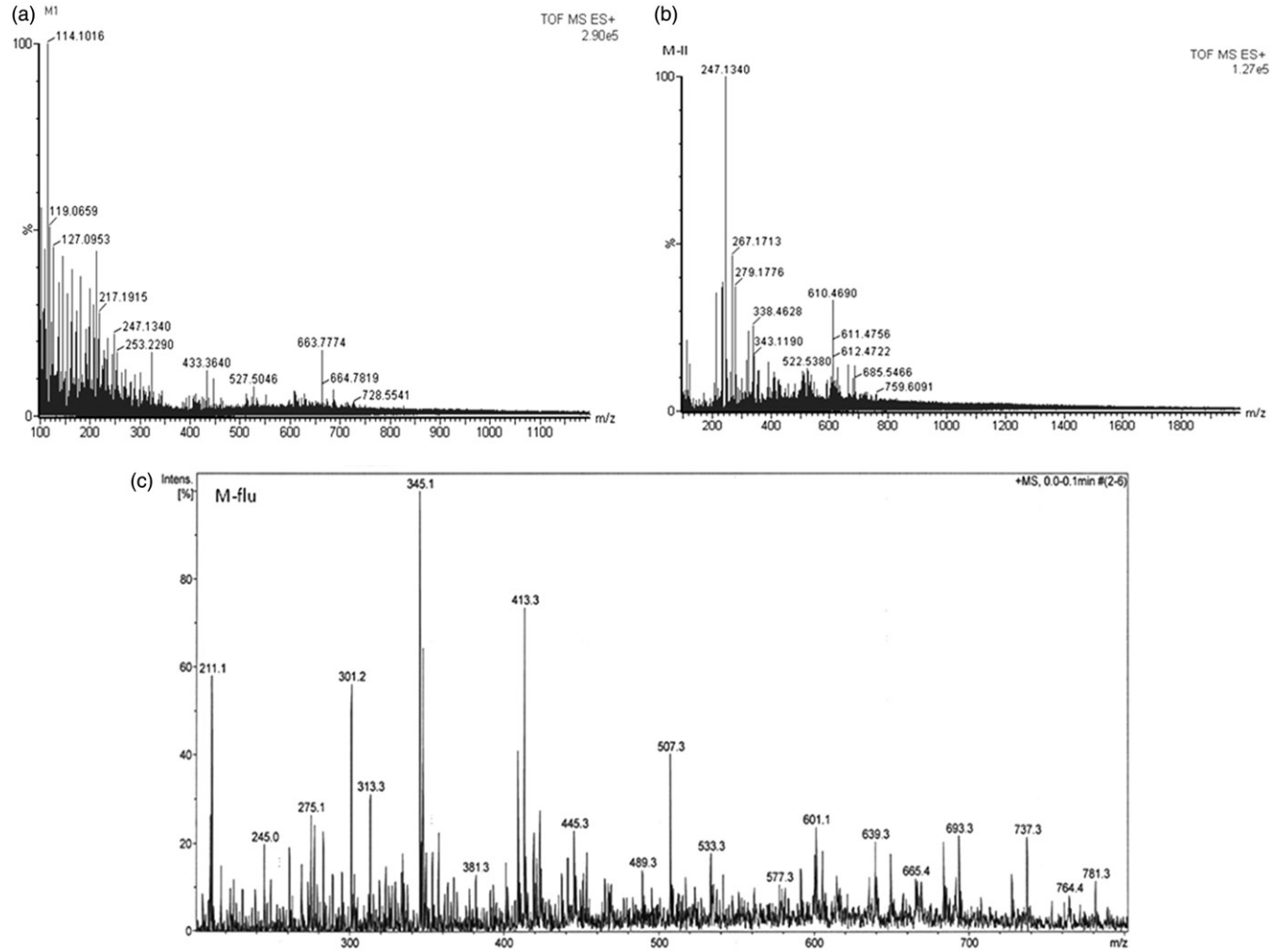

Figure 6. Mass spectra of the TLC fractions of *Myrothecium* (M1-CA-102). (a) MS of TLC fraction M-I. (b) MS of TLC fraction M-II. (c) MS of TLC fraction M-flu.

Studies on reactive oxygen species revealed that it plays a critical role in skin damage, aging, and several neurodegenerative ailments (Lopaczynski & Zeisel, 2001). In our study, the crude extract of *Myrothecium* and the TLC fractions at a concentration as low as 50 µg/mL had the capacity to scavenge the ·OH radicals produced by Fenton's reagent protecting the pBR322 plasmid DNA. The absence or reduction of Form II plasmid DNA specified a remarkable safeguard offered by the extracts. Most of the anticancer drugs work either by reducing the free radicals or through direct interaction with the DNA (Akhdar et al., 2012). This study clearly implies the protection of DNA by these extracts.

Cytotoxic and antitumor activities of endophytic fungi from the Chinese medicinal plant *Actinidia macrosperma* against brine shrimp and five types of tumor cells were studied by Lu et al. (2012). Li et al. (2005a) reported that among the 130 endophytes isolated from 12 Chinese traditional medicinal plants tested for antitumor and antifungal activities by MTT assay on human gastric tumor cell line BGC-823, 9.2% of the endophytic isolates exhibited antitumor activity. An endophytic fungus *Colletotrichum gloeosporioides* (strain JGC-9) isolated from the medicinal plant, *Justicia gendarussa*, was screened for the production of taxol. Taxol or paclitaxel is a registered antitumor agent and used by physicians in tumor treatments (Gangadevi & Muthumary, 2008). In the present study, the endophytic crude extracts and its TLC fractions showed cytotoxicity against the HeLa cell line. The crude extract of *Myrothecium* showed the strongest cytotoxicity whereas among the TLC fractions, M-I, illustrated a robust cytotoxic activity showing 92% cell inhibition.

HPLC analysis of the crude extract such as *Myrothecium* and its TLC fractions such as M-I, M-II, and M-flu were carried out. The HPLC analysis showed that the TLC fractions were purified as it displayed single major peak. The biological activity displayed by the TLC fractions, M-I and M-flu, acclaims to be potent and these will be further investigated for their structural analysis of the bioactive compounds.

Conclusion

Exploring novel endophytes and their related secondary metabolites should also be directed towards plants that are commonly used in traditional medicines. It is feasible that these plants harbor microbes which imitate the chemistry of their respective hosts producing similar bioactive(s) or derivatives that may be more effective than those of their

hosts (Strobel, 2003). The results from this study evidently indicate that the high diversity of endophytic fungi of *C. apetalum* is a promising source of novel bioactive compounds with therapeutic value. Endophytic fungi associated with the endemic tree species of Western Ghats (India) is a kind of poorly studied microorganisms, which are now recognized as a novel reservoir of bioactive metabolites with a wide array of curative properties. These findings will continue to drive the research of biological evaluation and chemical characterization in this field.

Declaration of interest

The authors declare there is no conflict of interest.

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