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RESEARCH ARTICLE

Antioxidative properties of phenolic compounds isolated from the fungal endophytes of *Zingiber nimmonii* (J. Graham) Dalzell.

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BACKGROUND: The microbes living *in planta* termed 'endophytes' is bestowed with the potential to produce bioactive substances. The aim of this investigation was focused on the isolation and molecular identification of the fungal endophytes from *Zingiber nimmonii* (J. Graham) Dalzell., an endemic medicinal plant species of the 'Western ghats', a hotspot location in southern India and characterization of the secondary metabolites responsible for the antioxidant and DNA protective capacity using chromatography and mass spectrometry techniques.
METHODS: Endophytic fungi were isolated and identified by sequencing the Internal Transcribed Spacer (ITS). The secondary metabolites were extracted with ethyl acetate and evaluated for the total phenolic, flavonoid and antioxidant capacities. The isolates with potential antioxidative property were further analyzed for the DNA protection ability and the presence of bioactive phenolic compounds by High Performance Liquid Chromatography (HPLC) and Electrospray Ionization-Mass Spectroscopy/Mass Spectroscopy (ESI-MS/MS) techniques.
RESULTS: Endophytic fungi belonging to 11 different taxa were identified. The total phenolic content of the extracts ranged from 10.8 ± 0.7 to 81.6 ± 6.0 mg gallic acid equivalent/g dry extract. Flavonoid was present in eight extracts in the range of 5.2 ± 0.5 to 24.3 ± 0.9 mg catechin equivalents/g dry extract. *Bipolaris specifera*, *Alternaria tenuissima*, *Aspergillus terreus*, *Nectria haematococca* and *Fusarium chlamydosporum* extracts exhibited a potentially high antioxidant capacity. Characterization of the extracts revealed an array of phenolic acids and flavonoids. *N. haematococca* and *F. chlamydosporum* extracts contained quercetin and showed DNA protection ability.
CONCLUSION: This study is the first comprehensive report on the fungal endophytes from *Z. nimmonii*, as potential sources of antioxidative and DNA protective compounds. The study indicates that *Z. nimmonii* endophytes are potential sources of antioxidants over the plant itself.

Keywords endophytic fungi, *Zingiber*, Western Ghats, phenolic acids, flavonoid, DNA protection

Introduction

Reactive oxygen species (ROS) are continuously generated from the cells because of endogenous (aerobic metabolism) and exogenous (UV irradiation, environmental pollutants, diets etc.) elicits. ROS includes superoxide, hydroxyl ion, hydrogen peroxide, alkoxyl, nitric oxide, sulfhydryl etc. It is well known that excess of ROS is toxic and causes damage to the cell components, including nucleic acids, proteins and lipids. In addition, it causes apoptosis or necrosis (Thannickal and Fanburg, 2000). Oxidative damage to cell components plays a vital role in human diseases *viz.*, diabetes, cardiovascular, cancer, neurodegenerative disorders, Alzheimer's disease, kidney disease etc (Maldonado et al., 2005). Antioxidants are important compounds with potential therapeutic benefits against these oxidative damages. Antioxidants can inhibit free radicals by binding to the oxidation promoter or by scavenging the free radical chain reaction at early stage, the mechanism is very diverse. Antioxidant compounds can be thiols, ascorbic acids or phenolics.

Bioactive antioxidants are being investigated from various plants since long. Microbes are also gaining importance for the production of various bioactive metabolites as plants. Fungal endophytes are microorganisms which live within the plant tissues without causing any apparent infections (Wilson, 1995). These are not only rich sources of bioactive metabolites as plant itself; exploitation of such organisms

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also does not harm the biodiversity. The endophytic fungi produce a plethora of substances with potential application in modern medicine, agriculture and industry such as antioxidants, novel antibiotics, antimycotics, immunosuppressants and anticancer compounds (Strobel and Daisy, 2003). Endophytes are known to produce a broad variety of bioactive secondary metabolites with unique structures, including alkaloids, benzopyranones, chinones, flavonoids, phenolic acids, quinones, steroids, terpenoids, tetralones, xanthones etc. (Tan and Zou, 2001). Phenolic acids and flavonoids are isolated as major bioactive components from the endophytic fungi (Huang et al., 2007a, 2007b). Phenolic acids have been documented recently from the endophytic fungus *Annulohypoxylon stygium* BCRC34024 (Cheng et al., 2014).

Zingiber nimmonii (J. Graham) Dalzell., is an endemic species of the Western Ghats, a biodiversity hotspot of Southern India. The bioactive potential of the plant is well established (Sabulal et al., 2006; Finose and Gopalakrishnan, 2014). *Z. nimmonii* is a wild congener of *Z. officinale* (Kavitha et al., 2010). Zingiberaceous species have earlier been subjected to endophytic fungal isolations with the emphasis on the diversity and bioactive potentials of the endophytes (Bussaban et al., 2001; Nongalleima et al., 2013). Despite these findings, attempts are limited on documentation and characterization of the bioactive compounds from the endophytic fungi responsible for the bioactivity.

Therefore, the primary aim of the present study was to isolate and characterize the fungal endophytes from the plant parts of *Z. nimmonii* and the characterization of secondary metabolites of fungi responsible for the antioxidant activity and DNA protectivity by HPLC and ESI-MS/MS techniques. This study is important in the search for new sources of antioxidants from the endophytic fungi.

Materials and methods

Chemicals

All phenolic acids and flavonoid standards, trolox, ascorbic acid, ABTS [2, 2'-azino-bis(3- ethylbenzthiazoline-6-sulphonic acid)], DPPH (1, 1-diphenyl-2- picrylhydrazyl), Calf thymus DNA were purchased from Sigma Aldrich (St. Louis, MO, USA). Folin-Ciocalteu's reagent was purchased from SRL Pvt. Ltd. (Mumbai, India). Solvents used for HPLC analyses were of HPLC grade. Sodium hypochlorite, potassium ferricyanide, trichloroacetic acid (TCA), ferric chloride, sodium nitrite, aluminum chloride, potassium persulphate, all other general chemicals and solvents were of analytical grade.Triple distilled water was used wherever necessary.

Collection of the plant material

Z. nimmonii was collected from the Talacauvery sub cluster (012°17′ to 012°27′ N and 075°26′ to 075°33′E) of Western Ghats, in Kodagu district, Karnataka state, India, during November, 2011 (Fig. 1) and identified taxonomically (Gamble, 1928). The plant parts such as roots, rhizomes, leafy stem, leaves, and inflorescences were collected in ziplock polyethylene bags and brought to the laboratory and processed for the isolation within 24 h of collection. A herbarium specimen has been preserved in the Department of Studies in Botany, University of Mysore.

Figure 1 *Z. nimmonii* (J. Graham) Dalzell., from the Western Ghats. (A) Aerial portion; (B) Roots and spikes arising directly from root stock; (C) Flower with yellow lip.

Isolation of endophytic fungi from the plant parts

Endophytic fungal isolations was carried out under aseptic conditions, according to Tejesvi et al. (2005). All plant parts were surface sterilized with ethanol (70%, 1 min), sodium hypochlorite (3.5%, 3 min) and then washed 3-4 times in sterile distilled water. The dried plant parts were cut into 1.0 cm × 0.1 cm × 0.1 cm pieces and 900 plant fragments were placed on water agar media (2%, w/v) for the isolation of endophytic fungi (Strobel et al., 1996), supplemented with the antibiotic streptomycin (50 mg/L) to inhibit bacterial growth. The effectiveness of the surface sterilization was confirmed (Schulz et al., 1998). The plates were sealed with Cling-wrap™ and incubated at 28±2°C with 12 h of light and dark cycles for 4–6 weeks. The fungal hyphae emerged on the fragments were further inoculated onto potato dextrose agar (PDA) slants and incubated at 28±2°C for 10-15 days and maintained as pure cultures at 4°C for further use.

Identification of the fungal endophytes

Microscopic slides of each endophyte were prepared by using lactophenol cotton blue stain. The slides were examined under the light microscope (Labovision, India) for morphological analysis. Based on the cultural characteristics and spore structure, 11 endophytic fungi were selected for the molecular identification and further analysis of their metabolites.

Molecular identification of fungal endophytes

Actively grown mycelial plugs from 11 morphologically different endophytic fungi were inoculated into potato dextrose broth (PDB, Hi Media, Mumbai, India). The isolates were grown in still culture at 28±2°C for 7-10 days. The genomic DNA was extracted from the freeze-dried fungal mats by cetyltrimethyl ammonium bromide (CTAB) method with slight modifications (Ausubel et al., 1994). The DNA concentration was estimated by measuring the absorbance at 260 and 280 nm (Thermo Scientific Nanodrop 2000/2000c, Bangalore, India). Target regions of the rDNA ITS 1 and 2 regions and 5.8 rRNA gene were amplified using primers ITS 1 and ITS 4. The amplification was performed in a total reaction volume of 25 µL containing 200 µmol/L dNTP, 10 pmol/µL of each primer and 50 ng template DNA. The amplification conditions consisted of an initial denaturation step at 95°C for 3 min, followed by 35 cycles of 92°C for 1 min, 50°C for 1 min, 72°C for 2 min and final extension at 72°C for 10 min. The amplified product was subjected to sequencing at Chromous Biotech Pvt. Ltd. Bangalore, India. The endophyte sequences were aligned with the reference sequences using the BLAST algorithm and submitted to the NCBI GenBank nucleotide collection.

Fermentation and extraction of metabolites

The pure cultures of 10 day old isolates were inoculated into 500 mL of PDB contained in Erlenmeyer flasks in duplicates and kept for incubation for 3 weeks at 28±2°C. The fermentation broth of each endophyte was extracted with ethyl acetate thrice at room temperature and further concentrated in a Rotary flash evaporator (Superfit Model, PBU-6D, India). The residue obtained was designated as the crude dry extract and stored in glass vials, until use.

Determination of the total phenolic content

The total phenolic content (TPC) was assessed according to the Folin–Ciocalteau (FC) method of Liu et al. (2007) with some modifications. One mL of FC reagent and 2 mL of sodium carbonate (20%, w/v) was mixed with the crude extracts. The mixture was incubated for 45 min in the dark. The absorbance was read at 765 nm (T- 60, TTL Technology, India). The total phenolic content of the extracts was expressed as mg of gallic acid equivalent (GAE)/g of the extract.

Determination of total flavonoid content

The total flavonoid content (TFC) was determined according to the method of Barros et al. (2007). The fungal extract was mixed with sodium nitrite (5%, 75 µL). After 5 min, aluminum chloride (10%, 150 µL) and sodium hydroxide (1 M, 500 µL) were added. The absorbance was measured at 510 nm. The content of flavonoid was calculated using calibration curve of catechin and the results were expressed as mg of catechin equivalent (CE)/g of the extract.

Determination of antioxidant properties

$ABTS^+$ radical scavenging assay

The $ABTS^+$ scavenging capacity was determined by the method of Re et al. (1999). $ABTS^+$ was generated by the mixing of ABTS (7 mM) and potassium per-sulfate (2.45 mM). The working solution was prepared by diluting with methanol to obtain an absorbance of 0.70 at 734 nm. The activity was expressed as Trolox equivalent antioxidant capacity (mg TE/g dry extract).

DPPH radical scavenging assay

The quenching ability of DPPH was measured according to the procedure of Liu et al. (2007) with modifications. A methanolic solution of DPPH (0.001 mM) was added to the fungal extract. The absorbance was read at 517 nm after 20 min of incubation. The scavenging activity was expressed as IC_{50} (µg/mL). Ascorbic acid was used as the standard. The scavenging ability of the DPPH radical was calculated by the formula:

$$\% \text{ scavenging} = [(A_{control} - A_{sample})/A_{control}] \times 100.$$

Reducing power assay

The reducing power was measured by the method of Oyaizu (1986) with modifications. The fungal extracts were mixed with phosphate buffer (0.2 M, pH 6.5) and potassium ferricyanide (1%, 0.5 mL). The mixture was then incubated at 50°C for 20 min. After incubation, TCA (10% w/v, 0.5 mL) was added and centrifuged at 3000 rpm for 10 min. To the supernatant same volume of distilled water and ferric chloride (0.1%, 300 µL) was added and the absorbance was measured at 700 nm. The activity was expressed as mg ascorbic acid (AA)/g dry extract.

Inhibition of lipid peroxidation capacity

Inhibition of lipid peroxidation in the egg yolk of hen was determined according to the procedure of Ohkawa et al. (1979) with modifications. The egg yolk of hen (0.5 g) was emulsified with phosphate buffer (0.1 M, pH 7.4) to achieve the final volume of 25 g/L. Then, 0.5 mL of the egg homogenate was mixed with 0.1 mL of each sample. The volume was made up to 1 mL with distilled water. In this mixture 0.05 mL ferrous sulfate (0.07 M) was added and incubated for 30 min at room temperature to induce lipid peroxidation. After incubation, 1.5 mL of acetic acid (20%), 2 mL of TBA (1% w/v) in 1.1% sodium dodecyl sulfate (SDS) and 0.05 mL TCA (20%) was added and vortexed. This mixture was again incubated for 60 min in a boiling water bath. After cooling, 5.0 mL of butanol was added and centrifuged at 3000 rpm for 10 min. The organic upper layer was taken in a separate tube and the absorbance was measured at 532 nm.The inhibition of lipid peroxidation capacity was expressed as IC_{50} (µg/mL). Catechin was used as the standard. The ability to inhibit lipid peroxidation was calculated by the following formula:

$$\text{Inhibition of lipid peroxidationcapacity } (\%) = [(A_{control} - A_{sample})/A_{control}] \times 100$$

DNA protection assay

DNA protection assay was conducted using calf thymus DNA (Lee et al., 2002). Calf thymus DNA (5 µg) was incubated with Fenton's reagent (30 mM H_2O_2, 50 mM ascorbic acid and 80 mM $FeCl_3$) and fungal extracts (150 µg/mL). The mixture was analyzed by 1% agarose gel electrophoresis after 30 min incubation at 37°C. The positive control reaction contained calf thymus DNA and Fenton's reagent. Negative control consisted of the calf thymus DNA. The results were documented using XR + Molecular Imager Gel documentation system (Bio Rad, USA).

HPLC analysis

Phenolic acids and flavonoids were separated by the reverse phase analytical HPLC using Shimadzu LC-8A (Shimadzu Corporation, Tokyo, Japan) HPLC fitted with C_{18} column (25 cm × 4 mm length, 5 µm, Kromasil, India) and a diode array detector. An isocratic mobile phase consisting of water: formic acid: acetonitrile: (78:2:20 v/v) was delivered at a flow rate of 1 mL/min to elute phenolic acids. The absorbance of phenolic acids was recorded at 280 nm. Flavonoids were eluted with a gradient of solvent A (water adjusted with acetic acid to pH 2.8) and solvent B (acetonitrile) as described by Das and Singh (2016). The gradient was linear to 10% B in 5 min, 23% B in 31 min, and 35% B in 43 min. The column was washed with 100% B for 6 min and equilibrated for 6 min at 100% A to start the next sample. The absorbance was recorded at 260 nm. The peaks were identified by comparison with the standards. The HPLC water was purified by a Milli-Q System. The LC fragments of all individual peaks were collected in separate vials and used for ESI-MS/MS analysis.

ESI-MS/MS analysis

ESI-MS and MS/MS were performed using quadrupole time of flight (Q-TOF) mass spectrometer (Micromass Waters, Milford, MA, USA) with electrospray ionization (ESI). The instrument was calibrated through a mass range of 100–1000 and operated at negative mode $[M-H]^-$. The capillary voltage was 3 kV; source and desolvation temperatures were 120 and 300°C, respectively; cone gas (argon) and desolvation gas (nitrogen) flow rates were 50 L/h and 500 L/h, respectively. MS/MS spectra were acquired using collision energy of 20 V. The *m/z* ratio as well as fragmentation patterns were used for the confirmation of the phenolic acids and flavonoids.

Data and statistical analysis

The colonization frequency (CF) was calculated by the following formula: % $CF = [N_{col}/N_t] \times 100$, where, N_{col} is number of tissue segments colonized by a fungus; N_t is total number of tissue segments plated (Nalini et al., 2014).

The relative species frequency (RF) was calculated by the following formula: % $RF = [I_0/I_t] \times 100$, where, I_0 is number of isolates of one species; I_t is total number of isolates (Huang et al., 2007b).

Data reported as mean±standard deviation (SD) of three replicate, were analyzed with one-way ANOVA and Tukey-Kramer multiple comparisons test to determine significant differences between means using GraphPadInStat 3.0. Data were considered statistically significant at $p < 0.05$ and denoted with different superscripts.

Results

Isolation and identification of fungal endophytes

Fungal endophytes (471) isolated from 900 plant fragments

were distributed in 11 different taxa. The isolates were identified morphologically and with the DNA sequence analysis of the ITS region. The strains with their genbank accession numbers, colonization and relative frequency (%RF) are depicted in Table 1. *Fusarium equiseti* showed the highest RF (18.5%) followed by *F. chlamydosporum* (17.8%) and *F. solani* (17.4%). The genus *Fusarium* was present in all the plant parts, except rhizome.

Total phenolic content

TPC of the extracts is represented in Fig. 2. TPC of the extracts ranged from 10.17±0.7 to 81.58±6.0 mg GAE/g dry extract. *B. specifera* extracts showed highest total phenolic content (81.58±6.0 mg GAE/g dry extract) followed by *A. terreus*, *A. tenuissima*, *N. hematococca* and *F. chlamydosporum* extracts (52.46±1.3, 47.01±2.6, 28.82±0.7 and 26.64±1.3 mg GAE/g dry extract respectively).

Total flavonoid content

Among the 11 strains, flavonoid was detected in eight strains (Fig. 2). TFC ranged from 5.2±0.5 to 24.3±0.9 mg CE/g dry extract. *N. hematococca* exhibited high flavonoid content (24.3±0.9 mg CE/g dry extract). TFC of *F. chlamydosporum* and *A. tenuissima* was recorded as 22.9±1.9 and 19.8±0.5 mg CE/g dry extract respectively. The lowest TFC was recorded for *F. equiseti*.

Antioxidant properties

$ABTS^+$ radical scavenging assay

The total antioxidant capacity of endophytic extracts was determined by $ABTS^+$ scavenging capacity. The scavenging capacity of the extracts varied considerably, ranging from 10.93±0.26 to 70.68±0.26 mg TE/g dry extract (Table 2). Among the extracts, *B. specifera*, *A. terreus*, *A. tenuissima*, *N. hematococca* and *F. chlamydosporum* showed high scavenging capacity (70.68±0.26, 68.0±0.26, 67.45±2.24, 62.20±0.17 and 58.18±1.03 mg TE/g dry extract, respectively) in consistence with the TPC.

DPPH radical scavenging capacity

The DPPH radical is almost stable and used for antioxidant activity widely. The radical scavenging activity is presented as 50% scavenging capacity (IC_{50}) in Table 2. As depicted, the IC_{50} value of the fungal extracts varied considerably (1337.7±213.6 μg/mL to 96.9±2.4μg/mL). *A. tenuissima* showed highest scavenging capacity with 96.9±2.4 μg/mL, followed by *A. terreus* (123.3±7.6 μg/mL), *N. hematococca* (133.4±5.3 μg/mL) and *F. chlamydosporum* (226.9±23.7 μg/mL). Although, high TPC was exhibited by *B. specifera*, the scavenging capacity of DPPH radical was found to be very less (1057.2±122.3 μg/mL) in the extract.

Reducing power assay

Antioxidant compounds have the reductive ability to transform Fe^{3+} to Fe^{2+} through their functional groups which are susceptible to transfer electron. The reducing power of ferric ion to ferrous ion by the fungal extracts is represented in terms of ascorbic acid equivalent (Table 2). The values of reducing power assay ranged from 18.8±0.5 to 110.4±1.1 mg AA/g dry extract. *B. specifera* showed highest activity (110.4±1.1 mg AA/g dry extract) followed by *A. tenuissima* (86.9±0.9 mg AA/g dry extract) and *A. terreus* (74.9±2.9 mg AA/g dry extract). The reducing power activity of *N. hematococca* and *F. chlamydosporum* was 68.0±2.2 and 63.1±0.5 mg AA/g dry extract, respectively.

Inhibition of lipid peroxidation capacity

The peroxidation reaction was induced with Fe^{2+}. The

Table 1 Taxonomic identification,percent colonization and relative frequency of the fungal endophytes isolated from *Z. nimmonii*.

Endophytic Fungi	Code	Accession No.	Colonization frequency (%) of isolates					Relative frequency of isolation (%)
			Leaf*	Root*	Leafy stem*	Inflorescence†	Rhizome*	
Alternari atenuissima	ZN-WG-01	KJ547594	28.5	–	–	–	–	12.1
Trichoderma harzianum	ZN-WG-02	KJ547595	–	12	–	–	–	5.1
Fusarium solani	ZN-WG-03	KJ547596	–	7.5	14.5	38	–	17.4
Alternaria consortiale	ZN-WG-04	KM114288	13.5	–	–	–	–	5.7
Bipolaris specifera	ZN-WG-06	KM114290	–	13	–	–	–	5.5
Fusarium chlamydosporum	ZN-WG-07	KM396301	15.5	26.5	–	–	–	17.8
Hypocrea lixi	ZN-WG-08	KM396302	–	5.5	–	–	–	2.3
Aspergillus terreus	ZN-WG-09	KM396303	–	–	–	29	–	6.2
Nectria haematococca	ZN-WG-10	KM396304	–	11.5	–	–	–	4.9
Sarocladium kiliens	ZN-WG-11	KM396305	10.5	–	–	–	–	4.5
Fusarium equiseti	ZN-WG-12	KM396306	14.5	–	29	–	–	18.5
Total			82.5	76	43.5	67	–	100

– Indicates the absence of the endophytic fungi; * 200 fragments were plated from each part; † 100 fragments were plated from the inflorescence

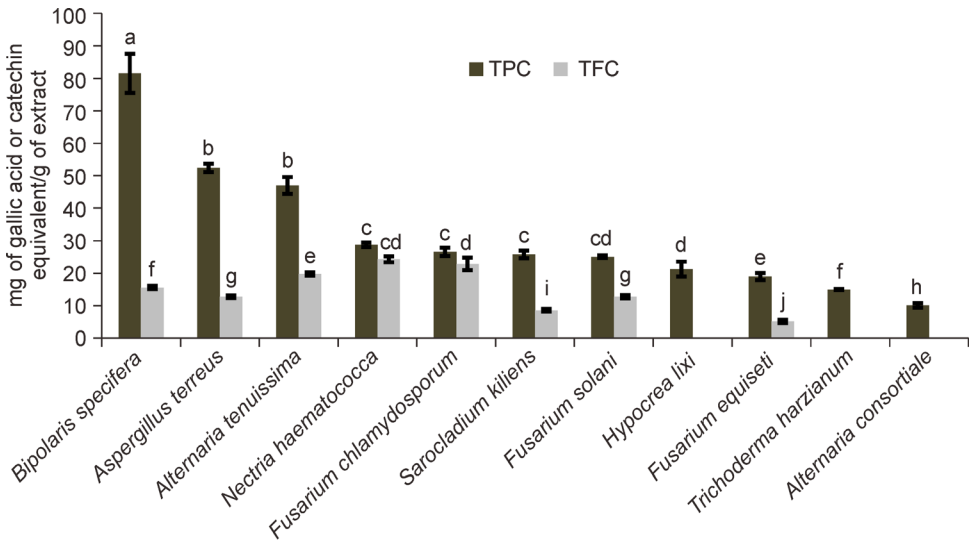

Figure 2 Total phenolic content (TPC) and total flavonoid content of fungal endophytes. Data are reported as mean±SD of three independent analyses ($n = 3$). Values with the different letters in the data labels are significantly different ($p < 0.05$).

Table 2 Antioxidant capacity of fungal endophytes isolated from *Z. nimmonii*

Fungal strains	$ABTS^{+}$ scavenging capacity (mg TE/g dry extract)	Reducing power (mg AA/g dry extract)	DPPH radical scavenging capacity (IC_{50} µg/mL)	Inhibition of lipid peroxidation capacity (IC_{50} µg/mL)
Bipolaris specifera	70.68 ± 0.26^{a}	110.4 ± 1.1^{a}	1057.2 ± 82.3^{g}	75.3 ± 2.75^{b}
Aspergillusterreus	68.0 ± 0.26^{b}	74.9 ± 2.9^{b}	123.3 ± 7.6^{c}	110.0 ± 3.3^{c}
Alternariatenuissima	67.45 ± 2.24^{b}	86.9 ± 0.9^{c}	96.9 ± 2.4^{b}	147.0 ± 5.5^{d}
Nectria haematococca	62.20 ± 0.17^{c}	68.0 ± 2.2^{d}	133.4 ± 5.3^{c}	159.8 ± 6.2^{e}
Fusarium chlamydosporum	58.18 ± 1.03^{d}	63.1 ± 0.5^{e}	226.9 ± 23.7^{d}	224.8 ± 5.2^{f}
Sarocladium kiliens	30.86 ± 2.07^{e}	45.3 ± 1.9^{f}	344.9 ± 21.2^{e}	332.1 ± 11.5^{g}
Fusarium solani	37.39 ± 2.83^{e}	42.7 ± 0.3^{f}	326.2 ± 20.6^{e}	349.4 ± 15.1^{g}
Hypocrea lixi	25.38 ± 1.8^{f}	39.2 ± 0.4^{g}	498.9 ± 40.9^{f}	565.3 ± 17.4^{h}
Fusarium equiseti	20.68 ± 1.47^{g}	28.3 ± 0.2^{h}	1082.3 ± 3.3^{g}	803.9 ± 15.4^{i}
Trichoderma harzianum	14.83 ± 1.29^{h}	23.8 ± 0.5^{i}	546.7 ± 25.7^{f}	897 ± 25.7^{j}
Alternaria consortiale	10.93 ± 0.26^{i}	18.8 ± 0.5^{j}	1337.7 ± 113.6^{h}	1035 ± 30.8^{k}
		Ascorbic acid	7.7 ± 0.02^{a}	30.7 ± 2.1^{a}

Data are reported as mean±SD of three independent analyses ($n = 3$). Mean with the different superscript within a column are significantly different ($p < 0.05$).

inhibition of lipid peroxidation capacity of fungal extracts is presented in Table 2. The capacity of the extracts ranged from 75.3±2.75 µg/mL to 1035±30.8 µg/mL. The extract of *B. specifera* showed highest inhibition capacity (75.3± 2.75 µg/mL) followed by *A. terreus* (110.0±3.3 µg/mL). The inhibition of lipid peroxidation capacity proceeded in the similar pattern as in case of ABTS scavenging capacity.

The extracts of *B. specifera*, *A. terreus*, *A. tenuissima*, *N. hematococca* and *F. chlamydosporum* were found to have high TPC and antioxidant capacity. The light micrograph of these fungal spore and hyphae depicted in Fig. 3. These fungal extracts were further examined for DNA protection assay. The phenolics present in the extracts were characterized by HPLC and ESI-MS/MS techniques.

DNA protection assay

Among the five isolates, *N. hematococca* and *F. chlamydosporum* extracts had visibly shown the DNA protection ability by inhibiting its fragmentation through the scavenging of –OH radicals generated by Fenton's reagent (Fig. 4).

HPLC, ESI-MS and MS-MS analysis

The extracts of selected five strains were characterized for the compounds. HPLC analysis revealed that these isolates contained various phenolic acids and flavonoids (Fig. 5). All these compounds were isolated individually and subjected to ESI-MS and MS/MS analyses for the confirmation of their presence (Fig. S1). The concentrations of individual phenolic

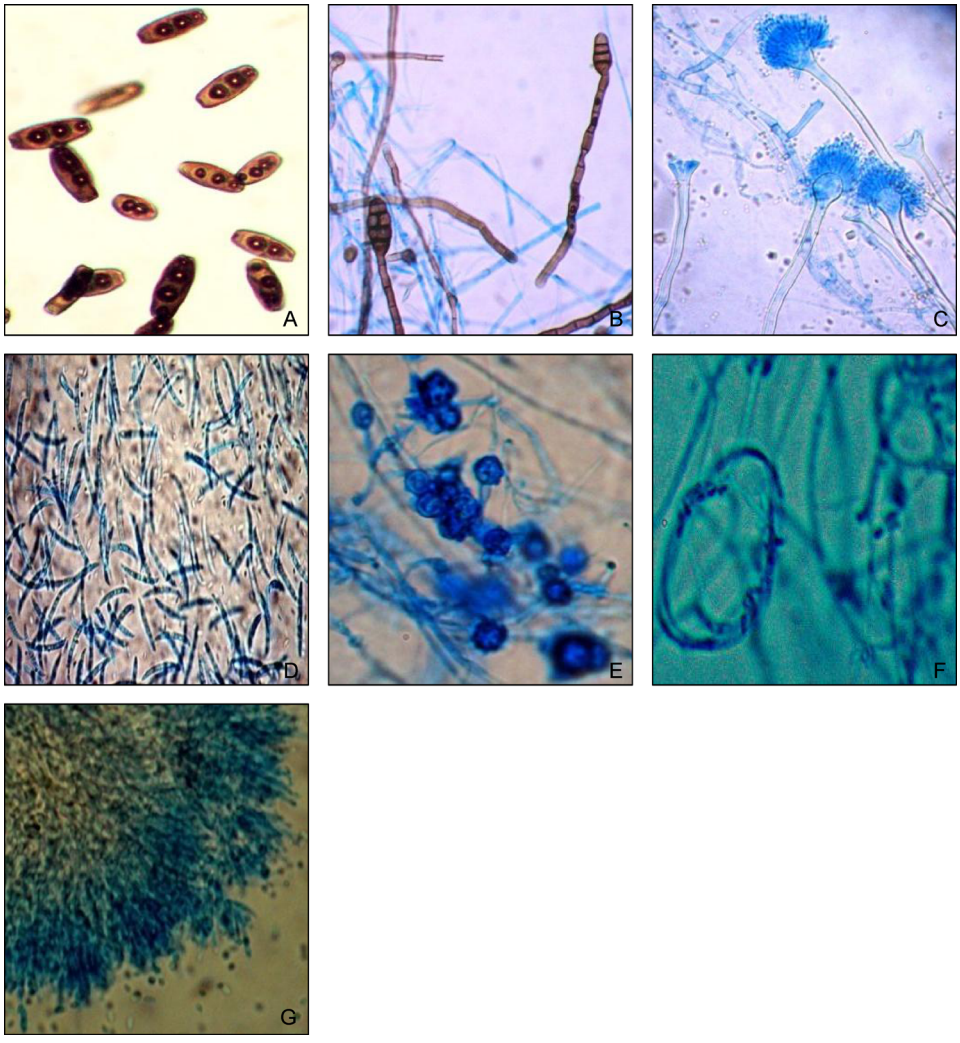

Figure 3 Light micrography of fungal endophytes with high antioxidant capacity at 40X. (A) *B. specifera*; (B) *A. tenuissima*; (C) *A. terreus*; (D) *F. chlamydosporum*; (E) Chlamydospores of *F. chlamydosporum*; (F) Hyphal coil of *F. chlamydosporum*; (G) *N. hematococca*.

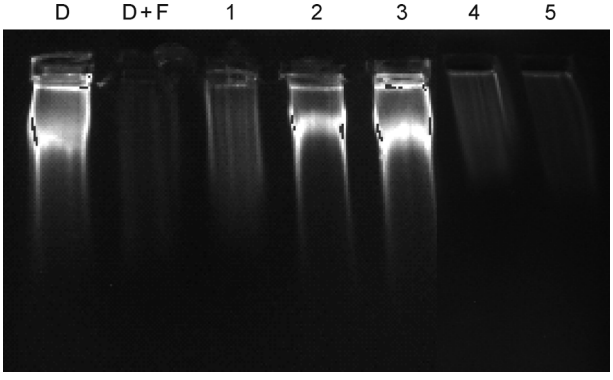

Figure 4 DNA protection assay of extracts from respective endophytic fungus. D. Calf thymus DNA; (D + F). DNA + Fenton's reagent; 1. *A. tenuissima*; 2. *F. chlamydosporum*; 3. *N. hematococca*; 4. *B. specifera*; 5. *A. terreus.*

compounds in the extracts of selected strains are presented in Table 3. Phenolic acids *viz*., caffeic (CA), *p*-hydroxybenzoic (*p*HBA), *p*-coumaric (*p*CA) were identified from *A. tenuissima*. Syringic acid (SA) was present only in *B. specifera* in a very low concentration (0.018±0.0003 mg/g of extract). Chlorogenic (ChA), vanillic (VA), ferulic (FA) and protocatechuric acids (PA) were identified in *A. terreus*. The flavonoid compound, quercetin was identified in the extracts of *N. hematococca* (5.82±0.2 mg/g of extract) and *F. chlamydosporum* (7.31±0.1 mg/g of extract). Catechin (2.42±0.07 mg/g of extract) was identified only from the extract of *A. tenuissima,* while kaempferol (3.32±0.07 mg/g of extract) from *N. hematococca*.

The details of the identified compounds and MS/MS fragmentation patterns are depicted in Table 4. The peak at the retention time (RT) 4.2±0.3 min showed a major ion of 353

Table 3 Concentration (mg/g of extract) of various phenolic compounds in five selected extracts of endophytes

Phenolic compounds	*B. specifera*	*A. terreus*	*A. tenuissima*	*N. haematococca*	*F. chlamydosporum*
Caffeic acid	2.34±0.09	nf	2.96±0.1	nf	nf
p-hydroxybenzoic acid	1.48±0.04	Nf	0.004±0.0001	nf	nf
p-Coumaric acid	2.89±0.09	Nf	0.52±0.01	nf	nf
Vanillic acid	nf	2.77±0.1	nf	nf	nf
Chlorogenic acid	nf	2.52±0.07	nf	nf	nf
Syringic acid	0.018±0.0003	nf	nf	nf	nf
Ferulic acid	nf	2.01±0.04	nf	nf	nf
Protocatechuic acid	nf	1.77±0.03	nf	nf	nf
Catechin	nf	nf	2.42±0.07	nf	nf
Quercetin	nf	nf	nf	5.82±0.2	7.31±0.1
Kaempferol	nf	nf	nf	3.32±0.07	nf

Data are reported as mean±SD of two independent analyses ($n = 2$); nf – not found

Table 4 ESI-MS/MS analysis of HPLC fragments of endophytic fungal extracts

RT	*m/z* $[M-H]^-$	MS/MS fragment	Compound identified	Fungal strains
3.8±0.1	289	245, 205	Catechin	*A. tenuissima*
4.2±0.3	353	191	Chlorogenic acid	*A. terreus*
5.3±0.3	179	134	Caffeic acid	*B. specifera*, *A. tenuissima*
5.9±0.1	137	93	p-hydroxybenzoic acid	*B. specifera*, *A. tenuissima*
6.0±0.3	167	152,123	Vanillic acid	*A. terreus*
7.0±0.5	197	182,153	Syringic acid	*B. specifera*
8.2±0.5	163	119	p-Coumaric acid	*B. specifera*, *A. tenuissima*
11.3±0.1	193	178,149,134	Ferulic acid	*A. terreus*
28±1.3	153	109	Protocatechuic acid	*A. terreus*
42.8±3.1	300	151,179	Quercetin	*F. chlamydosporum*, *N. haematococca*
49.6±0.02	285	257	Kaempferol	*N. haematococca*

Data are reported as mean±SD of two independent analyses ($n = 2$)

m/z and the ion corresponding to the deprotonated quinic acid 191 *m/z*, was confirmed as ChA. CA was identified at RT 5.3± 0.3 min with a major ion 179 *m/z* and a fragment of 135 *m/z* [M-H-44] due to loss of a –COO group *i.e.* decarboxylation. The peak at RT 5.9±0.1 min showed a major ion of 137 *m/z* with a fragment of 93 *m/z* due to decarboxylation, was confirmed as *p*HBA. The peak at RT 6.0±0.3 min had a major ion of 167 *m/z* and a fragment of 152 *m/z* due to loss of a CH_3 moiety *i.e.* demethylation [M-H-15] on the aromatic benzene ring, thus confirmed as VA. SA was identified at RT 7.0±0.5 min with the major ion 197 *m/z* and a fragment of 182 *m/z* due to demethylation and 153 *m/z* due to decarboxylation. *p*CA (RT 8.2±0.5 min) was identified by major ion of 163 *m/z* and fragments of 119 *m/z* due to decarboxylation. FA was confirmed at RT 11.3±0.1 with major ion of 193 *m/z* and 3 fragments of 178 *m/z* (demethylation), 149 *m/z* (decarboxylation) and 134 *m/z* (both demethylation and decarboxylation). Likewise, PA (RT 28.7±1.3 min) was confirmed with its major ion of 153 *m/z* and a decarboxylated ion of 109 *m/z*. Three flavonoid molecules, catechin, quercetin and kaempferol were identified. The peak at 3.8±0.1 (289 *m/z*) was found to produce fragments 246 *m/z* and 205 *m/z*. The ion was produced by the loss of $(CH)_2OH$ moiety (Pérez-Magariño et al., 1999). MS/MS spectra showed that peak at RT 42.8±3.1 produced fragments of 151 and 179 *m/z* as a result of cleavage of the heterocyclic C-ring by Retro-Diels-Alder rearrangement (Sun et al., 2007) and confirmed as quercetin. Similarly, kaempferol (RT 49.6±0.02) was identified with its major ion of 285 *m/z* and a fragment of 257 *m/z* which is consistent with the previous report (Sun et al., 2007). The structure of phenolic compounds shown in Fig. S1.

Discussion

The Western Ghats, extending along the west coast of India comprises the 34th biodiversity hotspot as recognized by Conservation International (www.conservation.org). This area is extremely rich in endemic plant species of which,

many of them are of medicinal importance. Over exploitation of these medicinal plants is already a threat to biodiversity. Both herbaceous plants and shrubs have been examined for the presence of endophytes from this region (Nalini et al., 2014; Yashavantha Rao et al., 2015; Samaga and Rai, 2016). Therefore, bioprospecting of the endophytes from Western Ghats region is the main focal point of our investigation. The endophytes are a treasure house of diverse bioactive compounds. In the present study, the bioactive compounds of fungal endophytes were isolated and identified. Totally 11 representative isolates were identified morphologically (mycelial characters, spore morphology) as well as with the help of DNA sequence analysis of the ITS region. The isolates showed 99-100% similarity to their assigned taxa. *F. equiseti* showed the highest CF (18.5%) followed by *F. chlamydosporum* (17.8%). *F. solani* was isolated from all the plant parts except leaves and exhibited 17.4% CF. The genus *Fusarium* was present in all the plant parts except rhizome. *Fusarium* is one of the most frequently isolated endophyte from ginger species (Nongalleima et al., 2013). The rhizome sampled in our study of endophytic fungi was devoid of any fungal endophytic association. Although in few studies *Fusarium oxysporum* was the only endophyte isolated from rhizome of zingiberaceous species *viz. Zingiber zerumbet* (Nongalleima et al., 2013), *Curcuma amada* (Tiwari et al. 2014). It is apparent from the phytochemical studies that rhizomes of zingiberaceous species contains volatile oils. *Z. nimmonii* in particular contains caryophyllene-rich volatile oils which have antifungal activity (Sabulal et al., 2006). In another study, an active antifungal compound phenazine 1-carboxylic acid was reported from the endophytic bacteria isolated from the rhizome of *Z. officinale*, a congener of *Z. nimmonii* (Jasim et al., 2014). The presence of antifungal agents in the rhizome of '*Zingiber*' could be the probable reason for obtaining not a single endophytic fungus from it.

All 11 strains were tested for TPC, TFC and antioxidant capacities. Finose and Gopalakrishnan (2014) reported the antioxidant activity of *n*-hexane extract of *Z. nimmonii*. According to their study, the DPPH radical scavenging capacity of the extract was IC_{50} 270 μg/mL whereas, in the present study, the endophytic fungi isolated from *Z. nimmonii* showed higher antioxidant capacity in terms of scavenging DPPH radical. *A. tenuissima* exhibited highest scavenging activity with IC_{50} 96.9±2.4 μg/mL, followed by *A. terreus* (IC_{50} 123.3±7.6 μg/mL) and *N. hematococca* (IC_{50} 133.4± 5.3 μg/mL). This clearly indicates that the scavenging potentials of *A. tenuissima* is nearly three times more than the plant extract while in case of *A. terreus* and *N. hematococca*, it is nearly two times more. In another study, the DPPH scavenging activity of ethyl acetate extract of *Z. zerumbet* endophytes was reported as IC_{50} 182 μg (Nongalleima et al., 2013).

In stressed condition, highly reactive OH^- radical reacts with polyunsaturated fatty acids. Due to lipid peroxidation, various products like malondialdeyde (MDA), 4-hydroxyl 2-nonenal, hydrocarbons, volatile ketones and lipid polymersare produced which react with the cell macromolecules and effect cellular functions and biochemistry (Tuma and Casey, 2003; Onyema et al., 2006). Therefore, the inhibition of lipid peroxidation is necessary for cell viability in stressed conditions. The extracts of *B. specifera* exhibited the highest activity (75.3±2.75 μg/mL) which is twofold lesser than 50% of standard ascorbic acid (30.7±2.1 μg/mL).

Based on the antioxidant capacity, five isolates with higher capacities were selected for the study of DNA protection assay and the characterization of phenolic compounds present in the extracts by HPLC, ESI-MS and MS/MS fragmentation method. The MS/MS fragmentation pattern of phenolics was consistent with the previous study (Sun et al., 2007).

High TPC as well as $ABTS^+$ scavenging capacity was observed in the extract of *B. specifera* among all the isolates, but with very low DPPH scavenging activity. On the other hand *A. tenuissima* exhibited a high DPPH scavenging capacity, although its TPC was lower than *B. specifera.* In addition, only *N. hematococca* and *F. chlamydosporum* extracts had the DNA protection ability. Due to this variability in activity, the extracts of strains were selected for the characterization of compounds. HPLC analysis revealed that these isolates contained various phenolic acids (Fig. 5).

The presence of bulky side chains (Jing et al., 2012) and the number of hydroxyl moieties attached to the aromatic ring of the benzoic or cinnamic acid molecules (Karamac et al., 2005) were favorable for the DPPH radical scavenging activity of phenolic acids. The descending order of radical scavenging activities of phenolic acids is: SA > CA > PA > FA > VA > *p*CA > *p*HBA. *B. specifera* extract contained decarboxylated CA, SA, *p*HBA and *p*CA whereas, *A. tenuissima* contained CA, *p*HBA, *p*CA and catechin. CA with two –OH groups and a bulky side chain (CH = CHCOOH) may be responsible for the high scavenging activity of *A. tenuissima* extract apart from the presence of catechin in it. However, in *B. specifera* the concentration of *p*CA and *p*HBA was higher (2.89±0.09 and 1.48±0.04 mg/g extract respectively) than CA. SA was found in the extract but in traces (0.018±0.0003 mg/g extract). *p*HBA and *p*CA are less active in scavenging (Karamac et al., 2005). The extract of *A. tenuissima* found to contain very less concentration of *p*HBA (0.004±0.0001 mg/g extract). These reasons had collectively resulted in the low DPPH scavenging capacity of *B. specifera* and high capacity of *A. tenuissima.*

The extract of *A. terreus* also exhibited high scavenging capacity after *A. tenuissima*. Nevertheless, its phenolic composition was different than that of *A. tenuissima*. The presence of PA, FA, VA and ChA had collectively contributed to its scavenging activity. ChAwas also reported from the extracts of the endophytic fungi by Huang et al. (2007a, 2007b). Deng et al. (2013) reported the anti-cancer activity of secondary metabolites of endophytic *A. terreus* isolated from mangrove. As it is also known that the oxidative stress

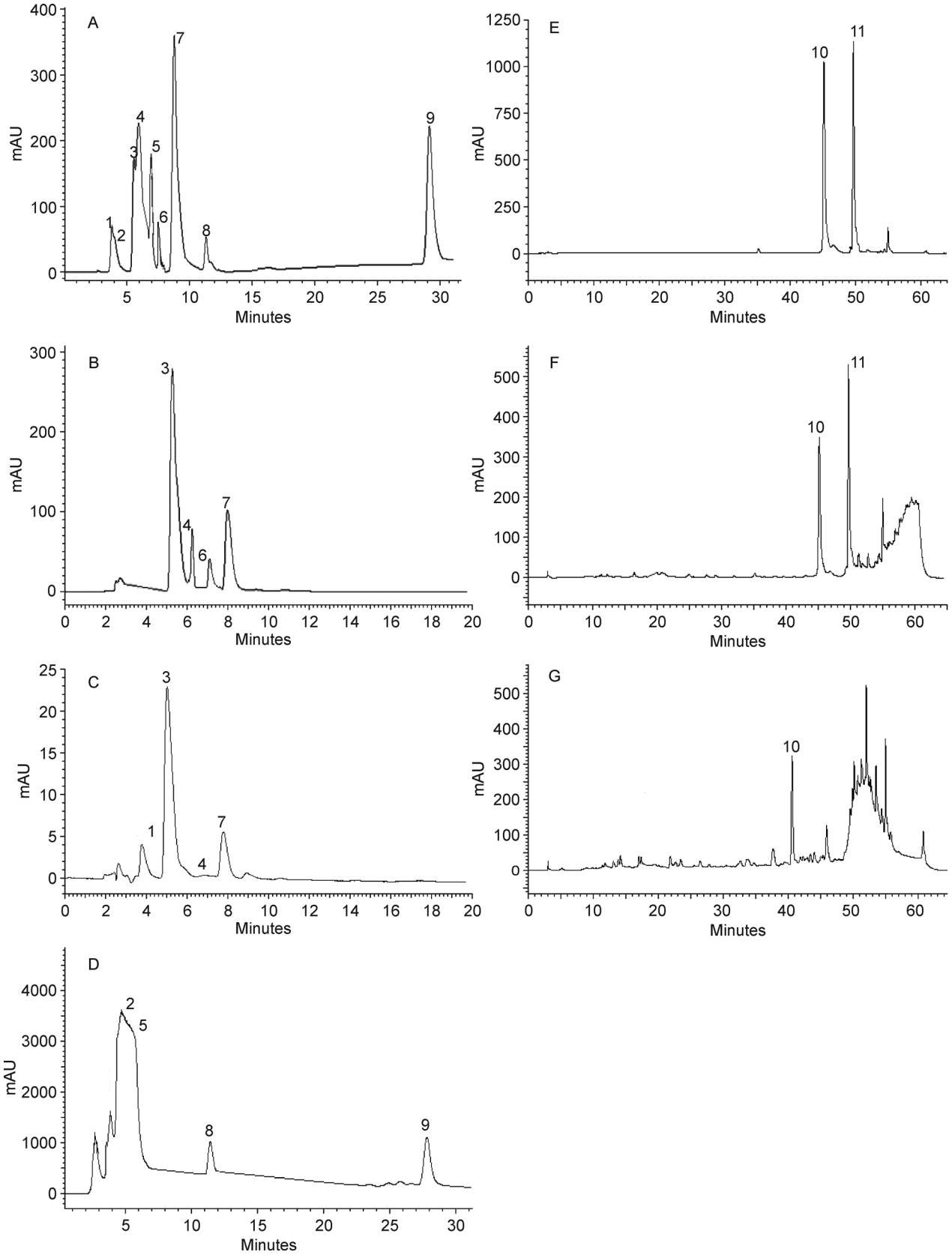

Figure 5 HPLC chromatograms of phenolics. (A) Separation of phenolic acids and catechin in standard mixture (at 280 nm); (B) *B. specifera*; (C) *A. tenuissima*; (D) *A. terreus*; (E) Separation of quercetin and kaempferol in standard mixture (at 260 nm); (F) *N. hematococca*; (G) *F. chlamydosporum*. (1- Catechin, 2- Chlorogenic acid, 3- Caffeic acid, 4- *p*-hydroxybenzoic acid, 5- Vanillic acid, 6- Syringic acid, 7- *p*-Coumaric acid, 8- Ferulic acid, 9- Protocatechuic acid, 10- Quercetin, 11- Kaempferol).

contributes to cell damage and uncontrolled cell proliferation, hence our findings on the presence of phenolic antioxidants in *A. terreus* augments its importance.

In the present study, we have studied the DNA protection ability of five strains. Our findings indicated that the extract of *N. hematococca* and *F. chlamydosporum* exhibited DNA protection ability against –OH induced DNA damage. It is well known that oxidative stress causes DNA damage and interrupt the cell cycle (Pizarro et al., 2009) which further contributes to several other diseases. Both the extracts exhibited high TFC (24.3±0.9 and 22.9±1.9 mg CE/g extract respectively). The DNA protective ability of the extracts of *N. hematococca* and *F. chlamydosporum* is due to the presence of quercetin, which has been reported to inhibit H_2O_2-induced (Duthie et al., 1997) and cadmium-induced DNA damage (Çelik and Arinc, 2010).

In summary, the present study is the first report on the fungal endophytes from *Z. nimmonii* (J. Graham) Dalzell. The Western Ghats, a biodiversity hotspot, is extremely rich in endemic medicinal plant species. Over exploitation of these medicinal plants is already a threat. Therefore, bioprospecting of the endophytes from Western Ghats region is the main focal point of our investigation. This study also reveals the phenolic profiles of the five selected isolates showing high antioxidant capacities. The varied phenolic profiles characterized from *Z. nimmonii* endophytes can be attributed to the differences in their antioxidant capacities. The concentrations of *p*HBA acid and *p*CA acid were found to be accountable for DPPH radical scavenging capacity whereas, the presence of quercetin is attributed to the DNA protection ability against –OH induced DNA damage. The present research implicates the role of fungal endophytes as renewable and new sources of bioactive components which can be useful in oxidative stress related diseases. Hence, *in vitro* findings presented herein can be useful in the war of protection against oxidative stress.

Abbreviations

ABTS- 2, 2'-azino-bis(3- ethylbenzthiazoline-6-sulphonic acid); CA- caffeic acid; CE- catechin equivalents; CF- colonization frequency; ChA- chlorogenic acid; CTAB –cetyltrimethyl ammonium bromide; DPPH-1, 1-diphenyl-2- picrylhydrazyl; FA – ferulic acid; FC- Folin–Ciocalteau; GAE-gallic acid equivalent; ITS- Internal Transcribed Spacer; NCBI – National Center for Biotechnology Information; PA – protocatechuic acid; *p*CA- p-coumaric acid; PDA- potato dextrose agar; PDB – potato dextrose agar; pHBA- p-hydroxybenzoic acid; RF- relative species frequency; RT – retention time; SA- syringic acid; SD – standard deviation; TEAC – Trolox Equivalent Antioxidant Capacity; VA – vanillic acid.

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Compliance with ethics guidelines

Madhuchhanda Das, Harischandra Sripathy Prakash and Monnanda Somaiah Nalini declare that they have no conflict of interest.

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