View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by University of Mysore - Digital Repository of Research, Innovation and Scholarship...

Academic Sciences International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 5, Suppl 3, 2013

Research Article

ANTIOXIDANT, ANTI-INFLAMMATORY, ANTIMICROBIAL AND CYTOTOXIC PROPERTIES OF FUNGAL ENDOPHYTES FROM GARCINIA SPECIES

KARMAKAR RUMA, KUMAR SUNIL, H. S. PRAKASH*

Department of Studies in Biotechnology, University of Mysore, Manasagangotri, Mysore 570006, Karnataka, India.
Email: prakashhs.biotech@gmail.com; hsp@appbot.uni-mysore.ac.in

Received: 15 July 2013, Revised and Accepted: 09 Aug 2013

ABSTRACT

Objective: Fungal endophytes of medicinal plants occupy a unique habitat, highly diverse and are important sources of natural metabolites of pharmaceutical importance.

Methods: The endophytes isolated from four species of *Garcinia* were subjected to fermentation in still culture and extracted using ethyl acetate. The crude extracts were screened for the biological activity through the assays such as antioxidant activity, 15-lipoxygenase/ human cyclooxygenase-2 inhibition, cytotoxicity, DNA nicking assay and antimicrobial activity.

Results: *Aspergillus fumigatus* and *Fusarium* sp. were most promising amongst the endophytes screened from *Garcinia* spp. The HPLC and MS profiling of *Aspergillus fumigatus* anticipated the presence of the compound phloroglucinol, which could be involved in the biological activities of this extract.

Conclusion: The results indicate that some of these endophytes isolated from *Garcinia* plants are a potential source for bioactives and could be further exploited to foster the identity of the novel molecule.

Keywords: Fungal endophytes; Secondary metabolites; Antioxidant activity; Antimicrobial activity; 15-LOX; Human COX-2; DNA protection studies; Cytotoxicity; RP-HPLC; MS.

INTRODUCTION

Natural products are metabolites derived from nature *viz.*, plants, microorganisms, or animals. Plants have been the major source of these compounds and are being exploited for human use for many years [1]. Bioactive compounds from natural resources have constantly been of immense importance to researchers working on several diseases [2]. Even though early drugs appeared from plants *viz.*, aspirin from willow tree, morphine from poppies, digitalis from foxglove, synthetic drug discovery has largely moved beyond natural products. Most of the cancer therapeutic drugs are derived from natural sources [3]. However, the loss of biodiversity reduces the raw material supply for the discovery of potential medicines. Hence, several recent studies have led to the breakthrough of significant plant secondary metabolites from fungal endophytes, thus hoisting the hope of using them as alternative resources for bioactive metabolites [4].

Endophytes colonize internal tissues of all plant species and are highly diverse in nature. Fungal endophytes produce various kinds of secondary metabolites, which have bioactive potential such as antioxidant, anti-inflammatory, antidiabetic, antitumor, antimicrobial properties [5, 6]. A current comprehensive study has specified that 51% of bioactive metabolites isolated from endophytic fungi were previously unidentified [7]. The spectacular discovery of the well known anti-cancer molecule, Paclitaxel (Taxol®) produced by the endophytic fungus *Taxomyces andreanae* from the yew plant *Taxus brevifolia,* verified that the latter being the original source of this vital drug [8].

Garcinia species finds application in traditional medicine to treat various infections [9]. In India, the fruits of *Garcinia indica* known as Kokum have been reported to have anti-scorbutic, cholagogue, cooling, anti-bilious, emollient and demulcent properties. The kokum butter is used for dysentery and is also applied externally to ulcerations and skin diseases, whereas the gum-resin of *G. morella* is a hydragogue, cathartic, anti-helmintic and amenorrhoea properties [10]. *Aspergillus*, *Botryosphaeria*, *Curvularia*, *Fusicoccum*, *Guignardia*, *Muscodor*, *Penicillium*, *Pestalotiopsis* and *Phomopsis* spp. are the endophytic fungi isolated from *Garcinia* species are shown to have such as, antimycobacterial, antimalarial, antiviral, antioxidant and cytotoxicity activities [11]. In the present study, *Garcinia gummigutta*, *G. indica*, *G. morella* and *G. xanthochymus* were used for the isolation of endophytic fungi and the extracts of the endophytes were screened against antioxidant, anti-inflammatory, anti-microbial and DNA protection activities.

MATERIALS AND METHODS

Chemicals and reagents

Linoleic acid, 1,1-Diphenyl-2-picrylhydrazyl (DPPH), 15-lipoxygenase (soybean), 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), phloroglucinol, catechin, quercetin, trypsin and MTT [3-(4,5-dimethylthylthiazol-2-yl)-2,5-diphynyl tetrazolium bromide], were purchased from Sigma-Aldrich (St. Louis, MO). COX-2 inhibition kit was obtained from Cayman Chemicals, Ann Arbor, USA. Ascorbic acid (AA), butylated hydroxyl toluene (BHT) chloramphenicol, 10% Foetal bovine serum, L-glutamine, sodium biocarbonate, non-essential amino acid and minimum essential medium Eagle were purchased from HiMedia, India. Plasmid pBR322 was purchased from Merck Biosciences, Bangalore, India. HeLa (human cervix) cell lines were obtained from National Centre for Cell Science, Pune, India. All the microbial strains of human pathogens used in the anti-microbial bioassay were procured from Institute of Microbial Technology (IMTECH), Chandigarh, India.

Isolation of endophytes

Fungal endophytes were isolated from the bark and twig samples of four endemic *Garcinia* trees of Western Ghats, India *viz.*, *G. gummigutta*, *G. indica*, *G. morella* and *G. xanthochymus* of the Clusiaceae family as described by Ruma *et al.* [12]. The bark and twig pieces were surface sterilized and dissected into ~0.5 x 1.0 cm. Two hundred bits of each species, were plated on water agar (15 g.l $^{-1}$) supplemented with chloramphenicol (100 mg.l $^{-1}$). The plates were incubated at 23 °C at 12h light / 12h dark cycles for 6 weeks. Each colony grown on the segments were transferred to potato dextrose agar (PDA) medium. The morphology of the isolated fungal endophytes was studied using Zeiss Advanced SteREO Discovery V20 Binocular Microscope. Identification was carried out based on the morphological and conidial characters using standard identification manuals [13].

Preparation of crude extract of endophyte culture broth

Endophytic fungal isolates were grown on potato dextrose agar at 27 ºC 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 *Acremonium* sp., *Aspergillus fumigatus*, *Botryodiplodea theobromae*, *Fusarium* sp., *F. verticillioides* and *Trichoderma* sp. were dissolved in methanol and used further for the assays.

Determination of Antioxidant Activity

DPPH scavenging assay

The free radical scavenging capacity of the endophytic extracts was determined by DPPH method described by Brand-Williams *et al.* [14] with minor modifications. The DPPH radical solution (300μM) was prepared in ethanol and 95μl of DPPH was added to each well of a microtitre plate. Different concentrations of test samples (5μl) 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). Ascorbic acid (AA), butylated hydroxytoluene (BHT) and quercetin (Q) were used as positive control. The results were expressed as 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 that is calculated by:

$$\%\text{DPPH radical scavenging} = \frac{(A_C - A) \times 100}{A_C}$$

ABTS Radical Cation Decolorization Assay

The antioxidant activity was analyzed by 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) method described by Re *et al.* [15]. ABTS was diluted in water to a 7 mM concentration. ABTS radical cations are generated by mixing 7mM 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 of endophytic extracts was mixed with 990 μl of ABTS solution and the absorbance was measured using a UV/visible spectrophotometer (Beckman Coulter, DU 730 Life Sciences) exactly after 5 min after initial mixing. Ascorbic acid (AA), butylated hydroxytoluene (BHT) and quercetin (Q) were used as positive control. A dose dependent curve was plotted to calculate the IC_{50} value.

Total Phenolic Content

The total polyphenols of the endophytic extracts were determined by Folin-Ciocalteu method as described by Singleton *et al.* [16]. Endophytic extracts (100 μl) were mixed with 500 μl of Folin-Ciocalteu reagent and incubated for 5 min at room temperature. Aqueous Na_2CO_3 (1.5ml) was added to solution and was mixed by vortexing. The solution was incubated at 37 ºC in dark for 2 hour. The absorbance was measured at 734 nm using UV/visible light spectrophotometer (Beckman Coulter, DU 730 Life Sciences). The results were analyzed in gallic acid equivalent using a (0-0.1 mg/ml) standard curve.

Anti-microbial activity

Gram negative bacteria, *Escherichia coli* (MTCC 724), *Klebsiella pneumoniae* (MTCC 661), *Salmonella typhi* (MTCC 733), *Shigella flexneri* (MTCC 1457); and Gram positive bacteria, *Staphylococcus aureus* (MTCC 96) and *Bacillus subtilis* (MTCC 441) were used for the assay. The cultures were maintained on nutrient agar slants and stored at 4 °C. These strains were sub-cultured on a fresh agar plate 24 h prior to antimicrobial testing.

Endophytic extracts were screened for antibacterial activity using disc diffusion method as described by Nostro *et al.* [17]. The target bacteria were cultured in nutrient broth and incubated for overnight. The overnight culture was centrifuged at 6000 g for 5 min and the pellet was resuspended in sterile water. The suspension of bacteria were plated on nutrient agar plates. The endophyte extracts (100 μg) were added to sterile filter discs and the discs were placed on the seeded agar plates. The plates were incubated at 37 °C for 16 hour. Chloramphenicol and methanol were used as positive and negative control respectively.

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

Soyabean 15-LOX inhibition was determined as described by Kemal *et al.* [18]. The substrate 0.2μM linoleic acid was prepared in 0.2M borate buffer (pH 9). Different concentrations of endophyte extracts were mixed with 15-LOX enzyme and incubated for 5 min at room temperature. The substrate was added to the mixture and the absorbance was measured at 243 nm using UV-Vis spectrophotometer (Beckman Coulter, DU 730 Life Sciences). Quercetin was used as positive control and methanol as 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, USA). The crude endophytic extracts were dissolved in methanol to a concentration of 25μg 50μg and 100μg were used for inhibition studies as per manufacturer's protocol. The absorbance at 415 nm was measured by using 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 [19]. Fenton reagent was prepared by mixing 30mM H_2O_2, 50 μM ascorbic acid and 80μM $FeCl_3$. Plasmid DNA was added to 10μl of endophyte extracts (5mg/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, USA).

Cytotoxicity assay

HeLa cell lines were maintained in Eagle's minimum essential medium (2mM L-glutamine and Earle's salts). The cytotoxicity was evaluated by MTT [3-4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] according to Mossmann [20]. HeLa cell cultures (5×10^5 cells / mL) were cultured in 96-well flat bottomed microtitre plate and incubated for 48 h at 37 ºC in a humidified 5% CO_2 incubator. Different concentrations of the endophytic extracts 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. MTT (5mg/ml) was prepared in phosphate buffered saline (PBS). MTT (10 μl) was added to each well 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). DMSO (100μl) and 0.1M glycine buffer (25μl, pH 10.5) were added to each well. The absorbances of the samples were measured at 570 nm after 15 min. Doxorubicin was used as positive control and methanol as negative control. The IC_{50} are the average of three assays with 6 concentrations.

HPLC analysis

Chromatographic RP-HPLC analysis of the endophyte extracts *viz.*, *Fusarium* sp. and *A. fumigatus* were performed using a HPLC system (Waters, Milford, USA) equipped with UV-Vis detector (Waters, 2489). The stationary phase was C18 Waters symmetry(R) column (4.6x250mm, 5 μm). An isocratic mobile phase consisting of acetonitrile: water: acetic acid :: 18:82:2 (v/v) was delivered at a flow rate of 1 ml/min and the elution profiles were read at 280 nm. Standard calibration curve was prepared using standard catechin

and phloroglucinol in a concentration range of 200 ppm (injection volume 10 μl) as described by Shen *et al.* [21].

MS Analysis

Mass spectrometry (MS-MS) was performed using a Waters Synapy G2 with UPLC Acquity System (Waters, Milford, MA, USA) to measure the mass of the metabolites of the crude extracts of *Aspergillus fumigatus* and *Fusarium* sp. Mass spectra data were acquired by electrospray ionization (ESI) in negative ion/ positive ion mode. The sample preparation was carried out in 1ml Acetonitrile + 1ml HPLC grade water + 2ul formic acid (0.1%). ESI was carried within a range of mass to charge (m/z) 100-1,000.

Statistical analysis

All determinations including antioxidant capacity by DPPH$^{\cdot}$, ABTS$^{\cdot+}$, measurements of total phenolic content, anti-microbial activity, anti-inflammatory ability by 15-lipoxygenase, human cyclooxygenase-2 inhibition assay and DNA nicking assay were conducted in triplicates. The reported value for each sample was calculated as the mean and SD of three independent experiments.

RESULTS

Fungal endophytes

Fungal endophytes were isolated from the bark and twigs of four different *Garcinia* spp. From the consortium of endophytes from *Garcinia* spp. *Acremonium* sp., *A. fumigatus*, *B. theobromae*, *F. verticillioides*, *Fusarium* sp. and *Trichoderma* sp. were selected for this study (**Fig. 1**).

Antioxidant Activity

Among the six extracts, 50 μg of *Fusarium* sp. and *A. fumigatus* extract exhibited higher DPPH activity of 72.42±2.75% and 60.62±1.10 % respectively. The IC_{50} value of *Fusarium* sp. and *A. fumigatus* were 20 μg/ml and 42.25 μg/ml for respectively. The IC_{50} for Quercetin, butylated hydroxytoluene and ascorbic acid were 6.1±1.24, 14.46 and 32.30 μg/ ml in DPPH assay respectively (**Table 1**). Six endophytes *Acremonium* sp., *A. fumigatus*, *B. theobromae*, *Fusarium* sp., *F. verticillioides* and *Trichoderma* sp exhibited potent DPPH scavenging activity.

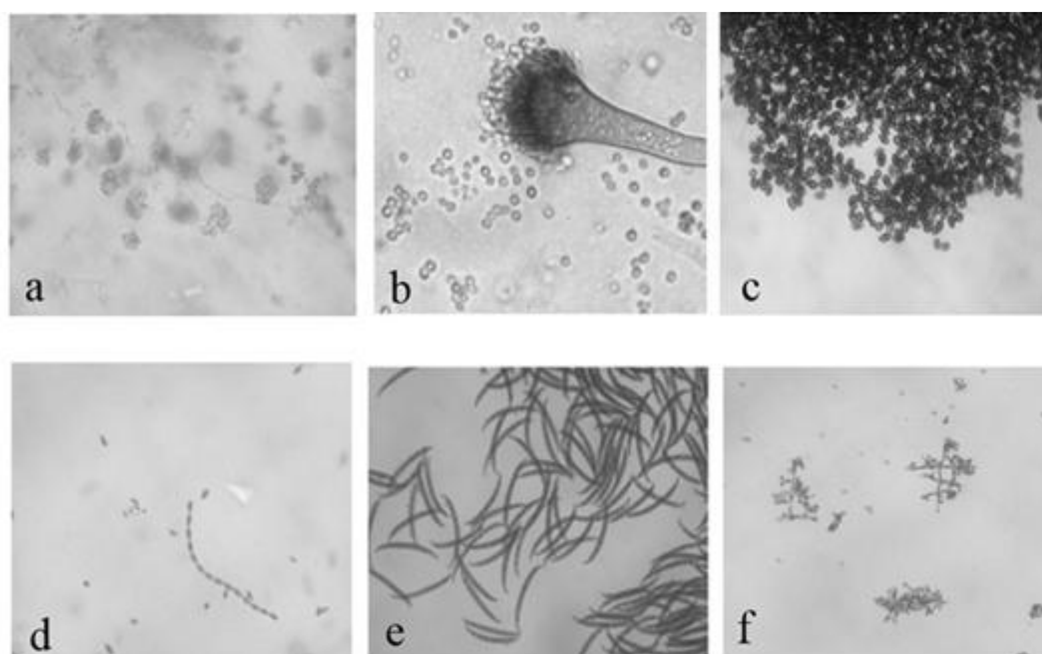

Fig. 1: Conidial characters of endophytic fungi isolated from the bark and twig of four different *Garcinia* species. (a) *Acremonium* sp.; (b) *Aspergillus fumigatus*; (c) *Botryodiplodea theobromae*; (d) *Fusarium verticillioides*; (e) *Fusarium* sp.; (f) *Trichoderma* sp.

In ABTS radical cation decolorization assay, 50 μg of *Fusarium* sp. extract showed a scavenging activity of 97.59±0.36 % whereas *A. fumigatus* showed 58.81±1.93 %. The IC_{50} value for the reference compounds, Quercetin, butylated hydroxytoluene and ascorbic acid were 7.03±0.66 μg/ml, 19.5±1.01 μg/ml and 75.2±0.13 μg/ml respectively (**Table 1**).

Table 1: Antioxidant activity and total phenolic content of fungal endophytes isolated from *Garcinia* spp.

Endophyte extract/ Reference compounds	Plant Name	DPPH (IC_{50}) (μg. ml^{-1})	ABTS (IC_{50}) (μg. ml^{-1})	Total Phenol content* (mg. ml^{-1})
Acremonium sp.	*G. gummigutta* (Twig)	120	102	4.00±0.01
Aspergillus fumigatus	*G. morella* (Twig)	42.25	45	100.24±0.03
Botryodiplodea theobromae	*G. xanthochymus* (Bark)	125	100	35.45±0.04
Fusarium sp.	*G. gummigutta* (Bark)	20	16	144.23±0.01
Fusarium verticillioides	*Garcinia morella* (Twig)	105	90	56.88±0.04
Trichoderma sp.	*G. indica* (Bark)	47	44	94.53±0.02
Ascorbic acid		32.30	75.2	
BHT		14.46	19.5	
Quercetin		6.1	7.03	

*Total phenolic content is expressed in mg gallic acid equivalent (GAE)/100g DW

Each result is expressed as mean ± S.D. (n = 3)

Total phenol

The total phenol content in different endophyte extracts varied considerably. The results are expressed as gallic acid equivalents (GAE). The total phenol content ranged from 4 mg to 144 mg GAE/1 mg extract. The highest content of total phenol was in *Fusarium* sp. with 144.23±0.01 mg GAE/1 mg extract whereas *A. fumigatus* contain 100.24±0.03 GAE/1 mg (**Table 1**). The correlation between the antioxidant activity and total phenolic content of the endophyte extracts had a correlation coefficient of R^2 = 0.905.

Anti-microbial activity

The extracts were most active against Gram negative bacteria. The inhibition zones observed ranged from 5-21 mm for different extracts (**Table 2**). The extracts of *Fusarium* sp. and *Trichoderma* sp. were most effective, exhibiting a zone of inhibition which ranged from 19 to 21mm for *K. pneumoniae*, *S. aureus*, *B. subtillis* and *S. flexneri*, where as the inhibition zone for *E. coli* ranged from 10 to 11 mm. The extract of *A. fumigatus* showed zone of inhibition ranging from 11 to 20 mm against *E. coli*, *K. pneumoniae*, *B. subtillis* and *S. flexneri*.

Table 2: Antimicrobial activity and the zone of inhibition of the ethyl acetate extracts from fungal endophytes

Bacterial Pathogens	**Zone of Inhibition (mm)**						
	***Acremonium* sp.**	***Aspergillus fumigatus***	***Botryodiplodea theobromae***	***Fusarium* sp.**	***Fusarium verticillioides***	***Trichoderma* sp.**	***Chloramphenicol***
Bacillus subtilis	5	16	11	21	15	19	27
Escherichia coli	0	18	10	11	10	11	11
Klebsiella pneumoniae	6	20	0	20	12	21	27
Salmonella typhi	5	0	0	11	0	16	15
Shigella flexneri	0	11	0	16	10	21	21
Staphylococcus aureus	9	16	7	16	13	12	21

Anti-inflammatory activity

15-lipoxygenase (LOX) inhibition

The anti-inflammatory activity was evaluated as % inhibition of LOX enzyme monitored as formation of hydroperoxylinoleic acid at 234 nm (**Table 3**). The endophyte extract showed dose dependent inhibition. *A. fumigatus* and *Fusarium* sp. at 100 µg concentration inhibited 80.74% and 75.58% LOX respectively. The IC_{50} for LOX inhibition activity for *A. fumigatus* and *Fusarium* sp. were IC_{50} of 62 µg/ml and 63 µg/ml respectively. The reference compound Quercetin exhibited IC_{50} of 1.45 µg/ml.

Human Cyclooxygenase-2 (COX-2) inhibition assay

The extracts were tested for its capacity to inhibit human cyclooxygenase-2 (COX-2). The enzyme COX-2 was inhibited in a dose dependent manner by the extracts. A 100 µg concentration of the extracts of *A. fumigatus* and *Fusarium* sp. showed 66.25% and 62.40% COX-2 inhibition respectively. The IC_{50} value of LOX inhibition activity for *A. fumigatus* and *Fusarium* sp. were 68 µg/ml and 74 µg/ml respectively (**Table 3**). The reference compound Quercetin exhibited IC_{50} of 3.8 µg/ml.

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 (**Fig. 2**). Incubation of the plasmid DNA with 50 µg of endophyte extracts for 30 min at 37°C and further exposure to Fenton's reagent under similar conditions as above, a concentration dependent shift was observed on the gel specifying no damage to the DNA.

Cytotoxicity assay

The extracts inhibited the proliferation of HeLa cells in a dose-dependent manner. The cytotoxicity of HeLa cell lines using MTT assay showed an IC_{50} of 92.2±0.23 and 88.54±1.23 µg/ml for the extracts of *Fusarium* sp. and *A. fumigatus* respectively (**Table 4**). The growth rate of the HeLa cell line in microtitre plate were determined and analyzed by counting the number of cells in each wells. The reference compound Doxorubicin exhibited IC_{50} of 16.2 µg/ml.

Table 3: Inhibition of 15-Lipoxygenase and Human Cyclooxygenase-2 by the extracts of endophytes isolated from *Garcinia* spp.

Endophyte extract	**Isolated from Plant**	**15-Lipoxygenase**				**Human Cyclooxygensae-2**	
		Final concentration of methanolic extract (µg.ml^{-1})	**Activity of lipoxygenase (U. mg $^{-1}$ LOX)**	**% inhibition**	**IC_{50} (µg)**	**% inhibition**	**IC_{50} (µg)**
Control			0.24 .10^{-1}				
Acremonium sp.	*G. gummigutta* (Twig)	25	1.62	18.97		11.52	
		50	1.47	35.81	120	28.50	101
		100	1.33	42.12		40.89	
Aspergillus fumigatus	*G. morella* (Twig)	25	1.867	22.17		18.85	
		50	1.415	41.04	62	40.45	68
		100	0.462	80.74		66.25	
Botryodiplodea theobromae	*G. xanthochymus* (Bark)	25	1.91	25.94		22.85	
		50	1.54	36.91	71	30.15	87
		100	0.787	68.12		55.65	
Fusarium sp.	*G. gummigutta* (Bark)	25	1.62	18.97		15.66	
		50	1.33	42.12	63	38.90	74
		100	0.56	75.58		62.40	
Fusarium verticillioides	*Garcinia morella* (Twig)	25	2.13	8.56		12.22	
		50	1.78	22.6	130	27.81	100
		100	1.39	37.18		41.21	
Trichoderma sp.	*G. indica* (Bark)	25	1.777	20.21		13.67	
		50	1.514	35.81	73	26.25	91
		100	0.765	67.17		55.02	

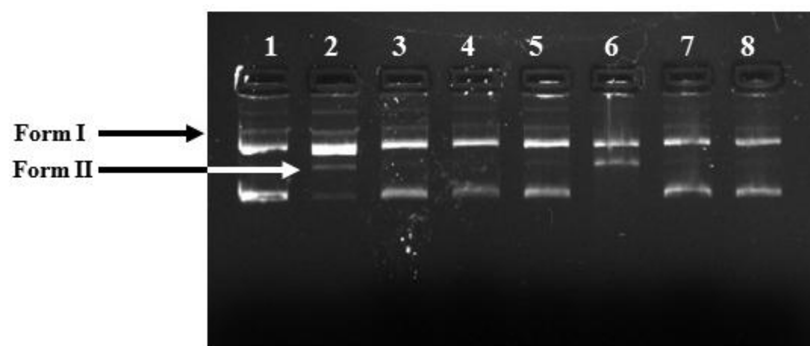

Fig. 2: Endophyte extracts assessed on plasmid pBR322 DNA treated by Fenton's reagent. Lane 1: pBR322 (native plasmid DNA); Lane 2: pBR322 DNA + Fenton's reagent; Lane 3: pBR322 + *Fusarium* sp. extract (50µg) + Fenton's reagent; Lane 4: pBR322 + *Aspergillus fumigatus* extract (50µg) + Fenton's reagent; Lane 5: pBR322 + *Trichoderma* extract (50µg)+ Fenton's reagent; Lane 6: pBR322 + *Acremonium* extract (50µg)+ Fenton's reagent; Lane 7: pBR322 + *Botryodiplodea theobromae* extract (50µg)+ Fenton's reagent; Lane 8: pBR322 + *Fusarium verticillioides* extract (50µg)+ Fenton's reagent.

Table 4: Cytotoxicity of endophyte extracts against HeLa cervix cancer cell lines

Extracts	Concentration (µg/ml)	Percent Cell Viability	IC_{50} (µg/ml)
Acremonium sp.	25	98.20	154.34
	50	85.49	
	100	72.66	
	200	34.27	
Aspergillus fumigatus	25	72.04	88.54
	50	63.11	
	100	48.90	
	200	25.26	
Botryodiplodea theobromae	25	75.14	118.31
	50	69.21	
	100	55.02	
	200	30.40	
Fusarium sp.	25	82.45	92.20
	50	70.12	
	100	46.32	
	200	22.92	
F. verticillioides	25	85.62	125.8
	50	78.00	
	100	59.26	
	200	28.35	
Trichoderma sp.	25	97.62	257.98
	50	93.09	
	100	81.42	
	200	63.77	

HPLC analysis

The activities have been supported by HPLC analysis. The HPLC chromatograms of the standard catechin and the extract *Fusarium* sp. showed specific peak at retention time 3.624 and 3.796 min respectively (**Fig. 3a & 3b**), where as the chromatograms of the standard phloroglucinol and extract of *A. fumigatus* showed specific peak at retention time 8.420 and 8.777 min respectively scanned on wavelength 280 nm (**Fig. 3c & 3d)**.

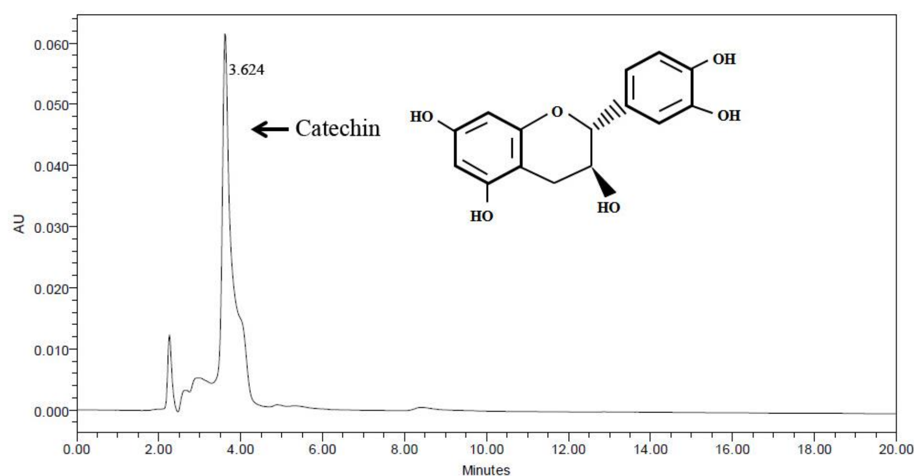

Fig. 3a: Analytical HPLC chromatogram of standard catechin separated on a semi-preparative RP-HPLC column.

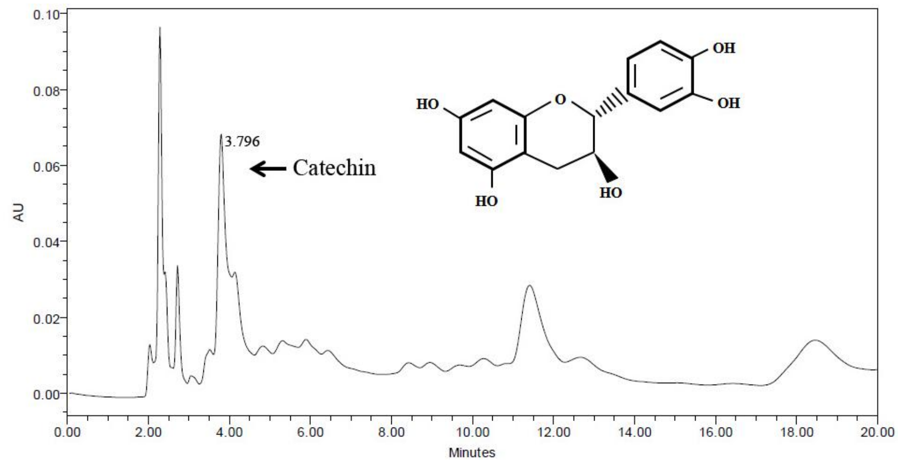

Fig. 3b: HPLC chromatogram of *Fusarium* sp. extract

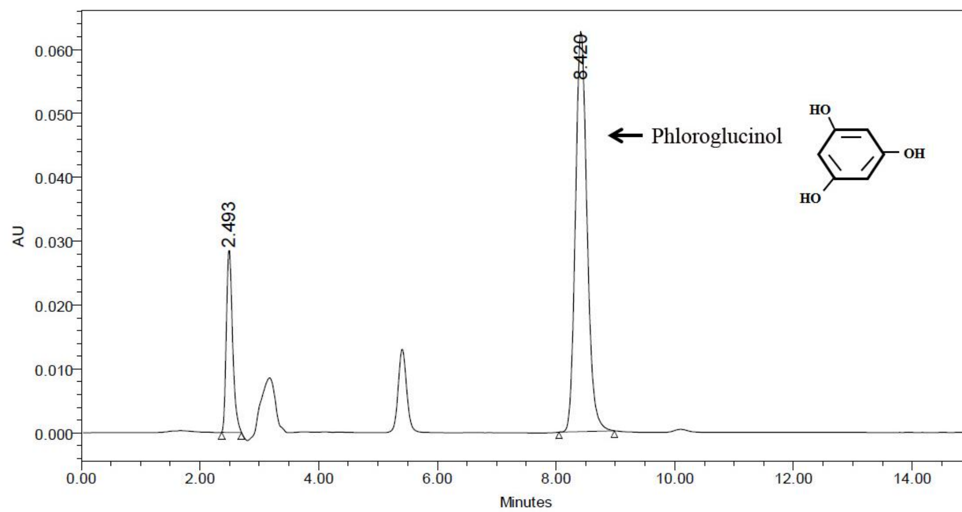

Fig. 3c: HPLC chromatogram of standard phloroglucinol

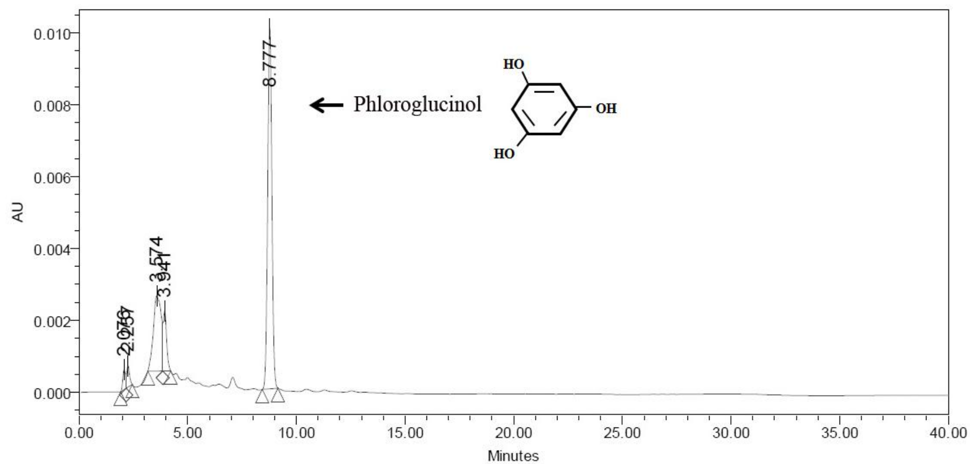

Fig. 3d: HPLC chromatogram of *Aspergillus fumigatus* extract

MS Analysis

The mass spectrometry technique facilitated to analyze the presence of different metabolites of diverse mass in the crude extracts of *Aspergillus fumigatus* and *Fusarium* sp. The MS spectrum of ethyl acetate extracts of *Aspergillus fumigatus* and *Fusarium* sp. exhibited intense peak 126.0018 m/z and 149.0143 m/z respectively (**Fig. 4a & 4b**).

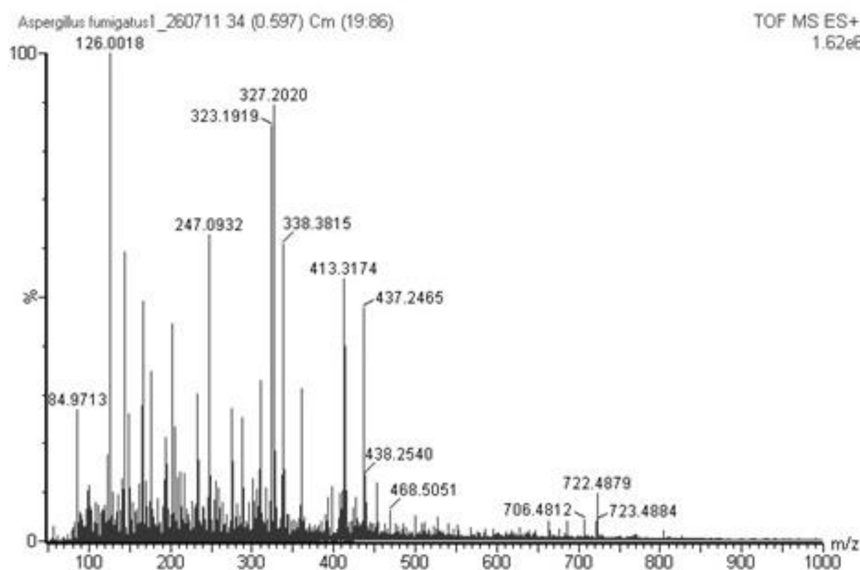

Fig. 4a: Mass spectra of *Aspergillus fumigatus* extract

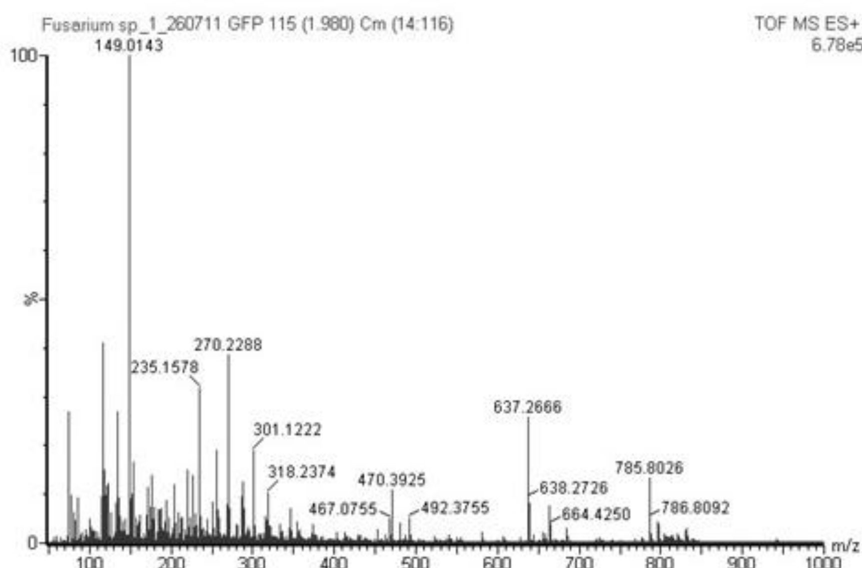

Fig. 4b: Mass spectra of *Fusarium* sp. extract

DISCUSSION

Endophytic fungi from plants are well known sources of bioactive secondary metabolites [7, 22]. Endophytes are reported to be a reservoir of novel metabolites with distinctive bioactivities [23]. Hence, screening of crude endophyte extracts is crucial for determining their potential for further analysis and characterization of bioactive molecules. *Aspergillus, Botryosphaeria*, *Phomopsis*, *Pestalotiopsis* spp. are some of the common endophytes occurring in a wide variety of distantly related host species [24]. Ruma *et al.* [12] reported such a recurrence with reference to the genera *Aspergillus*, *Trichoderma*, *Fusarium*, *Pestalotiopsis* spp. in *Garcinia* spp. Several bioactive secondary metabolites have been isolated and characterized from *Garcinia* species, few reports available on associated fungal endophytes and their bioprospecting [9].

Free radicals cause damage to the cell membrane, cell organelles and DNA through pairing with an unpaired electron which contributes to a variety of pathological effects [25]. The antioxidant activity of *Acremonium* sp., *A. fumigatus*, *B. theobromae*, *Fusarium* sp., *F. verticillioides* and *Trichoderma* sp. extracts was tested for the antioxidant potential by DPPH and ABTS radical scavenging ability. DPPH evaluates the inhibition of the stable radical $DPPH^{\cdot}$ whereas ABTS measures the loss of $ABTS^{\cdot +}$ generated through reaction between ABTS and potassium persulphate. Both ABTS and DPPH show bi-phasic kinetic reactions with many antioxidants, although they have difference in solubility. In the present study, *Fusarium* sp. and *A. fumigatus* were identified as potent sources of antioxidant with an IC_{50} value of 20 µg/ml and 42.25 µg/ml respectively against the DPPH radical and was taken for analysis of bioactive metabolites. Similar studies in the recent past have reported endophytes with potent antioxidant activity. *Phyllosticta* sp. isolated from *Guazuma tomentosa* showed potent antioxidant property with an EC_{50} (half maximum effective concentration) value of 580.02±0.57 µg/ml and 2030.25 ± 0.81 µg/ml against ABTS and DPPH radicals respectively [26]. The antioxidant property is generally associated with the presence of phenols in the extract. In addition, phenolic compounds

have been reported with potent anti-cancer, anti-bacterial, anti-viral or anti-inflammatory activities [27].

Endophytes produce secondary metabolites which are thought to bring out a confrontation mechanism to overcome the infection by pathogens [22]. The bioactive compound, 7-amino-4-methylcoumarin isolated from the extracts of the endophytic fungus *Xylaria* sp. YX-28 isolated from *Ginkgo biloba* L. presented broad-spectrum inhibitory activity against several pathogenic microorganisms [28]. Other bioactives reported to bear antimicrobial properties isolated from endophytes were 3-O-methylalaternin and altersolanol A, phomoenamide, phomodione, ambuic acid, isopestacin, and munumbicin A, B, C, D [29]. Bai and Krishnakumar [30] reported the antibacterial effect of different solvent extracts of marine microalgae *Tetraselmis suecica* against selected human pathogens such as *Vibrio cholerae*, *Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella* sp, *Proteus* sp., *Streptococcus pyogens*, *Staphylococcus aureus*, *Bacillus megaterium* and *Bacillus subtilis*. The results of the present investigation on antimicrobial activity revealed a broad spectrum antibacterial property. The extracts of *Fusarium* sp. and *Trichoderma* sp. exhibited potent antibacterial activity both against Gram positive and Gram negative bacteria.

The biosynthesis of leukotrienes (LTs) is catalyzed by a family of non-heme iron-containing dioxygenases *viz*., Lipoxygenases (LOXs) and Cyclooxygenase-2 (COX-2) which are reported to be initiators of inflammation. The inhibition of production of LTs by inhibiting LOX / COX-2 could alleviate the state of inflammation [29]. In the present study, the endophyte extracts of *A. fumigatus*, *Fusarium* sp. and *B. theobromae* showed potent 15-LOX and as well as human COX-2 inhibition. LOXs are perceptive to antioxidants as they restrain the formation of lipid hydroperoxide by scavenging lipidoxy- or lipidperoxy-radicals and decreasing the concentration of lipid hydroperoxide involved in the catalysis of LOX [31]. Earlier evidence exists on the presence of dual inhibition of LOX and COX enzymes for the efficient management of inflammatory disorders [32].

Earlier reports verifies that reactive oxygen species plays a critical role in skin damage, aging and neurodegenerative diseases [33]. The present study reports that the extracts as low as 50 µg/ml had the capacity to scavenge the ·OH radicals produced by Fenton's reagent protecting the pBR322 plasmid DNA. An absence or reduction in Form II recorded indicated a notable protection offered by the endophyte extracts. Fe^{2+}, the vital catalyst which generates free radical-driven ROS by Fenton's reaction has the potential to directly interact with DNA bases and results in DNA damage. The majority of the anti-cancer agents act by quenching the free radicals or by direct interaction with the DNA. The present study clearly suggests the protection of DNA by the endophyte extracts. All the five endophyte extracts prevented DNA damage as evident in the agarose gel except for the extract from *Acremonium* sp.

Screening of plant as well as endophyte extracts for cytotoxicity has been a long eminent concern in the discovery of cancer curatives. The crude organic extract of the endophyte, *Chaetomium* sp. isolated from *Salvia officinalis* was proven to be cytotoxic against L5178Y mouse lymphoma cells [34]. In the present study, amongst the six endophyte extracts tested for the antiproliferative activity on HeLa human cervix cancer cell lines, *A. fumigatus* and *Fusarium* sp. showed most potent activity. The ethyl acetate extract of the endophytic fungi, *Hypocrea lixii* VB1 isolated from mangrove showed strong anticancer activity for Hep2 and MCF7 cell line *in vitro* [35].

HPLC profiling of the extract of *Fusarium* sp. and *A. fumigatus*, was compared with the standards catechin and phloroglucinol. It is expected that the activity observed by the extracts could be due to the presence of these molecules. Earlier, Shen *et al*. [21] identified two lanostane type compounds i.e., dehydrosulfurenic acid and 15α-acetyl- dehydrosulfurenic acid using HPLC. In addition, Catechin, a phenol was reported with biological activities *viz*., lipoxygenase inhibition [36], antibacterial activity [37] and anticancer properties [38]. Whereas, Phloroglucinol, a benzenetriol have also displayed a vast array of biological activities which includes antifungal property, cytotoxic effects against various cancer cell lines [39]. Kang *et al*. [40] reported that phloroglucinol exhibited ROS scavenging activity, promoted cell viability, inhibited H_2O_2 induced apoptosis, activated extracellular signal regulated kinase (ERK) protein, and also enhanced the catalase activity.

Mass spectrum analysis of the extract *A. fumigatus* led to the conclusion that there may be the presence of the molecule phloroglucinol with a mass of 126.0018. Wicklow *et al*. [41] detected pyrrocidine B by LC-MS-MS in whole symptomatic maize kernels removed at harvest from ears of a commercial hybrid that were wound-inoculated in the milk stage with the endophyte, *Acremonium zeae.*

CONCLUSION

Endophytes have verified to be rich sources of novel natural compounds with a wide-spectrum of biological activities [29]. This study clearly demonstrated diversity and bioactive properties of endophytic fungi inhabiting *Garcinia* spp. The fungal extracts revealed their potential as a source of antioxidant, antiinflammatory and antimicrobial inhibitors which could have a role in the development of drugs for treatment of a wide spectrum of diseases. The endophytes could be further exploited to identify a novel lead molecule and use to develop the analogues.

ACKNOWLEDGEMENT

The authors acknowledge the support under Institution of Excellence Program of University of Mysore awarded by Ministry of Human Resource Development and UGC, Government of India.

CONFLICT OF INTEREST

The authors declare there is no conflict of interest.

REFERENCES

1. Strobel GA, Daisy B Bioprospecting for microbial endophytes and their natural products. Microbiol Mol Biol Rev. 2003; 67: 491–502.
2. Hemraj, Upmanyu N, Gupta A, Jindal A, Jalhan S Pharmacological activities of Stephania glabra, Woodfordia fruticosa and Cissempelos pareira – A review. Int J Pharm Pharm Sci. 2012; 4(3): 16-23.
3. Aggarwal BB, Ichikawa H, Garodia P, Weerasinghe P, Sethi G, Bhatt ID, Pandey, MK, Shishodia S, Nair MG From traditional Ayurvedic medicine to modern medicine: identification of therapeutic targets for suppression of inflammation and cancer. Expert Opin Ther Targets 2006; 10: 87-118.
4. Aly AH, Debbab A, Kjer J, Proksch P Fungal endophytes from higher plants: a prolific source of phytochemicals and other bioactive natural products. Fungal Divers. 2010; 41: 1–16.
5. Arora DS, Chandra P Assay of antioxidant potential of two *Aspergillus* isolates by different methods under various physio-chemical conditions. Braz J Microbiol. 2010; 41: 765-777.
6. Wiyakrutta S, Sriubolmas N, Panphut W, Thongon N, Danwiset-kanjana K, Ruangrungsi N, Meevootisom V Endophytic fungi with anti-microbial, anti-cancer and anti-malarial activities isolated from Thai medicinal plants. World J Microbiol Biotechnol. 2004; 20: 265-272.
7. Schulz B, Boyle C, Draeger S, Römmert AK, Krohn K Endophytic fungi: a source of novel biologically active secondary metabolites. Mycol Res. 2002; 106: 996-1004.
8. Stierle A, Strobel G, Stierle D, Grothaus P, Bignami G The search for a taxol-producing microorganism among the endophytic fungi of the pacific yew, *Taxus brevifolia*. J Nat Prod. 1995; 58: 1315–1324.
9. Phongpaichit S, Rungjidamai N, Rukachaisirikul V, Skayaroj J Antimicrobial activity in cultures of endophytic fungi isolated from *Garcinia* species. FEMS Immunol Med Microbiol. 2006; 48: 367-372.
10. Khare CP Indian medicinal plant: An illustrated dictionary (eds. Khare CP) Springer 2007.
11. Phongpaichit S, Nikom J, Rungjindamai N, Sakayaroj J, Hutadilok-Towatana N, Rukachaisirikul V, Kirtikara, K Biological activities of extracts from endophytic fungi isolated from *Garcinia* plants. FEMS Immunol Med Microbiol. 2007; 51: 517–525.

12. Ruma K, Shailasree S, Sampath KKK, Niranjana SR, Prakash HS Endophytic Fungal Assemblages in *Calophyllum* and *Garcinia* spp. of Clusiaceae Family in Western Ghats, India. Curr Biotechnol. 2012; 1: 109-114.
13. Barnett HL, Hunter BB. Illustrated Genera of Imperfect Fungi. Burgess Publishing Company, Minnaapolia, Minnaaota 1972.
14. Brand-Williams W, Cuvelier ME, Berset C Use of a free radical method to evaluate antioxidant activity. Lebensmittel Wiss Technol. 1995; 28: 25-30.
15. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Biol Med, 1999; 26: 1231–1237.
16. Singleton VL, Ortofehr R, Lamuela-Raventos RM Analysis of total phenols and other oxidation substrate and antioxidants by means of Folin-Ciolcalteau reagent. Methods Enzymol. 1999; 299: 152-178.
17. Nostro A, Germanò MP, D'angelo V, Marino A, Cannatelli MA Extraction methods and bioautography for evaluation of medicinal plant antimicrobial activity. Lett Appl Microbiol. 2000; 30: 379-384.
18. Kemal C, Louis-Flamberg P, Krupinsky-Olsen R, Shorter AL Reductive inactivation of soybean lipoxygenase I by catechols: a possible mechanism for regulation of lipoxygenase activity. Biochem. 1987; 26: 7064-7072.
19. Lee JC, Kim HR, Kim J, Jang YS Antioxidant activity of ethanol extract of the stem of *Opuntia ficus*-indica var. saboten. J Agric Food Chem. 2002; 50: 1619-1624.
20. Mossmann T Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983; 65: 55–63.
21. Shen YC, Chou CJ, Wang Y-H, Chen C-Fu, Chou Y-C, Lu M-K Anti-inflammatory activity of the extracts from mycelia of *Antrodia camphorata* cultured with water-soluble fractions from five different *Cinnamomum* species. FEMS Microbiol Lett. 2004; 231: 137-143.
22. Strobel GA Endophytes as sources of bioactive products. Microbe Infect. 2003; 5: 535–544.
23. Tan RX, Zou WX Endophytes: a rich source of functional metabolites. Nat Prod Rep. 2001; 18: 448-459.
24. Tejesvi MV, Mahesh B, Nalini, MS, Prakash, HS, Kini KR, Shetty HS Fungal endophyte assemblages from ethnopharmaceutically important medicinal trees. Can J Microbiol. 2006; 52: 427–435.
25. Seifried HE, Anderson DE, Fisher EI, Milner JA A review of the interaction among dietary antioxidants and reactive oxygen species. J Nutr Biochem. 2007; 18: 567-579.
26. Srinivasan K, Jagadish LK, Shenbhagaraman R, Muthumary J Antioxidant activity of endophytic fungus *Phyllosticta* sp. isolated from *Guazuma tomentosa*. J Phytol. 2010; 2: 37-41.
27. Tapiero H, Tew KD, Ba N, Mathe G Polyphenols: do they play a role in the prevention of human pathologies? Biomed Pharmacother. 2002; 56: 200-207.
28. Liu X, Dong M, Chen X, Jiang M, Lv X, Zhou J Antimicrobial activity of an endophytic *Xylaria* sp.YX-28 and identification of its antimicrobial compound 7-amino-4-methylcoumarin. Appl Microbiol Biotechnol. 2008; 78: 241–247.
29. Pimentel MR, Molina G, Dion´ısio AP, Junior MRM, Pastore GM The Use of Endophytes to Obtain Bioactive Compounds and Their Application in Biotransformation Process. Biotechnol Res Int. 2010; doi:10.4061/2011/576286.
30. Bai VDM, Krishnakumar S Evaluation of antimicrobial metabolites from marine microalgae *tetraselmis suecica* using gas chromatography – mass spectrometry (GC – MS) analysis. Int J Pharm Pharm Sci. 2013; 5(3): 17-23.
31. Ammon HPT, Annazodo MI, Safayhi H, Dhawan BN, Scrimal R Curcumin: a potent inhibitor of leukotriene B4 formation in rat peritoneal polymorphonuclear neutrophils (PMNL). Planta Medica 1992; 58: 226.
32. Rackova L, Oblozinsky M, Kostalova D, Kettmann V, Bezakova L Free radical scavenging activity and lipoxygenase inhibition of *Mahonia aquifolium* extract and isoquinoline alkaloids. J Inflamm. 2007; 4: 15-21.
33. Martel-Pelletier J, Lajeunesse D, Reboul P, Pelletier JP Therapeutic role of dual inhibitors of 5-LOX and COX, selective and non-selective non-steroidal anti-inflammatory drugs. Ann Rheum Dis. 2003; 62: 501-509.
34. Lopaczynski W, Zeisel SH Antioxidant, programmed cell death and cancer. Nutr Res. 2001; 21: 295-307.
35. Debbab A, Aly AH, Edrada-Ebel RA, Muller WEG, Mosaddak M, Hakiki A, Ebel R, Proksch P Bioactive secondary metabolites from the endophytic fungus *Chaetomium* sp. isolated from *Salvia officinalis* growing in Morocco. Biotechnol Agron Soc Environ. 2009; 13: 229-234.
36. Bhimba BV, Franco DAAD, Mathew JM, Jose GM, Joel EL, Thangaraj M Anticancer and antimicrobial activity of mangrove derived fungi Hypocrea lixii VB1. Chin J Nat Med. 2012; 10: 77-80.
37. Maqsood S, Benjakul S Comparative studies of four different phenolic compounds on *in vitro* antioxidative activity and the preventive effect on lipid oxidation of fish oil emulsion and fish mince. Food Chem. 2010; 119: 123–132.
38. Shimamura T, Zhao W-H, Hu Z-Q Mechanism of Action and Potential for Use of Tea Catechin as an Anti-infective Agent. Med Chem. 2007; 6: 57-62.
39. Demeule M, Michaud-Levesque J, Annabi B, Gingras D, Boivin D, Jodoin J, Lamy S, Bertrand Y, Béliveau R Green Tea Catechins as Novel Antitumor and Antiangiogenic Compounds. Curr Med Chem. 2002; 2: 441-463.
40. Mahmoud AA, Al-Shihry SS, Hegazy ME Biological activity of a phloroglucinol glucoside derivative from *Conyza aegyptiaca*. Z Naturforsch C. 2009; 64: 513-517.
41. Kang AhK, Lee KH, Chae S, Zhang R, Jung MS, Ham YM, Baik JS, Lee NH, Hyun JW Cytoprotective Effect of Phloroglucinol on Oxidative Stress Induced Cell Damage via Catalase Activation. J Cellular Biochem. 2006; 97: 609–620.
42. Wicklow DT, Roth S, Deyrup ST, James B, Gloer JB A protective endophyte of maize: *Acremonium zeae* antibiotics inhibitory to *Aspergillus flavus* and *Fusarium verticillioides*. Mycol Res. 2005; 109: 610–618.