Final Report of the work done on the Major Research Project

"ISOLATION OF NOVEL ANTIANGIOGENIC AND OTHER PHARMACOLOGICALLY SIGNIFICANT LEAD MOLECULES FROM ENDEMIC ENDOPHYTIC FUNGI".

Principal Investigator: **Dr. Chandrashekhar Gajanan Joshi**

UGC Reference No **: F.No. 42-671/2013(SR) dated 22nd March 2013**

Period of report : **01st April 2013-30th March 207**

Submitted to

UNIVERSITY GRANTS COMMISSION
BAHADUR SHAH ZAFAR MARG
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PROGRESS REPORT

Title of research project: "**Isolation of novel Antiangiogenic and other pharmacologically significant lead molecules from endemic endophytic fungi".**

Principal Investigator: **Dr. Chandrashekhar Gajanan Joshi**

Name of the Project Fellow: **Ananda. D**

UGC Reference No : F.No. 42-671/2013(SR) dated 22nd March 2013

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Objectives:

- Screening and selection of medicinally important endophytes based on traditional knowledge and literature.
- Checking the efficiency of the endophytes for antimicrobial, larvicidal and cytotoxic activities.
- Extraction of endophytic components using different solvent system.
- In vitro and in vivo studies of endophytic extracts for their antiangiogenic property.
- Elucidation of the structure of lead molecule/s.

Results

In total, 16 endophytic fungi were isolated from *T. involucrata* and *Z. officinale* rhizome. Nine and seven endophytic fungi were isolated from *T. involucrata* and *Z. officinale* rhizome the stem of the respectively(Table.1). The pure cultures were isolated from different colonies of endophytic fungi grown from the host plants and colony characters were studied. An attempt was made to isolate phytochemicals from the fungal cultures. We have successfully isolated the phytochemicals from endophytes of *T.involucrata*. The extracts were subjected to phytochemical analysis and found to contain flavonoids.

Table1.Details of endophytic fungi isolated from *T. involucrata* and rhizome of the *Z.officinale.*

	7th Day	**8th Day**	**9th Day**	**10th Day**	**11th Day**	**19th Day**	**Total Colony**
A-(T)	2 colony (Brown & white)	1 colony Green	-	-	-	-	3
C(T)	1 colony (Green white)	-	1 colony (Green)	-	-	-	2
D-(T)	-	-	1 colony (black)	-	1 colony (white Brown)	-	2
E-(T)	-	-	-	-	1 colony (white Brown)	-	1
F-(T)	-	-	-	-	-	1 colony (Brown)	1
B-(Z)	1 colony (Brown & white)		1 Colony (Brown)	1 Colony (Brown)	1 Colony (Brown)		4
G-(Z)		1 colony (White)	1 colony (Pink)	1 colony (White)			3
			Total edophytic fungal colonies isolated				**16**

Total polyphenolic content in the endophytic extracts were measured by Folin ciocalteau's method. Phenolic content was found to highest in C2 while it was least in D1. The total flavonoid content in the fungal extracts were expressed as the mg equivalents of qurcitin. Flavonoid content varied in different extracts and it was highest in D2 extract. Total antioxidant activities of each extracts were measured by phosphomolybdenum method and the results were expressed as mg equivalents of ascorbic acids. All the extracts showed significant total antioxidant activity.D2 extract showed significant radical scavenging activity in DPPH method.

Table 2. Identification of fungal strains isolated from the *Tragia involucrata* and *Zinger officinal* based on morphological characteristics as well as DNA analysis of the internal transcribed spacer (ITS) region. The closest relatives in GenBank according to BLAST search were presented.

Medicinal plant	Endophytic fungi isolate	Nucleotide sequence	NCBI accession number	NCIM accession number	Sequence analysis	
					Closest NCBI database match with accession number	Percentage of identity
Tragia involucrata	CGJ-C1	584	KT780618	NCIM-1391	*Penecillium citrinum* (CGJ-C1)	99
Tragia involucrata	CGJ-C2	745	KP739821	NCIM-1390	*Penecillium citrinum* (CGJ-C2)	99
Tragia involucrata	CGJ-D1	787	KP739822	NCIM-1392	*Cladosporium* species (CGJ-D1),	99
Tragia involucrata	CGJ-D2	563	KT780619	NCIM-1389	*Cryptendoxyla hypophloia* (CGJ-D2)	99
Zinger officinal	CGJ-B3	584	KT780617	NCIM-1393	*A. austroafricanus* (CGJ-B3)	99

Ethyl acetate extracts of the four endophytic extracts were tested for the antimicrobial activity. All the extracts showed significant antimicrobial activity and it was comparable to the standard antibiotics

The ethyl acetate extracts of endophytic fungi isolated from *T.involucrata* showed significant larvicidal activity. All the extracts were active against larva even at 100ppm with D2 extract showing the highest activity. The toxicity towards the larva was showing a positive relation with the time of incubation.100 percent larval death was observed in all the extract when the concentration was scaled up to 500ppm. All the larvae were almost dead at 500ppm in C1 and D1 extract after 12h incubation.

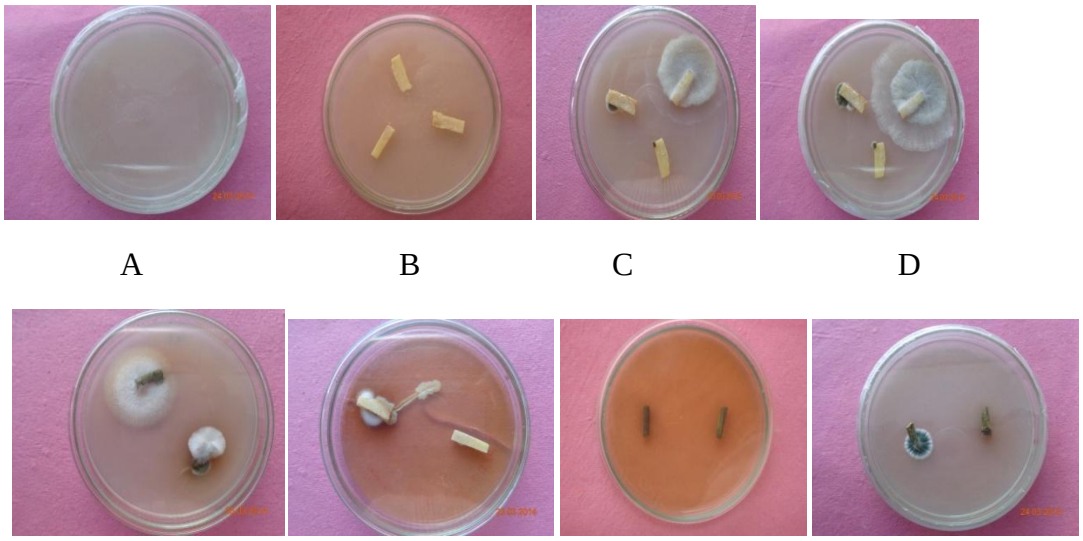


Figure 1. Endophytic fungus isolated from rhizome of the *Z. oficinale* and *T involucrata*

1. Control plate, (B-E) Growth of the endophytic fungi of Z. *officinale* and(F-H) Growth of the endophytic fungi of *T-involucruta*.

Figure 2. Pure cultures of endophytic fungi isolated from the *T Involucrata.*

MCF -7 and MOLT-4 cell line were selected for invitro antiprolefarative study by MTT assay. The endophytic fungal extract C2 was showing a significant anticancer effect on MCF-7 breast cancer cell line compared to other extracts. The antiproliferative activity of the extracts was

dose dependent. D1 did not show anticancer effect on MCF-7 cell line. The cell proliferation capacity of MCF-7 was drastically reduced with the incubation of the extract with cell lines for 72 hr. C2 extract was active against even MOLT-4 cell line. D1 extract was least toxic to MOLT-4 cells .Even in MOLT -4 cells the cell death was dependent on the dose of extract and incubation period with extract.

Antiangiogenic activity of the ethyl acetate extracts were tested by chick chorioallantoic membrane (CAM) assay. All the extracts showed significant antiangiogenic activity.

The fungal extracts were subjected to HPLC, FT-IR and UV-Vis sphetrophotometric analysis to find out the lead molecules. These studies confirmed presence of poly phenols and flavonoids.These molecules may be playing a crucial role in the different biological activity mentioned so far.

Extraction of secondary metabolites was carried out according to the procedure explained by Higginbotham et al (2013) with slight modification. EtoAc extract of five endophytic fungi were tested against *in vivo* CAM and peritoneal angiogenesis assay (data not shown). We found that the ethyl acetate extract of *P. citrinum* CGJ-C2 showed potent activity compared to other fungi. The EtoAc fraction was eluted from silica gel column and yielded four fractions (Fraction 1: 2: 3: and4). Fraction 2 (200 mg) was applied for preparative TLC and purified F3 lead (The RF value of the F3 lead was 0.36) dissolved in DMSO for the treatment.

IR abortion peak at 1409 cm^{-1} and 1451 cm^{-1} band at 1021cm^{-1} confirms the presence of aromatic C=C stretching and C-H bending respectively. A broad peak at 3338cm^{-1} confirms the presence of O-H / COO-H group. A sharp peak 1658cm^{-1} confirms the presence of –C=O group.

The multiplet peak at □ 6.816-6.761 in ^{1}H-NMR spectra further confirms the presence of aromatic ring in F3. A broad peak at □12.1 and at □ 3.27 confirms the presence of –COOH and –OH groups. Based on the IR and ^{1}H-NMR spectra it is clear that F3 is a phenolic acid compound.

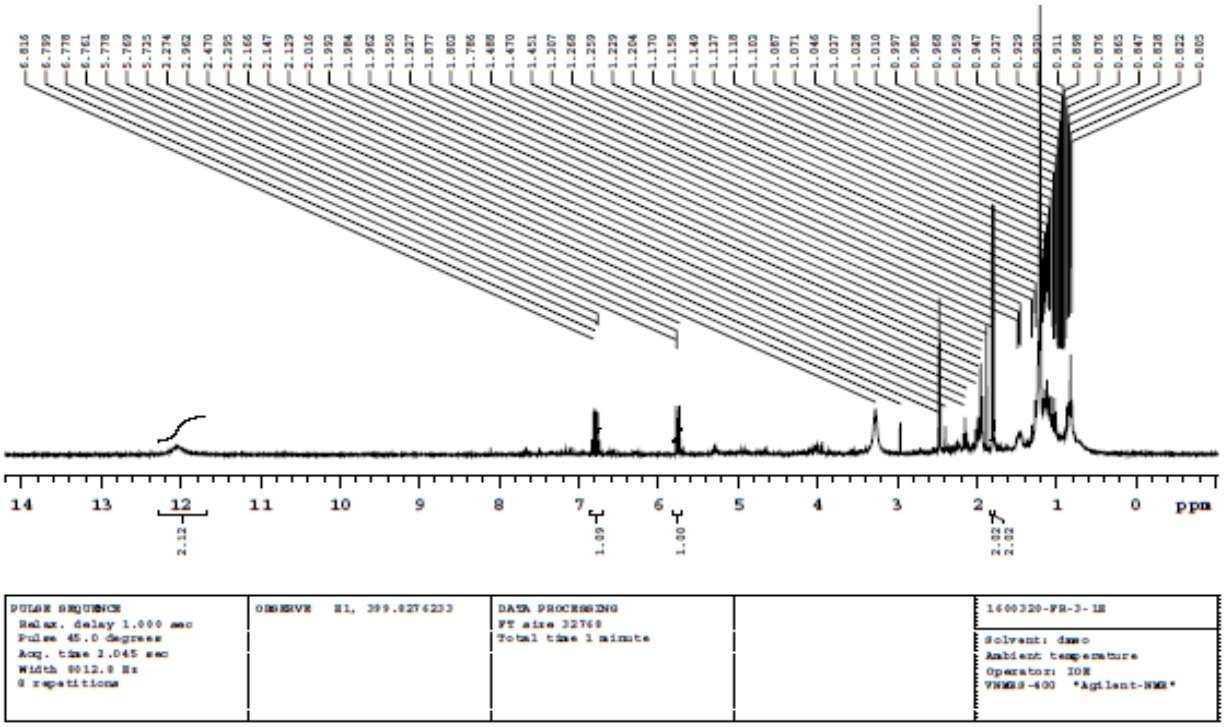


Figure. 19 H NMR Analysis of F3 compound

EAC cells were cultured *in vivo* and administered with 100 mg/kg (i.p.) of F3, Cisplatin, F3+Cisplatin (50:50) for three doses and inhibits the proliferation of ascites carcinoma cells as well as ascites fluid volume *in vivo* compared to control (Figure 20). Body weight of tumor bearing mice depicting the tumor regression.

MAJOR CONTRIBUTIONS OF THE PROJECT

1. Five new medicinally important endophytic strains were isolated from the plants.
2. A lead molecule has been isolated from one of the endophytic fungi.
3. Understanding the mechanism of action of lead molecule isolated from endophytic fungi.
4. Three papers published in international peer reviewed journals and two are under preparation.
5. The findings were presented in two national conferences.
6. This project work leading to the Ph.D degree to project fellow.

List of Publications

1. Ananda Danagoudar, Chandrashekhar G Joshi, R. Sunil Kumar, Jagadeesha Poyya, T Nivya, Manjunath M. Hulikere & KA Anu Appaiah (2017): Molecular profiling and antioxidant as well as anti-bacterial potential of polyphenol producing endophytic fungus-*Aspergillus austroafricanus* CGJ-B3, Mycology, DOI: 10.1080/21501203.2017.1281358.

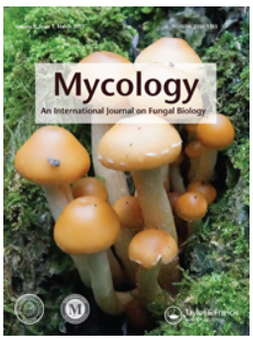
2. Ananda Danagoudar, Chandrashekhar G. Joshi, Nivya M.T., Manjunath H.M., Jagadeesha Poyya and Sunil Kumar R. (2017): Antimicrobial and Larvicidal potential of Endophytic fungi isolated from *Tragia involucrata* Linn. Annals of Plant Sciences, 6.01:1494-1498.

3. Ananda Danagoudar, Chandrashekhar G Joshi, R. Sunil Kumar, Rohit Kumar RG, KA Anu Appaiah and Ramesh BR (2017): Antioxidant and Cytotoxic Potential of Endophytic Fungi Isolated from Medicinal Plant *Tragia involucrata* L. Pharmacognosy Research 10(2):188-194.

Conference Presentation

1. Oral presentation on National Conference on “Role of biopharmaceutical in achieving health by 2020” in Dr. NGP Arts and Science college, Coimbatore, Tamil Nadu.

2. Poster presentation in National Conference on “Natural Bioactive Compounds and Potential Effects on Health” 27th January 2017. ST. ALOYSIUS COLLEGE, Mangalore, Karnataka.



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Molecular profiling and antioxidant as well as anti-bacterial potential of polyphenol producing endophytic fungus-*Aspergillus austroafricanus* CGJ-B3

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ABSTRACT

Fungal endophytes are less studied group of microorganisms with vast therapeutic potential. A polyphenol producing endophyticfungus was isolated from *Zingiber officinale* rhizome. The ethyl acetate extract of *Aspergillus austroafricanus* (EAE) was tested against five human pathogenic bacteria by disc diffusion method. Antioxidant activity of EAE was determined by DPPH, H_2O_2 and nitric oxide radical-scavenging methods. DNA protection from the OH radicals was tested by agarose gel electrophoresis. High-performance liquid chromatography was used to determine the total yield of polyphenols. The identity of the endophytic fungus was established as *A. austroafricanus* CGJ-B3 (GenBank accession No. KT780617) based on rDNA and phylogenetic analysis. EAE showed significant antioxidant, antimicrobial activity and DNA damage protection capacity. The HPLC analysis showed the presence of polyphenols such as *p*-coumaric acid, ferulic acid and cinnamic acid and the content was about 0.392 ± 0.08 µg/mg, 4.35 ± 0.16 µg/mg and 1.976 ± 0.11 µg/mg, respectively. *A. austroafricanus* CGJ-B3 isolated from *Z. officinale* is a promising potential pharmaceutical agent and can be used as an alternative source of polyphenols like *p*-coumaric acid, ferulic acid and cinnamic acid.

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Endophytic fungi; *Aspergillus austroafricanus* CGJ-B3; *Zingiber officinale*; polyphenols; antimicrobiol activity; secondary metabolite

1. Introduction

Plants are the reservoir of therapeutic compounds like phenolics, flavonoids, terpenoids and alkaloids. Amongst these, polyphenols and flavonoids play a very vital role in the antioxidant, free radical-scavenging and other pharmacological activities (Gangwar et al. 2014). So, the polyphenol producing medicinal plants are serving the needs of pharmaceutical industries since many years (Hosseinzadeh et al. 2015).

Zingiber officinale is one of the important tropical medicinal plants which belongs to the family Zingiberaceae. This plant was used as an ingredient in Ayurveda and Siddha medicine since ages for treating various ailments (Kumar et al. 2011). The rhizome of this plant is one of the ingredients in food, beverages, medicines and cosmetics. Different parts of *Z. officinale* have been reported to show an array of medicinal properties such as analgesic, anti-inflammatory, antiulcer, antioxidant (Ma et al. 2004; Chrubasik et al. 2005; Ghasemzadeh et al. 2010; Nanjundaiah et al. 2011a), anticancer (Miyoshi et al. 2003; Shukla & Singh 2007), antimicrobial (Aghazadeh et al. 2016), anticoagulant (Srivas 1984), fibrinolytic, prebiotic (Helal et al. 2014), immunomodulatory (Gupta & Chaphalkar 2015), hepatoprotective, larvicidal (Kumar et al. 2011) and anti-*Limnatisnilotic* activities (Bahmani et al. 2013). It is a potent inhibitor of proton–potassium ATPase activity as well as the ulcer-causing organism, *Helicobacter pylori* (Siddaraju & Dharmesh 2007).

Endophytes are the microorganisms that inhibit the plant organs and can colonise internal plant tissue without causing any apparent harm to the host (Petrini et al. 1993). These organisms harbour numerous therapeutic molecules like phenolics, flavonoids, terpenoids and alkaloids (Kaul et al. 2012). According to Zhao et al. (2010), endophytic fungi have the capacity to produce similar compounds as that of the host plants. Isolation of potent bioactive compounds from the endophytic fungi is in great demand as it has several beneficial applications in pharmaceutical, agricultural and pharmaceutical industries. Endophytic fungal extracts have been reported to possess antimicrobial, anticancer,

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antimalarial (Wiyakrutta et al. 2004; Santos et al. 2012), antitumor (Li et al. 2005), antidiabetic (Ushasri & Anusha 2015), antimicrobial (Supaphon et al. 2013; Hussain et al. 2014; Shen et al. 2014; Rao et al. 2015) and antioxidant activity (Liu et al. 2007; Yadav et al. 2014). Many endophytes have been isolated from *Z. officinale* and these endophytes have been reported to possess antimicrobial (Ginting et al. 2013), antioxidant activity (Bussaban et al. 2003). Anisha and Radhakrishnan (2015) have isolate D-gliotoxin-producing endophytic fungus, *Acremonium* sp. from *Z. officinale*. Even though some of the endophytes have been isolated from *Z. officinale*, still many are underexploited for the therapeutic potential. So, the aim of our current investigation was to isolate the novel endophytes from *Z. officinale* and to evaluate the antioxidant, antimicrobial, DNA protection activity. We had successfully isolated the endophytes from the rhizomes of *Z. officinale*. The fungus was identified as *Aspergillus* sp. which was capable to produce polyphenols and flavonoids which is a rarely reported in the literature.

2. Materials and method

2.1. Chemicals used

Agarose and Folin–Ciocalteu reagent were purchased from Sisco Research Laboratories. -DNA was purchased from Bangalore Genei, India. Ferrous sulfate heptahydrate ($FeSO_4{\cdot}7H_2O$), hydrogen peroxide, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, TRIS base, butylated hydroxyanisole, ethidium bromide, gallic acid, ethylene diaminetetraacetic acid (EDTA) and thiobarbituric acid were purchased from Sigma Chemicals (USA). All other chemicals used were of analytical grade.

2.2. Collection of plant material

Z. officinale rhizome was collected in the month of February 2014, Somawarpet (T), Kodagu (D) Karnataka, India. The Latitude and longitude is 12.46700N 75.96700E and a plant was authenticated by Dr. Jagat Timmaiah, Assistant professor, Dept. of Botany, Mangalore University, Cauvery campus, Madikeri, Karnataka, India.

2.3. Isolation of endophytic fungus from **Z. officinale** rhizome

The rhizome of *Z. officinale* was washed with running tap water to remove soil debris followed by washing with distilled water. The samples were cut into small pieces (0.6–0.8 cm) and rinsed with 70% alcohol. The alcohol washed pieces were again rinsed with 0.5% sodium hypochloride followed by washing thrice with sterile distilled water. The sterilised samples were transferred aseptically to PDA media containing 150 mg/L chloramphenicol (Higginbotham et al. 2013).

2.4. Scanning electron microscopic observation of Aspergillus austroafricanus CGJ-B3

The pure cultures of isolated endophytic fungal colony characters were studied and observed under the Scanning Electron Microscope (Model No. LEO 425 VP, Electron Microscopy Ltd. (Cambridge, UK). The methods mainly referred to (Qiu et al. 2010) with slight modification. The specimens were first fixed with 2.5% glutaraldehyde in phosphate buffer (PBS, 0.2 M, pH 7.0) for 6 h and then washed three times with PBS. They were postfixed with 1% (w/v) osmium tetroxide (OsO_4) in PBS for 1 h and again washed three times with PBS. The specimens were dehydrated by a graded series of ethanol (10–100%) for about 10–15 min at each step; finally, they were dehydrated in critical point dryer and coated with gold palladium and observed the microscopic images under SEM (Qiu et al. 2010).

2.5. Molecular identification

Molecular identification was achieved by sequencing the partial sequence of 28S rDNA. The fungus was grown and cultured in potato dextrose broth at 28 ± 2°C temperature for 4 days. The genomic DNA was isolated and the 28S rDNA was amplified using forward 5 ACC CGC TGA ACT TAA GC 3 and reverse 5 GGT CCG TGT TTC AAG ACG G 3 primers.

2.6. Phylogenetic analysis

The evolutionary history was inferred using the neighbour-joining method (Saitou & Nei 1987). The bootstrap consensus tree inferred from 1000 replicates was

taken to represent the evolutionary history of the taxa analysed (Felsenstein 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The evolutionary distances were computed using the maximum composite likelihood method (Tamura et al. 2004) and are in the units of the number of base substitutions per site. The analysis involved 10 nucleotide sequences. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA6 Software (Tamura et al. 2013).

2.7. Preparation of fungal mat culture

The endophytic fungal isolate was cultured in 1000 mL conical flask containing 350 mL potato dextrose broth and 10% of starter culture inoculum was inoculated and incubated in an incubate shaker at 100 rpm at 28 ± 1°C for 3 days followed by incubation 28 ± 1°C for18 days in a static condition (Cui et al. 2011).

2.8. Extraction of secondary metabolites

Extraction of secondary metabolites was carried according to the procedure explained by Higginbotham et al. (2013), with slight modification. Liquid culture with evident growth was mixed with an equal volume of ethyl acetate (Merck, India). The mixture was blended with the help of pastel and mortar. The resulting homogenate was filtered with Whatman® qualitative filter paper, Grade one and extracted thrice with an equal volume of ethyl acetate. The aqueous and organic layer were dried and stored at −20°C for further use (Higginbotham et al. 2013). The extract was labelled as endophytic ethyl acetate extract of *A. austroafricanus* (EAE).

2.9. Preliminary phytochemical analysis

Freshly prepared EAE was subjected to standard methods of phytochemical analyses to detect the presence of phyto-constituents, viz. flavonoids, carbohydrates, glycosides, saponins, tannins and alkaloids (Poojary et al. 2015).

2.10. Determination of total phenolic content

Total phenolic content was measured according to the method of (Yadav et al. 2014), with minor modifications. Standard tannic acid (0.1 mg/mL) and EAE (1 mg/mL) were taken in a tube and 0.5 mL of Folin–Ciocalteu reagent (1:1) and 2.5 mL of sodium carbonate (20%) were added. The final volume was made up to 10 mL by adding the distilled water. The absorbance of the reaction mixture was measured at 760 nm in UV spectrophotometer (Optima Tokyo, Japan). Gallic acid was used as standard and total phenolic content was expressed as milligram equivalents of gallic acid.

2.11. Total flavonoids content

The total flavonoids contents in EAE were determined spectrophotometrically according to the method of Qiu et al. (2010). Standard quercetin was employed to make the standard curve. EAE (1 mg/1 mL) was mixed with 1 mL of 2% aluminium chloride ($AlCl_3{\cdot}6H_2O$) methanolic solution. The reaction mixtures were incubated at 25 ± 1°C for 15 min, and then the absorbance was measured at 430 nm in UV spectrophotometer. The total flavonoids content was expressed as milligram quercetin equivalent.

2.12. Antioxidant activity

2.12.1. Reducing power assay

Reducing power was determined according to the method of Poojary et al. (2015) with modifications. Standard (0.1 mg/mL) and diluted EAE (1 mg/mL) were taken in different tubes. Phosphate buffer (2.0 mL, 0.2 M, pH 6.6) and 2.5 mL of 1% potassium ferricyanide were added to reaction mixture and incubated at 50°C for 20 min. Trichloroacetic acid (2.5 mL, 10%), 2.5 mL distilled water and 0.5 mL of 0.1% ferric chloride were added and mixed well. The absorbance of the reaction mixture was measured at 700 nm UV spectrophotometer.

2.12.2. Total antioxidant activity by phosphomolybdate reagent

Total antioxidant capacity of the EAE was determined according to the method of Kanner et al. (1994), with slight modifications. The EAE (1 mg/mL) was taken in a test tube and mixed with a mixture of 3 mL of phosphomolybdate reagent (0.6 M H_2SO_4, 4 mM ammonium molybdate and 28 mM sodium phosphate) and incubated at 95°C for 90 min. The absorbance of the solution was measured at 695 nm in a

UV spectrophotometer (Optima, Tokyo). The total antioxidant content of the EAE was calculated as the ascorbic acid equivalent.

2.12.3. Determination of free radical-scavenging activity by DPPH method

The free radical-scavenging activity by DPPH method is carried out by using the procedure as explained by Brand-Williams et al. (1995), with slight modifications. Standard ascorbic acid (0.1 mg/mL) and diluted EAE (0.1 mg/mL) were taken different tubes. The volume of each test tube was made up to 1 mL using methanol and 3 mL of 0.1 M DPPH (Sigma, USA) was added and mixed thoroughly with the help of vertex machine (REMI, India). The reaction mixture was incubated at dark for 30 min and absorbance was measured at 517 nm in UV spectrophotometer (Optima, Tokyo). DPPH activity was calculated by using the following equation,

$$\text{Inhibition}\,(\%) = \left[\frac{(A_{\text{control}} - A_{\text{test or standard}})}{A_{\text{control}}}\right] \times 100$$

2.12.4. Hydroxyl radical-scavenging activity

Hydroxyl radical-scavenging activity was assayed by deoxyribose method (Halliwell et al. 1987), with slight modifications. Hydroxyl radicals were generated by using Fenton reagent (Ascorbate-EDTA-H_2O_2-Fe^{3+} method). In this method, the total reaction mixture containing 2-deoxy-2-ribose (2.6 mM), ferric chloride (20 M), H_2O_2 (500 M), as well as phosphate buffer (100 M, pH 7.4) was mixed with ascorbic acid (100 M) and EAE (100–500 g/mL). The reaction mixture was incubated in 37°C for 1 h to initiate the reaction. After incubation, 0.8 mL of the reaction mixture was added to the 2.8% TCA (0.8 mL), followed by 1% TBA (1 mL) and 0.1% SDS (0.2 mL); the reaction mixture was then heated at 90°C for 20 min to obtain colour, later cooled and 1 mL of double distilled water was added. The absorbance was read at 532 nm and the percentage inhibition was calculated by following equation:

The scavenging activity on hydroxyl radicals was expressed as

$$\text{Hydroxyl radicals inhibition}\,\% = \left(1 - \frac{A}{A_0}\right) \times 100$$

where A_0 is the absorbance of the negative control (without sample) at 532 nm, and A is the absorbance at 532 nm of the reaction mixture containing the sample.

2.12.5. Nitricoxide radical-scavenging assay

Nitric oxide radical inhibition was estimated using Griess Illosvory reaction Patel et al. (2010). The reaction mixture (3 mL) containing 2 mL of 10 mM sodium nitroprusside, 0.5 mL saline phosphate buffer and 0.5 mL of standard solution or EAE extract (100–500 g/ml) was incubated at 25°C for 150 min. After incubation, 0.5 mL of the reaction mixture was mixed with 1 mL sulfanilic acid reagent (0.33% in 20% glacial acetic acid) and allowed to stand for 5 min for the completion of the reaction of diazotisation. After this, a further 1 mL of the naphthylethylenediamine dihydrochloride was added, mixed and was allowed to stand for 30 min at 25°C. The concentration of nitrite was assayed at 546 nm and was calculated with the control absorbance of the standard nitrite solution. Buffer was used as a blank and ascorbic acid was taken as the standard solution. The percentage inhibition was calculated using the formula:

$$\%\,\text{scavenging activity} = \left[\frac{(A_{\text{control}} - A_{\text{test}}\ \text{or}\ A_{\text{std}})}{A_{\text{control}}}\right] \times 100$$

where A_{control} is the absorbance of control and A_{test} or A_{std} is the absorbance of test or standard, respectively.

2.13. Antimicrobial activity

Antimicrobial activity of EAE was tested by disc diffusion method against five pathogenic strains namely, *Escherichia coli* (ATCC-118), *Bacillus subtilis* (ATCC-441), *Staphylococcus aureus* (ATCC-7443), *Staphylococcus epidermidis* (ATCC-435) and *Pseudomonas aeruginosa* (ATCC-424) (Arora & Kaur 1999). The plates containing brain heart infusion medium (Himedia, India) were spread with 0.3 µL of 24 h starter culture inoculum. Discs made of Whatman® qualitative filter paper, grade 1 (6 mm diameter) containing 100, 200 and 400 µg/disc of EAE were placed on the bacterial lawn. The plates were incubated at 37°C for 24 h. The zone of inhibition was measured.

2.14. λ-DNA nicking assay

Oxidative -DNA damage prevention potential of EAE was assayed according to the protocol of Ghanta et al. (2007). -DNA (0.5 µg) with and without EAE (200 µg) was incubated with Fenton

reagent (1 mM $FeSO_4$, 25 mM H_2O_2 in tris buffer 10 mM, pH 7.4) in a final reaction volume of 30 µL for 1 h at 37°C. The relative difference between oxidised and native DNA was analysed on 1% agarose gel prepared in tris-acetate-EDTA buffer (pH 8.5). The gel was run at 50 V for 3 h at room temperature and documented (Uvitec Company, software platinum 1D, UK) and the band intensity was determined.

2.15. HPLC analysis

EAE samples were filtered with 0.45 µm PS membrane filter prior to high-pressure liquid chromatography (HPLC) analysis. HPLC was done according to the Le et al. (2007), with slight modifications. The system was equipped with a photodiode array detector (Agilent-Model 1200 series). The analysis was performed on a grace smart reverse phase C-18 column (250 mm × 4.6 mm; 5 m) at a flow rate of 1 mL/min, using an injection volume of 20 µL and detected at 280 nm. The mobile phases employed were water/methanol/acetic acid (83:15:2). The standard phenolic compounds such as gallic acid, *p*-coumaric acid, cinnamic acid, ferulic acid, gentisic acid, syringic acid and vanillic acid were used for the identification and quantification of phenolic acids present in the EAE.

2.16. Statistical analysis

Each experiment was conducted in triplicate and the data were reported as mean ± SD with the help of graph pad prism 5.0 software. The difference between the control and treated groups is determined by one-way ANOVA test (Bonferroni multiple comparison test).

3. Results

3.1. Isolation and characterisation of endophytic fungus

A polyphenol and flavonoid producing fungus was isolated from the rhizome of *Z. officinale*. Greenish to greyish white colonies of the fungal strain grown up to 3–4 cm diameter on a PDA plate after 7 days of incubation at room temperature. The rear side of the plate was yellowish due to the secretion of pigment by the fungus (Figure 1).

3.1.1. Scanning electron microscopic study of endophytic fungus

Based on the SEM images of mycelium and conidia, fungus was identified as the species of the genus *Aspergillus*. The conidial spores were 0.3–0.4 µm size in showing similarity to conidial spores of *Aspergillus* sp. (Figure 2).

3.1.2. Molecular identification

The identity of the endophytes was established by comparing the 28S rDNA sequence. The phylogenetic tree was constructed with the help of Bootstrap values (1000 replications) based on multiple sequence alignment using the MEGA-6 software. The endophytic fungus was identified as *A. astroafricans* as it shared 99% similarity with the sequence data deposited in GenBank (GenBank Accession No.: KT780617) (Figure 3).

3.2. Phytochemical analysis

The phytochemical screening of EAE revealed the presence of phenolics and flavonoid. However, the extract did not show any positive results for alkaloid, saponins, glycosides, carbohydrates and steroids.

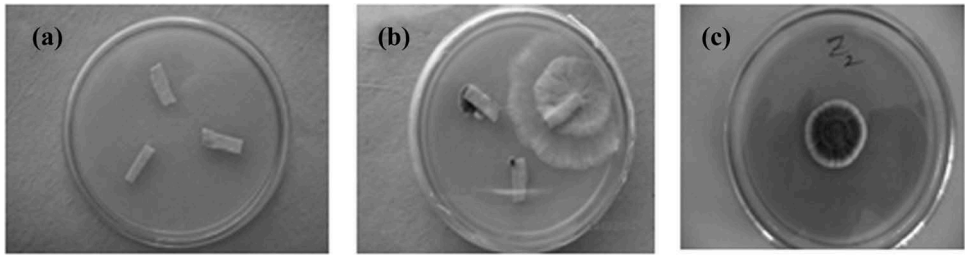

Figure 1. Isolation and pure culture of endophytic fungus from *Z. officinale*. (a) Control plate. (b) Growth of the endophytic fungi on *Z. officinale*.

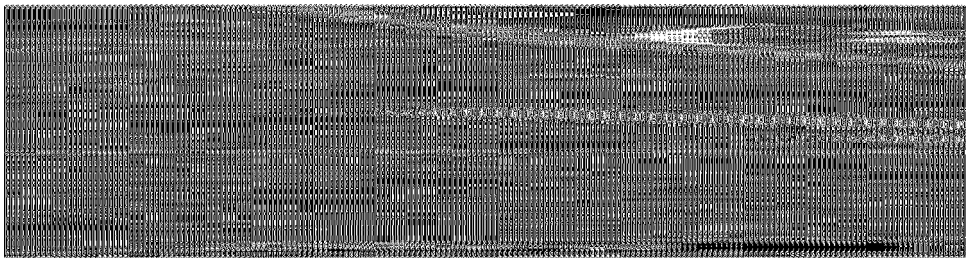

Figure 2. Scanning electron micrographs of *A. austroafricanus* CGJ-B3. (a) conidiospores (scale bar = 20 µm), (b) mycelial hyphae (scale bar = 10 µm) and (c) spore (scale bar = 1 µm).

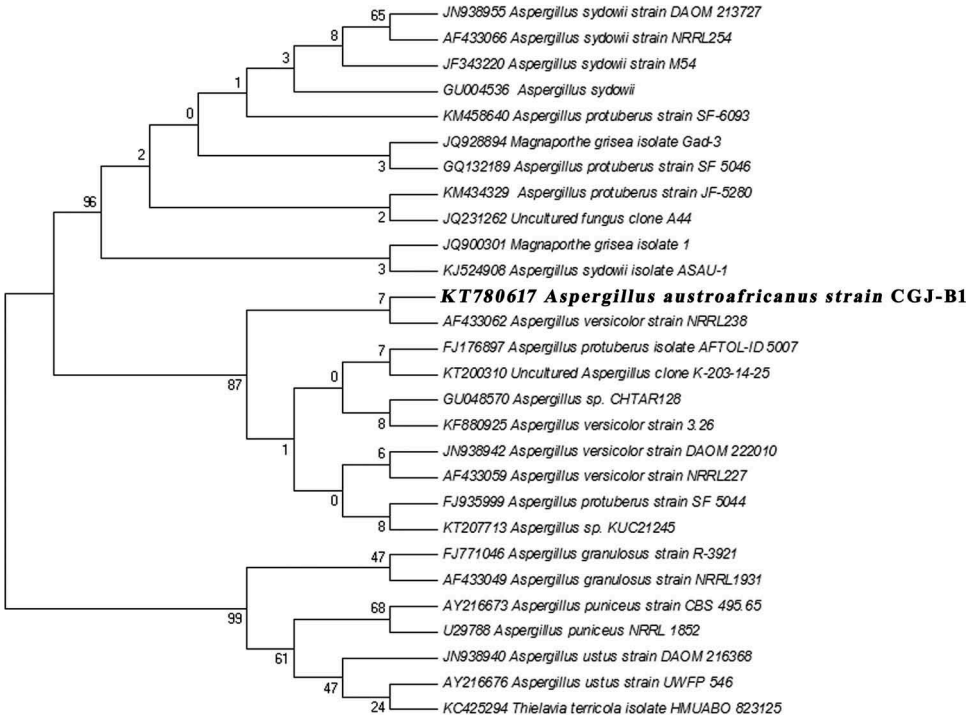

Figure 3. Phylogenetic tree: phylogenetic tree derived from neighbour joining (NJ) analysis showing the evolutionary relationship of *Aspergillus austroafricanus* CGJ-B3 with its closest BLAST hits. Bootstrap values (1000 replications) based on multiple sequence alignment using the MEGA-6 software.

3.3. Antioxidant activity

The EAE was analysed for the total polyphenol, flavonoid content. Polyphenol was estimated using Folin–Ciocalteu's reagent method and the flavonoid was estimated by making it to react with aluminium chloride. Tannic acid and quercetin were used as the standard for total phenol and flavonoid content determination, respectively. A volume of 80 ± 11.50 µg/mg of phenol and 16.0 ± 0.46 µg/mg of flavonoid were present in EAE. The EAE also showed significant reducing power (15 ± 0.81 µg/mg) as well as total antioxidant activity (82 ± 0.78 µg/mg) (Figure 4).

EAE was also studied for its free radical-scavenging activity by three methods namely, DPPH, H_2O_2 and nitric oxide radical-scavenging activity. Ascorbic acid was used as the standard in DPPH and nitric oxide-scavenging activity while mannitol was used as a standard in H_2O_2 radical-scavenging study. EAE showed varying degree of antioxidant activity in the three methods used for the study. DPPH radical-scavenging activity of the EAE was comparable to the standard ascorbic acid. The radical-scavenging activity of the EAE was concentration dependent (Figure 5).

3.4. Antimicrobial activity

The EAE was also tested for antimicrobial activities against a panel of four bacterial and a fungal

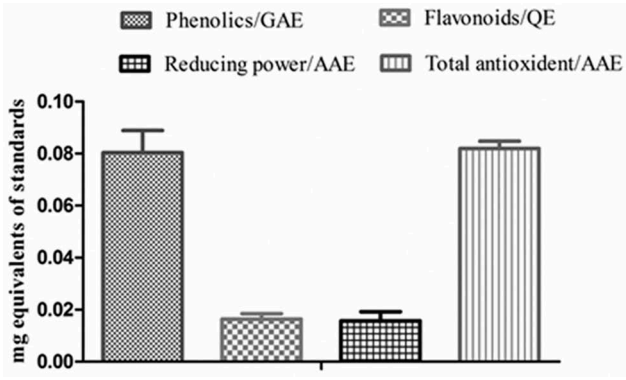

Figure 4. Total phenolic content, flavonoids, reducing power and total antioxidant of the EAE.

pathogen by disc diffusion method. Results of the antimicrobial activity were shown in Figure 6. The antimicrobial potential of EAE was found to be in the range of moderate to a significant level. The antimicrobial activity of the fungal extract was found to be dose dependent. The zone inhibition was ranging from 7 to 24 mm.

3.5. Protection of λ-DNA from the free radicals

Incubation of Fenton radicals with -DNA for 1 h resulted in the total disappearance of DNA bands on 1% agarose gel compared to DNA control and DMSO vehicle (Lane 2). However, the addition of 200 µg of EAE to the mixture of -DNA and Fenton reagent prevented the radical induced DNA damages (Lane 3) as shown in Figure 7.

3.6. HPLC analysis

The polyphenols in EAE were identified by using reverse phase C-18 column in HPLC. Different peaks with respect to retention time confirmed the presence of polyphenols in EAE (Figure 8). The EAE showed the presence of *p*-coumaric acid (0.392 ± 0.08 µg/mg), ferulic acid (4.35 ± 0.16 µg/mg) and cinnamic acid (1.976 ± 0.11 µg/mg). The fungal extract was quantitated for the presence of phytochemicals and the quantity of individual chemical.

4. Discussion

Endophytes present in the medicinal plants share the therapeutic properties of the host plants (Zhao et al. 2010). These characters of the endophytes make them the best source of medicinal compounds instead of plants as they have several advantages over plants (such as the purification and mass cultivation). *Z. officinale* is a medicinal plant and its medicinal properties are exploited since ages. This plant

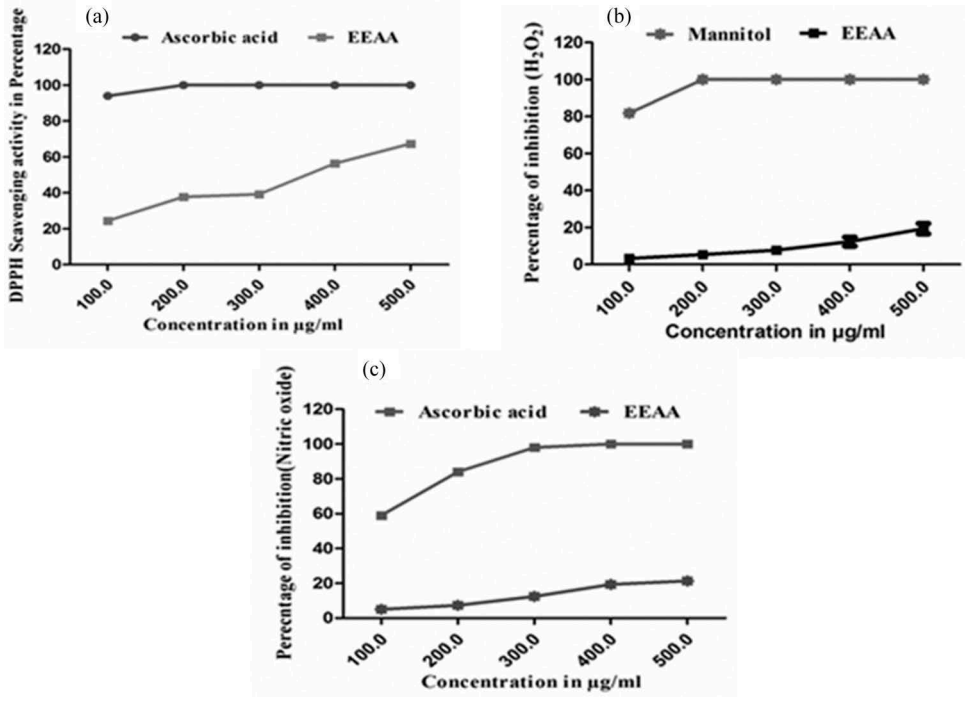

Figure 5. Antioxidant activity of EAE. (a) DPPH, (b) H_2O_2 and (C) nitric oxide-scavenging methods.

Figure 6. Antimicrobial activity of EAE.

Figure 7. DNA protection assay of EAE. Line 1: λ DNA (0.5 µg), line 2: λ-DNA (0.5 µg) + Fenton's reagent, line 3: λ-DNA (0.5 µg) + EAE (200 µg) + Fenton's reagent.

Figure 8. Separation of phenolic compounds of EAE with HPLC system. (1) *p*-Coumaric acid, (2) ferulic acid and (3) cinnamic acid.

has been shown to be the source of therapeutic molecule against various ailments (Chrubasik et al. 2005; Nanjundaiah et al. 2011a).

The phytochemical analysis of EAE showed the presence of polyphenol and flavonoids as the major constituents. Phenols and flavonoids are well-documented phytochemicals responsible for the antioxidant potential of the natural products (Ghasemzadeh et al. 2010; Nanjundaiah et al. 2011a). Different groups of researchers have reported contradictory results about the correlation between the total phenolic content and antioxidant potential of the extract. Yadav et al. (2014) have described about the positive correlation between the antioxidant capacities of the extract to the phenolic constituents. Even our study showed a good relation between the phenolic content and antioxidant capacity. The hydroxyl group present in the phenols may be involved in radical-scavenging. This is in agreement with the findings of Yadav et al. (2014) and Huang et al. (2005).

Even though the antioxidant activity was measured with various protocols, DPPH method is extensively used to assess the radical generation inhibition potential of the molecules. The accuracy and repeatability of DPPH method makes it a method of choice for most of the researchers (Brand-Williams et al. 1995). This method primarily works on the power of the molecule to scavenge proton radical. The characteristic absorbance of DPPH decreases with the increase in proton radical scavengers (Singh & Rajini 2004). In our study, EAE showed a dose-dependent DPPH radical-scavenging activity. This trend may be due to the hydrogen donating capability of the compounds present in EAE. It is well established that the compounds scavenge DPPH radical by donating hydrogen (Chen & Ho 1995).

Many researchers have reported the relation between the antioxidant activity and reducing power. The reducing power of the extract is due to the presence of reductons which have the capacity to stop the free radicals by donating hydrogen as well as by preventing peroxide formation (Singh & Rajini 2004). Our data on reducing power may be attributed to the hydrogen donation capacity as the extract is rich in phenols and flavonoids.

Even though H_2O_2 presents in small quantities in the human body, it will rapidly decompose to produce hydrogen radicals. These radicals have deleterious effects on biomolecules such as lipids and DNA (Gülçin et al. 2005). The EAE showed hydrogen peroxide-scavenging activity. Our results are in agreement with the studies of Saeed et al. (2012). The

phenolic groups present in the extract could neutralise the H_2O_2 by donating the electron. EAE also showed the nitric oxide radical-scavenging potential. Nitric oxide radicals have the capability to alter the structure as well as the function of biomolecules. The reaction of nitric oxide (NO) with superoxide will result in a more toxic peroxy nitrite amines which undergo decomposition to OH^- and NO_2 (Pacher et al. 2007; Awah 2010).

Secondary metabolites derived from fungi were used as a model structure to develop several antimicrobial agents. In the present study, the EAE showed moderate antimicrobial activity against the pathogens. According to Hussain et al. (2014), lower antimicrobial activity against the microorganisms may be due to the presence of active compounds in lesser amounts (Hussain et al. 2014). Our study is in agreement with the observation of Hussain et al. (2014).

Photolysis of H_2O_2 release hydrogen radicals which intern damage the DNA. The radicals generated may break single or double strand (Patel et al. 2010). Chemicals that prevent the generation of free radical can protect the DNA damage. Antioxidant secondary metabolites of endophytic fungus are the key mediators of protection of DNA damage by free radicals. Similar results have been reported by Ma et al. (2004) and Ruma et al. (2013).

The HPLC analysis revealed the presence of various phytochemicals such as *p*-coumaric acid, ferulic acid, cinnamic acid etc. These phytochemicals are known to be antioxidant molecules and are used in many therapeutic studies. Interestingly Siddaraju and Dharmesh (2007) have reported the chemical contents of *Z. officinale* and it biological properties. Our study also reports a similar result highlighting the association of microorganisms with medicinal plants and medicinal properties.

5. Conclusion

We have successfully isolated an endophytic fungus *A. austroafricanus* CGJ-B3 from the inner tissues of *Z. officinale* rhizome. The preliminary study on this fungus showed its capacity to produce polyphenol and flavonoids which are rare in the fungus *Aspergillus* sp. This is the first report on the isolation of polyphenol and flavonoid producing *Aspergillus* sp. from the rhizomes of *Z. officinale*. The fungal extract showed antioxidant, antimicrobial and DNA damage protection potential. The medicinal properties of this endophytic fungus may be attributed to the different phenolic compounds such as *p*-coumaric acid, ferulic acid, cinnamic acid etc. Our study showed the possibility of *A. austroafricanus* CGJ-B3 in the development of a potent drug against free radical-related disorders such as aging, arthritis, diabetes, cancer etc. Further studies on this organism at the molecular level will aid in optimising the production of bioactive compounds in a larger scale.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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ORIGINAL ARTICLE

Antioxidant and Cytotoxic Potential of Endophytic Fungi Isolated from Medicinal Plant *Tragia involucrata* L.

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ABSTRACT

Objective: The aim of the present study was to test the antioxidant and cytotoxic and DNA protection activities of ethyl acetate extracts of endophytic fungi isolated from *Tragia involucrata* Linn. (Euphorbiaceae). **Materials and Methods:** The 1, 1-diphenyl-2-picrylhydrazyl scavenging, reducing power, and total antioxidant assay were used to evaluate the antioxidant activity. Cytotoxic activity of endophytic fungal extracts against MCF-7 and MOLT-4 cell lines was carried out using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide method. **Results:** We have successfully isolated four endophytic fungi from *T. involucrata,* namely *Penicillium citrinum* CGJ-C1 (GenBank accession No. KT780618), *P. citrinum* CGJ-C2 (GenBank accession No. KP739821), *Cladosporium* sp. CGJ-D1 (GenBank accession No. KP739822), and *Cryptendoxyla hypophloia* CGJ-D2 (GenBank accession No. KT780619). The ethyl acetate extract of *P. citrinum* CGJ-C2 showed the highest antioxidant as well as cytotoxic activity among the four fungal extracts taken for the study. All the extracts showed moderate DNA protection activity. **Conclusions**: Further studies on the isolation and purification of the lead molecule will help in designing the novel therapy for different ailments associated with free radical generation.

Key words: Antioxidants, cytotoxicity, endophytes, *Tragia involucrata*

SUMMARY

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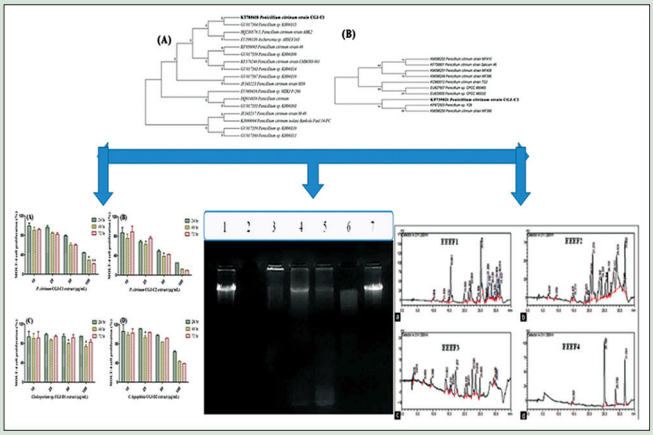

Abbreviations used: ???.

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INTRODUCTION

Endophytic fungi are microscopic organisms that grow within plant tissue without causing immediate symptoms of disease.[1] Natural products from fungal endophytes have vast applications in medicine as they exhibit diverse therapeutic properties.[2,3] Many antitumor agents such as taxol and beauvericin are extracted from the endophytic fungi such as *Pestalotiopsis microspore* and *Fusarium oxysporum* on *Taxus wallachiana* and *Ephedra fasciculate* plants, respectively.[4,5] Even though the endophytes are studied for various applications, still many are unexplored with respect to their therapeutic potential. According to Strobel and Daisy, each plant identified on earth hosts an endophyte.[6] Endophytes acquire the medicinal properties of the host plant in which they exist.[7] Hence, they are the reservoirs for various novel molecules with therapeutic efficacy.

Tragia involucrata belongs to Euphorbiaceae family and widely distributed in Asian subcontinent. This plant has been reported to contain several medicinal properties such as cytotoxic, antitumor, anti-fertility,[8-10] antimicrobial,[11] antidiabetic,[12] wound healing,[11] and anti-inflammatory activities.[13] Even though *T. involucrata* has been extensively studied for its medicinal activity, there are no reports on the therapeutic potential of endophytes of *T. involucrata*. To the best of our knowledge, no one has isolated and studied the medicinal properties of endophytes of *T. involucrata.* Hence, the aim of the present study was to isolate endophytic fungi from *T. involucrata* and to study their antioxidant, cytotoxic, and DNA protection potential.

MATERIALS AND METHODS

Collection of plant material

Healthy stems of *T. involucrata* were collected in the month of May 2013 in and around Sullia (T), Dakshina Kannada (D), Karnataka, India. The Global Positioning System locations were 12.5581° N and 75.3892° E. The plant was shifted to laboratory using sterile polyethylene bags at

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8°C. The plant was identified by Dr. Jagath Timmaiah, Department of Botany, Karnataka, India. The plant was authenticated by CSIR-National Institute of Science Communication and Information Resources, New Delhi. Herbarium was peevishly deposited by one of the authors to Gandhi Krishi Vignana Kendra, Bengaluru, which has been taken as a reference (Herbarium No. 3687).

Isolation and identification of endophytic fungi from *Tragia involucrata*

T. involucrata was washed with running tap water to remove excess debris followed by washing with distilled water. The samples were cut into small pieces (0.6–0.8 cm) and rinsed with 70% alcohol. The alcohol-washed pieces were again rinsed with 0.5% sodium hypochlorite followed by washing thrice with sterile distilled water. The sterilized samples were transferred aseptically to potato dextrose agar media containing chloramphenicol (150 mg/L) and incubated at (28.00°C ± 1°C) for 7 days.[14]

The genomic DNA was isolated and purified using fungal genomic DNA mini kit using the manufacturer's protocol (xcelgenCat No: XG2416-01 from Xceleries genomics Pvt. Ltd, India), and molecular identification was achieved by internal transcribed spacer (ITS) region and 18S rDNA analysis. The sequences were used as query sequences to search for similar sequences from GenBank using the basic local alignment search tool program. The most similar reference sequences with query sequences were obtained and used for subsequent phylogenetic analyses [Figure 1]. These sequences were aligned using the CLUSTALX program.[15]

Preparation of ethyl acetate extracts of endophytic fungi

Extraction of secondary metabolites was carried according to the procedure explained by Higginbotham *et al.* with slight modification. Each liquid culture with evident growth (incubation for 18 days in static condition at 25°C–28°C) on potato dextrose broth was mixed with an equal volume of ethyl acetate (100%). The mixture was blended using pestle and mortar. The resulting homogenate was filtered with Whatman® No. 1 filter paper and extracted twice with an equal volume of ethyl acetate. The aqueous and organic layers were dried and stored at −20°C until use.[14]

Total phenolic content

The total phenolic content of the fungal extract was estimated using Folin–Ciocalteu (FC) reagent. Different aliquots (0–1 mL) of gallic acid (Sigma-Aldrich, USA) and extracts were taken in tubes and the volume was made up to 1 mL with distilled water. One mL of (1:1 diluted) FC reagent and 1.5 mL of 20% sodium carbonate were added to each tube. After the addition of 6 mL of distilled water, the reaction mixture was incubated at room temperature for 30 min. The absorbance of the sample and tests was measured at 760 nm against blank in ultraviolet-visible (UV-Vis) spectrophotometer (Optima Tokyo, Japan). The total phenolics in fungal extract was calculated using standard curve and expressed as gallic acid equivalents.[16]

Total flavonoid content

Total flavonoid content of the fungal extract was estimated by the protocol published by Zhishen *et al.*[17] Different aliquots of endophytic extracts and quercetin (100 µg/ml) were taken in different tubes, and the volume in each tube was made up to 1 mL with distilled water. 1.5 mL of ethanol, 0.1 mL of 10% aluminum chloride, and 0.1 mL of 1 M potassium acetate and 2.8 mL of distilled water were added to each tube and the absorbance of the reaction mixture was measured at 450 nm in UV-Vis spectrophotometer (Optima Tokyo, Japan).

Total antioxidant assay

The total antioxidant assay was carried out using molybdenum reagent. Phosphomolybdate (3 mL) was added into tubes containing either ascorbic acid or extract and incubated at 95°C in boiling water bath for 90 min after shaking vigorously for 15 min at room temperature. The optical density was measured at 695 nm using UV-Vis spectrophotometer (Optima Tokyo, Japan). Total antioxidant potential of endophytic fungal extracts was expressed as ascorbic acid equivalents.[18]

1, 1-diphenyl-2-picrylhydrazyl radical scavenging activity

The free radical scavenging activity of the fungal extracts was studied by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) method based on the procedure of Brand-Williams *et al.*[19] with modifications. Standard ascorbic acid and extracts (100 µg/mL) were taken in different aliquots (0–1 mL) and volume was made up to 1 mL using methanol. Three mL of 0.1 M DPPH (Sigma, USA) was added and tubes were incubated in dark for 30 min. The absorbance was measured at 517 nm using UV-Vis spectrophotometer (Optima Tokyo, Japan). DPPH scavenging activity was calculated by using the following equation,

$$\text{DPPH scavenging (\%)} = (A_{Control}\text{-}A_{Extract}/A_{Control}) \times 100$$

where A denotes absorbance.

Cytotoxic activity

Assessment of cytotoxicity by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide assay

Cytotoxic effect of the endophytic fungal extracts was determined by the procedure explained by Kuriakose *et al.*[20] Cells were grown on 96-well flat-bottomed microtiter plates at a final volume of 100 µL culture medium per well and incubated at 37°C and 5% CO_2 for 24 h, 48 h, and 72 h. Supernatant was removed after incubation and the cells were washed. Fungal extracts (25–200 µg/mL for MCF-7 and 10–100 µg/mL for MOLT-4) were added into the respective labeled wells. After 24 h, 48 h, and 72 h incubation, 10 µL (5 mg/mL) 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) solution in phosphate-buffered saline was added to each well and incubated for 2 h in dark. Thereafter, medium was removed and 100 µL of dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan crystals. Plates were read at 570 nm using a microplate reader (Bio-Rad, USA) and results were expressed as viability percentage against the untreated control cells (100% of viability).

DNA protection ability

Oxidative λ-DNA damage was prevented by endophytic fungal extract and it was assayed.[21] λ-DNA (0.5 µg) with and without endophytic fungal extract (200 µg) was incubated with Fenton's reagent (1 mM FeSO4, 25 mM H_2O_2 in Tris buffer 10 mM, pH 7.4) at a final reaction volume of 30 µL for 1 h at 37°C. Relative difference between oxidized and native DNA was analyzed on 1% agarose gel prepared in tris-acetate-ethylenediaminetetraacetic acid buffer (pH 8.5) at 50 V for 3 h at room temperature. The gel was documented (Uvitec Company, software platinum 1D, UK) and the band intensity was determined.

High-pressure liquid chromatography analysis of endophytic fungal extracts

Extract samples were filtered with 0.45 µ membrane filter prior to high-pressure liquid chromatography (HPLC) analysis. The system was equipped with an auto-injector and photodiode array detector (Waters Milford USA). Analysis was performed on a Grace Smart reverse-phase chromatography – 18 column (5 µm, 250 mm × 4.6 mm), at a flow rate of

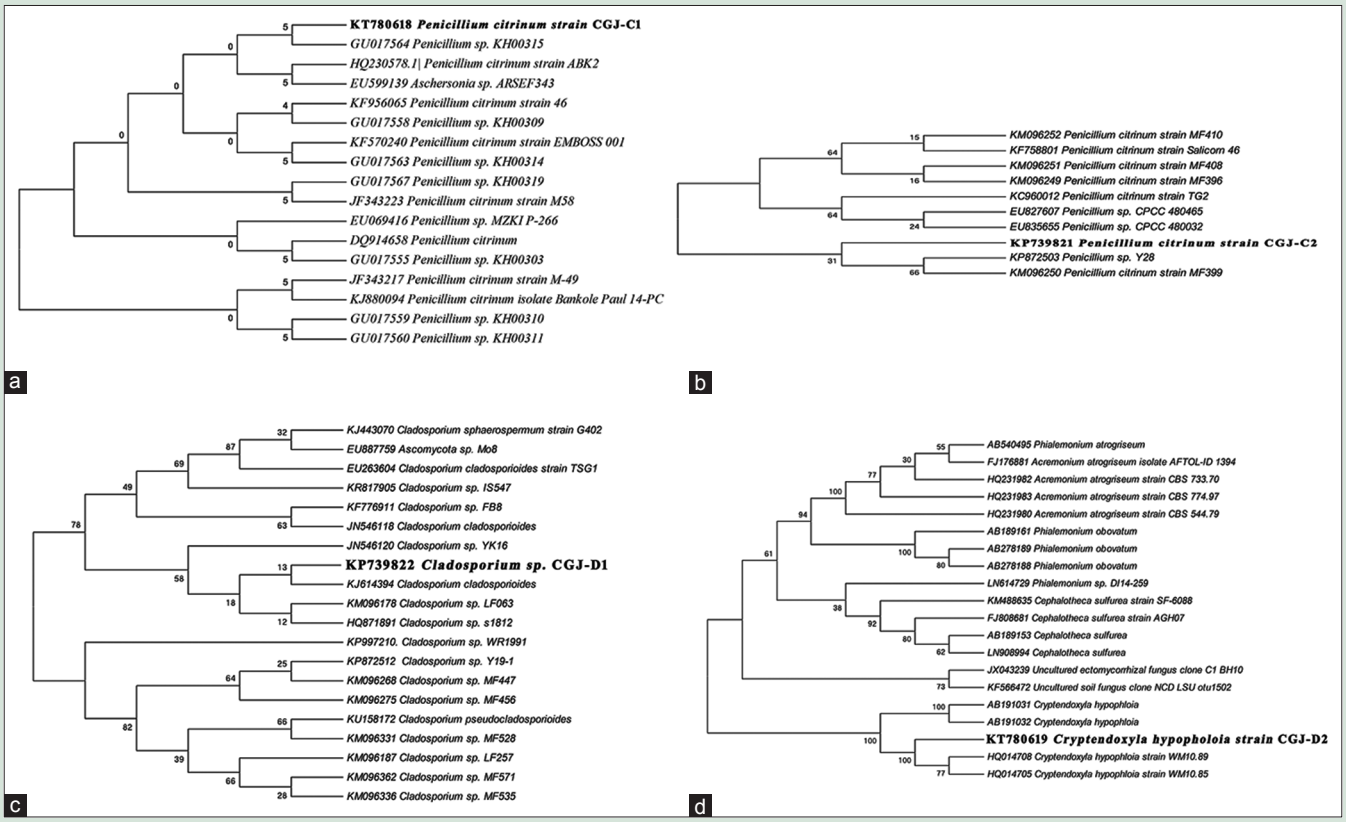

Figure 1: Neighbor-joining phylogenetic tree analysis of fungal endophyte sp. (a) *Penicillium citrinum* CGJ-C1, (b) *Penicillium citrinum* CGJ-C2, (c) *Cladosporium* sp. CGJ-D1, and (d) *Cryptendoxyla hypophloia* CGJ-D2. Confidence values above 50% obtained from a 1000-replicate bootstrap analysis are shown at the branch nodes. Bootstrap values from neighbor-joining method were determined

1 mL/min, using an injection volume of 20 µL, detection at 280 nm. The mobile phases employed were A (methanol 100%) and B (0.5% acetic acid). The gradient program followed was 0–28.6 min, 90%–40% B and 28.6–30 min, 40%–90% B, equilibrated till 35 min.

Statistical analysis

All the experiments were performed in triplicates and the data were compared with one-way analysis of variance using Graphpad prism software version 5.0 (GraphPad Software 7825 Fay Avenue, Suite 230 La Jolla, CA 92037 USA). A significant difference ($P < 0.05$) among the equation was evaluated by Turkey's test.

RESULTS

Isolation and identification of endophytic fungi from *Tragia involucrata*

Four endophytic fungi were isolated from *T. involucrata*. The identity of the endophytes was established by comparing the 18S rDNA sequence. These endophytic fungi were identified as *Penicillium citrinum* CGJ-C1 (GenBank accession number KT780618), *P. citrinum* CGJ-C2 (GenBank accession number KP739821), *Cladosporium* sp. CGJ-D1 (GenBank accession number KP739822), and *Cryptendoxyla hypophloia* CGJ-D2 (GenBank accession number KT780619) as shown in Figure 1.

Determination of total phenolics

Total phenolic content in the fungal extracts was measured by Folin–Ciocalteu's method. The total polyphenol and flavonoid content of each extract is shown in Figure 2a and b. *P. citrinum* CGJ-C2 extracts showed the highest amount of phenolic content (112.00 ± 4.12 mg/g GAE) followed by *C. hypophloia* CGJ-D2, *P. citrinum* CGJ-C1, and *Cladosporium* sp. CGJ-D1 extracts [Figure 3a].

Flavonoid content

The total flavonoid content of four endophytic extracts was depicted in Figure 3b. Flavonoid content varied in different extracts. Among the four extracts analyzed, the highest amount of flavonoids was found in *C. hypophloia* CGJ-D2 extract (40.00 ± 1.14 mg/g quercetin equivalent/g) compared to the other three extracts.

Total antioxidant activity

The total antioxidant activity of the endophytic extracts was measured spectrophotometrically by phospho-molybdenum method. The principle involved in this method is the reduction of molybdenum (Mo) (IV) to Mo (V) by the extracts, and the final green-colored Mo (V) end product is measured as absorption at 695 nm.[22] In our study, significant antioxidant activity was observed in *C. hypophloia* CGJ-D2 (260.00 ± 1.45 mg/g) extract. The three other extracts also showed considerable total antioxidant activity [Figure 3c].

1, 1-diphenyl-2-picrylhydrazyl radical scavenging activity

The free radical scavenging ability of ethyl acetate extracts of endophytic fungi was evaluated using DPPH substrate. DPPH is a stable free radical with characteristic absorption at 517 nm, and antioxidants react with DPPH and convert it to 2,2-diphenyl-1-picrylhydrazine.

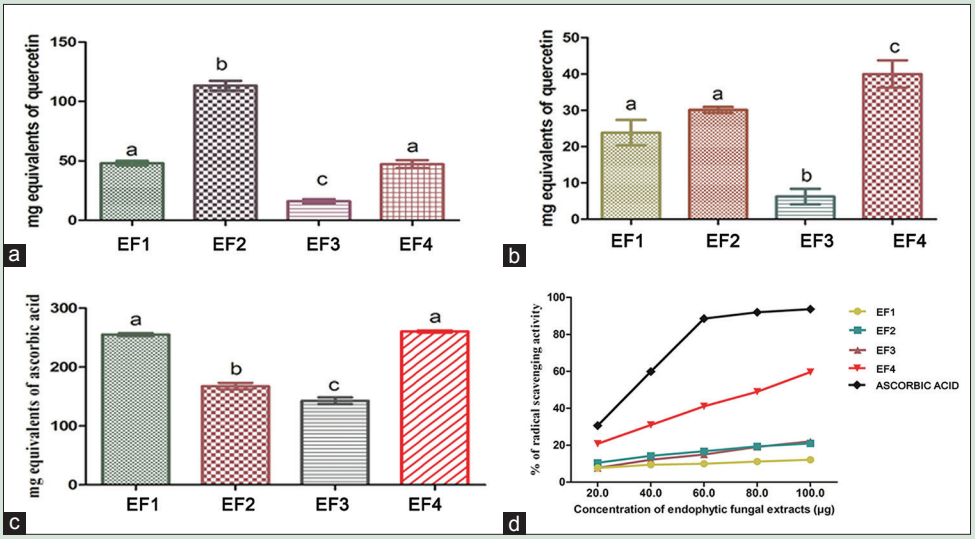

Figure 2: Cytotoxic activity of fungal extracts on MCF-7 cell line. (a) *Penicillium citrinum* CGJ-C1, (b) *Penicillium citrinum* CGJ-C2, (c) *Cladosporium* sp. CGJ-D1, and (d) *Cryptendoxyla hypophloia* CGJ-D2 in 48 h and 72 h. (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$)

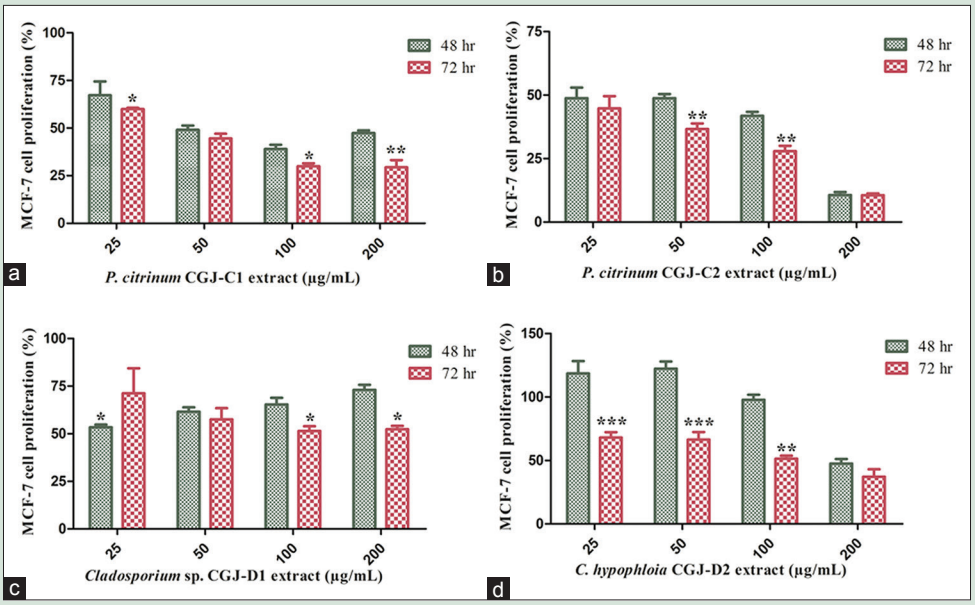

Figure 3: (a) Total phenolic content, (b) Total flavonoid content, (c) Total antioxidant of endophytic fungal extract, (d) 1, 1-diphenyl-2-picrylhydrazyl radical scavenging activity of the fungal extracts. All values are reported as means ± standard deviation ($n = 3$). * EF1 (*Penicillium citrinum* CGJ-C1 extract), EF2 (*Penicillium citrinum* CGJ-C2), EF3 (*Cladosporium* sp. CGJ-D1), and EF4 (*Cryptendoxyla hypophloia* CGJ-D2). All the experiments were performed in triplicates and the data were compared with one way analysis of variance using GraphPad prism software version 5.0. Different alphabets above the bars have mean values that are significantly different ($P < 0.05$).

DPPH-scavenging activities of all the extracts were comparable to standard ascorbic acid. *C. hypophloia* CGJ-D2 extract showed 50% radical scavenging activity at 4.01 µg/mL [Figure 3d and Supplementary Table 1].

Cytotoxic activity

Cytotoxic effect of the endophytic extract on MCF-7 and MOLT-4 cell line was assessed using MTT assay. The cytotoxic activity of the endophytic extracts is depicted in Figures 2 and 4. *P. citrinum* CGJ-C2 extract exhibited a significant cytotoxic effect on MCF-7 cell line in 48 h and 72 h compared to other extracts. The cytotoxic activities of all the four extracts were found to be dose dependent. In our study, *P. citrinum* CGJ-C2 extract showed significant cytotoxicity against MCF-7 as well as MOLT-4 cell lines. The IC_{50} value of the *P. citrinum* CGJ-C1, *P. citrinum* CGJ-C2, *Cladosporium* sp. CGJ-D1, and *C. hypophloia* CGJ-D2 on MCF-7 cells was 1.50, 1.09, 3.87, and 2.90 µg/mL, respectively, at 72 h of incubation after the treatment of extract. The IC_{50} value of the *P. citrinum* CGJ-C1, *P. citrinum* CGJ-C2, *Cladosporium* sp. CGJ-D1, and *C. hypophloia* CGJ-D2 on MOLT-4 cells was 2.97, 2.38, 8866, and 5.41 µg/mL, respectively [Supplementary Table 2].

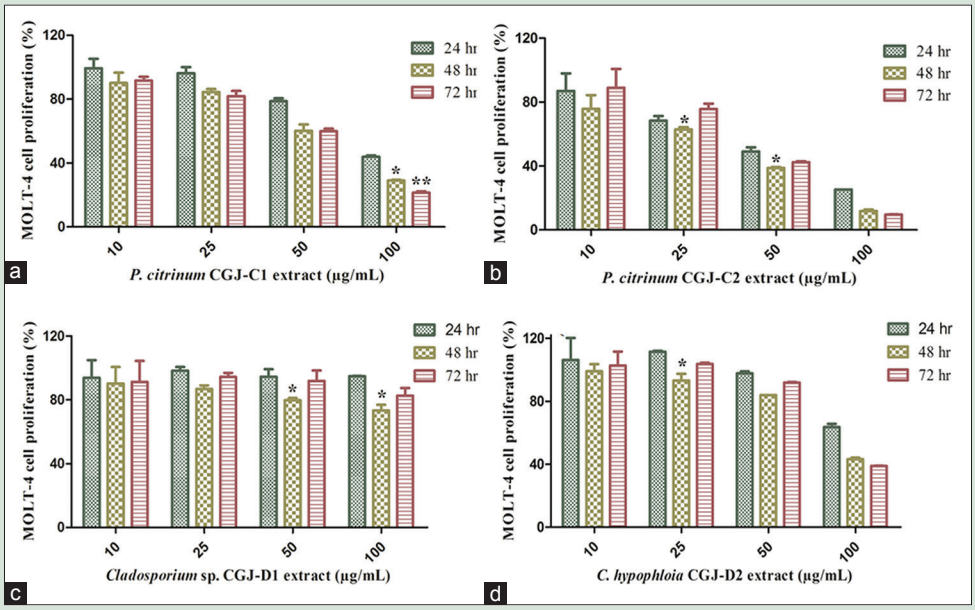

Figure 4: Cytotoxic activity of fungal extracts on MOLT-4 cell line. (a) *Penicillium citrinum* CGJ-C1, (b) *Penicillium citrinum* CGJ-C2, (c) *Cladosporium* sp. CGJ-D1, and (d) *Cryptendoxyla hypophloia* CGJ-D2 in 24 h, 48 h, and 72 h.(*$P \leq 0.05$; **$P \leq 0.01$)

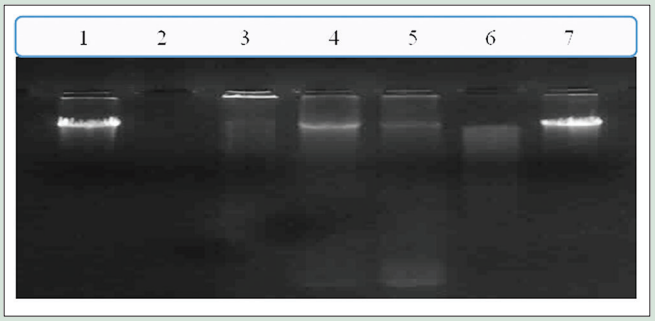

Figure 5: DNA protection by four isolated endophytic fungal extracts. Lane 1: λ DNA (0.5 µg), Lane 2: λ DNA (0.5 µg) + Fenton's reagent (10 µl), Lane 3: λ DNA (0.5 µg) + EF1 (200 µg) + Fenton's reagent (10 µl), Lane 4: λ DNA (0.5 µg) + EF2 (200 µg) + Fenton's reagent (10 µl), Lane 5: λ DNA (0.5 µg) + EF3 (200 µg) + Fenton's reagent (10 µl), Lane 6: λ DNA (0.5 µg) + EF4 (200 µg) + Fenton's reagent (10 µl), Lane 7: λ DNA (0.5 µg) + dimethyl sulfoxide. Fenton's reagent (30 mM H_2O_2, 50 mM ascorbic acid, and 80 mM $FeCl_3$). * EF1 (*Penicillium citrinum* CGJ-C1 extract), EF2 (*Penicillium citrinum* CGJ-C2), EF3 (*Cladosporium* sp. CGJ-D1), EF4 (*Cryptendoxyla hypophloia* CGJ-D2)

DNA protection assay

Incubation of radicals with λ-DNA for 1 h resulted in the total disappearance of DNA bands on 1% agarose gel compared to DNA control and DMSO vehicle (Lanes 1, 2, and 7). However, the addition of 200 µg of endophytic extracts to the mixture of λ-DNA and Fenton's reagent prevented the radical-induced DNA damages (Lanes 3–6) as shown in Figure 5.

High-pressure liquid chromatography analysis

The polyphenols present in the fungal extracts were identified by using reverse-phase C-18 column in HPLC. Different peaks with respect to retention time confirmed the presence of polyphenols in endophytic fungal extracts [Figure 6]. *P. citrinum* CGJ-C1 showed the presence of vanillin, caffeic acid, ferulic acid, quercetin, and coumaric acid. Each extract was quantitated for the presence of phytochemicals, and the quantity of individual chemical was varying with the species. Caffeic acid was found to be a major constituent in *P. citrinum* CGJ-C1 (15.22 ± 0.12 µg/mg), while quercetin (6.904 ± 0.44 µg/mg) and vanillin (1.70 ± 0.84 µg/mg) were present in abundant quantity in *P. citrinum* CGJ-C2 and *Cladosporium* sp. CGJ-D1 extracts, respectively, when compared to other extracts. The chemicals present in the *C. hypophloia* CGJ-D2 did not match any standards that were used for this study [Table 1].

DISCUSSION

The endophytes present in the plant produce similar secondary metabolites as that of the host plant. These secondary metabolites even exhibit the medicinal properties of the plant in which they exist. Endophytes isolated from medicinal plants have many advantages over the plant as they can be easily grown and harvested in laboratories. Even the process involved in the isolation of secondary metabolite is less complicated than medicinal plants.[7] This information prompted us to isolate the endophytes from *T. involucrate* and to test the antioxidant and cytotoxic activities as well as DNA protection potential

We have successfully isolated identified four endophytic fungi. Out of the four endophytic fungi, two were different strains of the same species *P. citrinum* CGJ-C1 (KT780618) and *P. citrinum* CGJ-C2 (KP739821). Organisms with 99% or more sequence similarity to be considered from the same species. If the sequence similarity between the organisms ranges from 93% and 98% ; the organisms to be considered from the same genus. However, the organism is considered to be previously unidentified if the ITS Sequence similarity is below 93%.[23] The endophyte had 99% similarity to *P. citrinum* CGJ-C1, *P. citrinum* CGJ-C2, *Cladosporium* sp. CGJ-D1, and *C. hypophloia* CGJ-D2.

According to Cui *et al.* (2011), medicinal properties of the endophytic fungi vary even though they belong to the same genus and isolated from the single host species.[24] Our study supported the observation of Cui *et al.* (2011) regarding the different bioactivity in different strains of the same species of endophytes (*P. citrinum* CGJ-C1 [KT780618] and

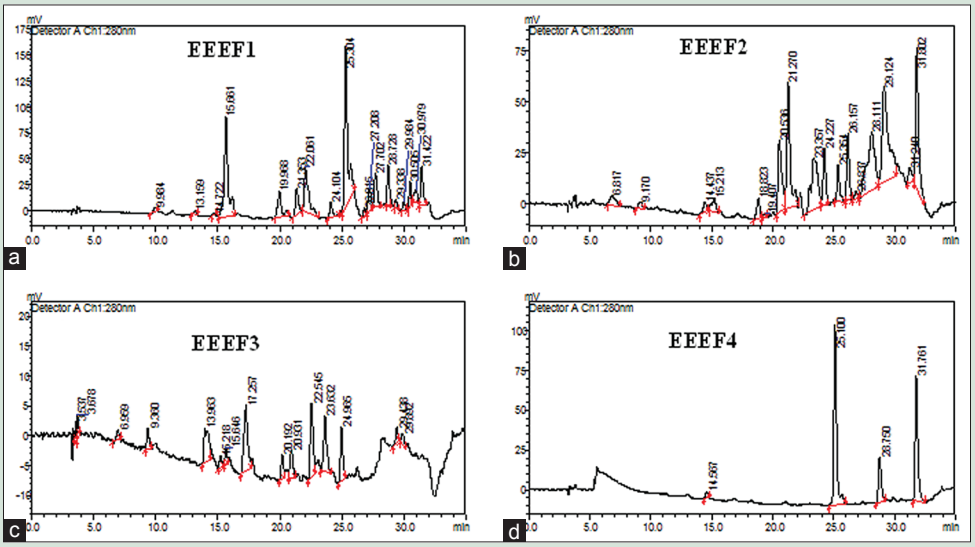

Figure 6: High-pressure liquid chromatography chromatograms of ethyl acetate extract of (a) *Penicillium citrinum* CGJ-C1, (b) *Penicillium citrinum* CGJ-C2, (c) *Cladosporium* sp. CGJ-D1, (d) *Cryptendoxyla hypophloia* CGJ-D2, detected at 280 nm

Table 1: Polyphenolic profiles of endophytic fungal extracts

Polyphenols	Amount of polyphenol (µg/mg)			
	***P. citrinum* CGJ-C1**	***P. citrinum* CGJ-C2**	***Cladosporium* spp CGJ-D1**	***C. hypophloia* CGJ-D2**
Vanillin	0.365±0.08	-	1.70±0.84	-
Caffeic acid	15.22±0.12	2.255±0.095	0.192±0.14	-
Ferulic acid	2.644±0.64	0.148±0.025	0.475±0.18	-
Quercetin	1.239±0.24	6.904±0.44	-	-
Coumaric acid	-	0.554±0.21	-	-

Different extracts were subjected to HPLC analysis along with phenolic acid standard and the peak area, concentrations of phenolic acids were calculated and expressed as mg/g. Values provided are mean of three replicates±SD. HPLC: High-pressure liquid chromatography; SD: Standard deviation; *P. citrinum*: *Penicillium citrinum*; *C. hypophloia*: *Cryptendoxyla hypophloia*

P. citrinum CGJ-C2 [KP739821] showed different bioactivity).[24]

Reactive oxygen species (ROS) such as O_2, H_2O_2, and OH− are generated as a result of metabolic process in living organisms. These ROS are involved in various pathophysiologies such as aging, cancer, and neurodegenerative processes. However, they are essential for certain vital functions in the body such as stress-response signaling and programmed cell death. ROS and antioxidants are the key mediators of symbiotic interactions between the host and microorganisms as they are ubiquitous and evolutionarily conserved.[25,26] The microorganism may produce the antioxidant molecules to protect the host plant from different harmful ROS.[27,28]

DPPH free radical scavenging activity is considered to be one of the basic, accurate, and widely used methods to find out the antioxidant potential of various natural products.[19] Electron donation capacity and free radical scavenging potential of a substance determine its reducing power. These properties of antioxidant compounds help them to prevent lipid peroxidation of the membrane as a consequence of a series of reactions of free radicals.[29,30] Some of the previous studies have reported about the linear relationship between total phenolic content and antioxidant activity.[31] However, some researchers have shown that there is no correlation between the phenolic content and antioxidant activity.[32,33] Our study supports the view of a nonlinear relationship between the phenolic content and antioxidant activity. This trend may be attributed to the presence of other phytochemicals along with the phenolics that could act as radical scavengers. Similar results were reported by Wang *et al.*[32] and Devi *et al.*[33]

Assessment of cytotoxicity of natural products is the first step in the development of anticancer compounds.[33] In our study, *P. citrinum* CGJ-C2 showed significant cytotoxicity against MCF-7 as well as MOLT-4 cell lines. All the endophytic extracts except *Cladosporium* sp. CGJ-D1 showed IC_{50} value much lesser than the National Cancer Institute (NCI) guideline values against *P. citrinum* CGJ-C2 in MCF-7 cell lines [Table 2]. According to the NCI guidelines, any crude extract showing IC_{50} value below 30 µg/mL can be considered as a promising anticancer substance.[34] Interestingly, the earlier work of this group have demonstrated the cytotoxic activity of the methanol extract of *T. involucrata* against KB (subline of the ubiquitous KERATIN-forming HeLa tumor cell line) and MCF-7 cell line, which was well below the NCI guidelines.[8] This finding supports the hypothesis of Zhao *et al.*[7] who proposed that the endophytes share the medicinal properties of a host plant. Our present study will be helpful in finding a new source for anticancer compounds from endophytes. Many studies on endophytes have shown their cytotoxic potential against various cell lines.[5,35] However the IC_{50} value of those endophytic extracts are not within the NCI-guided limits.

Ruma *et al.* reported the PBR-322 plasmid protection by the organic extracts of endophytes (isolated from *Garcinia* sp.).[36] 50 µg/mL was able to protect the plasmid DNA damage by free radicals. However, in the present study, much higher amount of the extract [Figure 5] was needed to protect the λ DNA from Fenton-induced OH− radicals. Polyphenolic compounds are known to have free radical scavenging activity.[35,37] Hence, the radical scavenging activity of different fungal

extracts in this study may be contributed by the phenolic compounds. Even though all the extracts showed significant cytotoxic activity against MCF-7 and MOLT-4 cell lines, extracts also prevented the λ DNA damages at higher concentrations. This contradictory trend can be attributed to the presence of various phytochemicals (as we have taken the crude extract) present in the endophytic extracts (as confirmed by HPLC analysis). As these extracts contain several phytochemicals that are responsible for radical scavenging activity; the prevention of Fenton radical generation might have prevented the DNA damage in fungal extract-treated group. Our observation is supported by the finding of Siddaraju and Dharmesh.[37]

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Conflicts of interest

There are no conflicts of interest.

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SUPPLEMENTARY TABLES

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Original Research Article

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Antimicrobial and larvicidal potential of endophytic fungi isolated from *Tragia involucrata* Linn.

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Abstract: Endophytic fungi are one of the untapped resources of therapeutic compounds for various diseases. The present study focused on the antimicrobial as well as larvicidal activity of ethyl acetate extract of endophytic fungi isolated from *Tragia involucrata*. The ethyl acetate extract of *Penicillium citrinum* CGJ-C1 (GenBankNo.KT780618), *Penicillium citrinum*CGJ-C2 (KP739821), *Cladosporium*sp. (KP739822), and *Cryptendoxyla hypophloia* CGJ-D2 (KT780619) was subjected to antimicrobial activity against a panel of microorganisms by disc diffusion method, larvicidal activity against *Culex quinquefasciatus*. All the extracts showed significant antimicrobial activity against the tested organisms ranging from 8±0.32 to 13±2.11mm (zone of inhibition). The extent of activity was comparable to the standard drugs. The larvicidal potential of the endophytes was superior to *T. involucrata* extract. The larvicidal activity was found to be dose and time dependent with LC_{50} value ranging from 4.25- 158.06 ppm after 24hrs of treatment. This is the first report on the bioactivity of the endophytes isolated from *T. involucrata*. Further studies on the bio-guided isolation of lead compound will benefit the people suffering from microbial diseases.

Key words: *Tragia involucrata*; Endophytic Fungi; Antimicrobial; Antiyeast; Larva.

Introduction

The demand for new antibiotic is escalating day by day due to the development of drug resistance even though many novel molecules are released to the market. Chemical synthesis and engineered biosynthesis are the major contributors to the several drugs. Although this method is extensively exploited for the new generic molecules, nature still astonishes us with the availability of versatile compounds. Even the chemical synthesis depends on the structure available in nature. So, an antibiotic discovery from nature is ever-increasing field of study (Supaphon *et al.,* 2013).

Most of the rural people in the developing and under developing countries still depend on natural products or its derivatives for primary healthcare (Tamokou *et al.,* 2013). Plants are the major providers of remedy for primary health associated disorders. They have been extensively used for treating various ailments since ages. Of late, researchers are focusing their attention on the myriads of untapped endophytic microorganisms symbiotically associated with plants *(Cui et al.,* 2011). Endophytic fungi have the capability to produce the secondary metabolites similar to that of the host plant (Zhao *et al.,* 2010). These microbes are reported to produce several therapeutic molecules which are studied against various ailments (Cui *et al.,* 2011; Higginbotham *et al.,* 2013; Strobel and Daisy, 2003). Endophytes have several advantages over the host plants in which they exist such as less complexity and easy to grow in mass culture. These properties make endophytes a new avenue for discovering novel drugs (Cui *et al.,* 2011).

Tragia involucrata L. (Indian stinging nettle, Family Euphorbiaceae) is a medicinal plant having multifarious uses. Different parts of this plant has been reported to show cytotoxic (Joshi *et al.,* 2011a), antitumor (Joshi *et al.,* 2011b), antifertility (Joshi and Gopal, 2011), antimicrobial, larvicidal (Bhattacharya and Chandra, 2014), and antidiabetic (Farook and Atlee, 2011). Even though *T. involucrata* have been extensively studied for various medicinal properties, not even a single report was available on theisolation and biological properties of endophytes associated with this plant. This observation prompted us to isolate the endophytic fungi from*T. involucrata* and to study antimicrobial and larvicidal activity.

Materials and Methods

Chemicals

Potato dextrose agar (PDA), chloramphenicol, agarose, Sodium hypochlorite, ethyl acetate, potassium acetate and dimethyl sulfoxide (DMSO), were procured from Merck, India. All other chemicals used for the study were of analytical grade.

Collection of plant material

Fresh stem parts of *T. involucrata* were collected from in and around Mangalore, Karnataka, India (2013) in order

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to study the endophytic fungi associated and transported to the lab in the sterile polyethylene bags. The Global Positioning System (GPS) locations were 12.5581˚ N, 75.3892˚ E.

Isolation of endophytic fungi from *T. involucrata*

Tragia involucrata stem was washed free of adhering foreign particles with running tap water and cut into small pieces (0.6-0.8cm). Stem samples were surface sterilized by 70% alcohol, 0.5% sodium hypochlorite followed by washing thrice with sterile distilled water. The samples were inoculated in PDA medium supplemented with chloramphenicol (150mg/L)and incubated at (28±1) ˚C for seven days (Higginbotham *et al.,* 2013).

Microscopic observation of endophytic fungi.

Colony characters of pure cultures of isolated endophytic fungi were studied and observed under the Inverted Microscope (Magnus, INVI, Japan.).

Preparation of fungal fermentation broth and Extraction of secondary metabolites from the endophytic fungal culture

Extraction of secondary metabolites was carried according to the procedure described by Higginbotham *et al.,* (2013) with slight modification. The liquid culture with evident growth of the fungus was extracted with an equal volume of ethyl acetate (100%). The homogenate was filtered and extracted twice with ethyl acetate. The ethyl acetate extracts of *P. citrinum* CGJ-C1*, P. citrinum* CGJ-C2*, Cladosporium* sp. CGJ-D1 and *C. hypophloia* CGJ-D2 were labeled as EF1, EF2, EF3, and EF4 respectively.

Antimicrobial Activity

Bacterial cultures used in the present studies were obtained from microbial type culture collection (MTCC) and yeast was isolated from coffee effluent and submitted to GenBank (Dr. Anuappaiah). The bacterial strains were *Escherichia coli* (MTCC 118)*, Staphylococcus aureus* (FRI722)*, Bacillus cereus* (F4433)*, Micrococcus luteus* (ATCC9341)*, Listeria monocytogenes* Scott A and reference strains of *Pichia kudriviavzevii*P1 (KC841145)*, Pichia kudriviavzevii* P2(KC841146), *Pichia kudriviavzevii* P3 (KC841147), *Candida tropicalis* P4 (KC841148).

Antimicrobial activity of the ethyl acetate extract of the four-endophytic fungi was tested by disc diffusion method. The plates containing brain heart infusion medium (Himedia, India) and yeast extract peptone dextrose medium were inoculated with a 24h grown inoculum of bacteria and yeast respectively. Paper discs impregnated with 200µg of endophytic fungal extracts were placed on the bacteria and yeast inoculated plates. The plates were incubated at 37˚±2˚C for bacteria and at 28˚C for yeasts for 24 h. The diameter of the zone of inhibition was measured (mm). Standard Ampicillin sodium salt (20µg) was used for antibacterial activity and Nystatin (50µg/disc) was used for the antifungal activity(Arora and Kaur, 1999; Supaphon *et al.,* 2013).

Collection and identification of Larvae of *C. Quinquefasciatus*.

Larvae (*Culex quinquefasciatus*) were collected from the stagnant water areas of Kodagu District, Karnataka, India (The Global Positioning System (GPS) locations were 12.46700 N 75.96700 E) in rectangular trays at an average temperature of 24˚C ± 2˚ C. The identification of morphological characters of larvae was determined by comparing with the literature (Darsie and Samanidou-Voyadjoglou, 1997; "Genus Culex - Florida Medical Entomology Laboratory," n.d.)

Larvicidal activity

Larvicidal activity of the endophytic fungal extract was carried out according to WHO protocol with slight modifications (Matasyoh *et al.,* 2011). The crude extracts of endophytic fungi were dissolved in dimethylsulphoxide (DMSO, analytical grade, Merck) and diluted to the required concentration with distilled water. The concentration of DMSO was kept below 1%. The colonies of larvae were maintained in plastic trays containing tap water. The experiments were carried out at 27 ± 2˚C and 75–85 % relative humidity under 14:10 h light and dark cycles. The bioassays were performed with third instar larvae of *Culex quinquefasciatus* and carried out in triplicate using 20 larvae for each replicate assay. The larvae were placed in 50 mL disposable plastic cups containing 15 mL of the test solution and were fed a diet of Brewer's yeast, dog biscuits, and algae in a ratio of 3:1:1, respectively. The number of larvae surviving at the end of 12, 24, 36, 48 and 60hrs was recorded and the percentage mortality was determined by using mentioned below formula.

The percentage of mortality =

(No. of larva dead /No. of larvae) X100

Results and Discussion

Plants protect themselves from the pests and pathogens by symbiotically associating with the endophytes where plants provide the shelter to endophytes and in turn endophytes protect the plants against the pathogens. Endophytes with defensive secondary metabolites are selected by the host (Katoch *et al.,* 2014). Antimicrobial secondary metabolites from microorganisms have several advantages such as no destruction of resources, sustainable use, large scale industrial productions and quality control (Liang *et al.,* 2012). In the present study, we have isolated four endophytic fungi namely, *P. citrinum* CGJ-C1 (GenBank No. KT780618), *P. citrinum* CGJ-C2 (GenBank No. KP739821), *Cladosporium* sp. CGJ-D1 (GenBank No. KP739822), and *C. hypophloia* CGJ-D2 (GenBank No. KT780619). Each fungus was extracted with ethyl acetate and the characteristics of the extracts are given in Table1. Ethyl acetate extract of endophytic fungi (EF) showed a different level of inhibition of pathogenic bacteria and fungi. EF2 showed broad spectrum activity against tested organisms than other extracts. EF3 was mild antibiotic against the tested organisms. However, the extent of antimicrobial activity of other two extracts was in between the EF2 and EF3

(Table 2). The different level of antimicrobial activity of the fungal extracts may be attributed the structure and composition of chemicals present in that fungus (Teke *et al.,* 2011). Our study is in agreement with the earlier studies on endophytic fungi containing secondary metabolites with different level of antimicrobial activity (Teke *et al.,* 2011).

Table 1: Characteristics of the ethyl acetate extracts of fungi isolated from *T.involucrata*

Fungal extract	% of yield (g)	Color	Odour	Nature
EF1	0.13	Light orange	Characteristic	Crystal
EF2	0.10	Yellow	Characteristic	Crystal
EF3	0.14	Colourless	Characteristic	Crystal
EF4	0.11	Brown	Characteristic	Sticky

Table 2: Antimicrobial activity of ethyl acetate extracts of endophytic fungi.

Name of the organism	Zone of inhibition (mm) (Mean±standard Deviation)					
	EF1	EF2	EF3	EF4	NYS	AP
E.coli	10 ± 0.12	10 ± 2.1	NS	NS	NT	NT
S. aureus	NS	NS	9 ± 0.25	12 ± 0.76	NT	NT
B.cereus	NS	NS	NS	12±1.43	NT	NT
M.luteus	11 ± 0.23	12 ± 3.0	11 ±0.83	11 ± 1.22	NT	NT
L.monocytogenes	10 ± 0.11	NS	NS	13±2.11	NT	NT
P.kudriviavzevii(P1)	11 ± 0.98	12 ± 0.41	NS	NS	18 ± 2.39	14 ± 0.43
P. kudriviavzevii (P2)	8 ± 1.20	10 ± 0.17	9 ± 0.56	NS	18 ± 1.43	15 ± 0.56
P. kudriviavzevii (P3)	8 ± 0.32	10 ± 0.67	9 ± 1.15	NS	18 ± 2.32	15 ± 1.32
C. tropicalis (P4)	NS	9 ± 0.42	9 ± 1.61	9 ± 0.76	24 ± 0.98	13 ± 1.76

NYS- Nystatin; AP-Ampicillin; NS-Not Sensitive; NT-Not tested. (n=3)

Table 3: Percentage mortality of mosquito larva (*Culex Quinquefasciatus*) by EF.

		Larvicidal activity of endophytic fungal extract				
EF	No hours	% Mortality *at different concentration (In ppm) (%, Mean ±standarddeviation) (n=3)				
		100ppm	200ppm	300ppm	400ppm	500ppm
EF1	12	0±0	5± 2.35	38.33±2.35	78.33±2.35	91.66±2.35
	24	0±0	36.66±2.35	61.66±2.35	90±4.08	100±0
	36	11.66±2.35	86.66±2.35	90±0	100±0	100±0
	48	16.66±2.35	95±4.08	100±0	100±0	100±0
	60	21.66±2.35	93.33±2.35	100±0	100±0	100±0
EF2	12	13.33 ±2.35	13.33±2.35	31.66±=2.35	61.66±2.35	70±0
	24	31.66±2.35	31.665±2.35	55±0	80±4.08	86.66±2.35
	36	33.3±2.35	40±0	60±4.08	81.66±2.35	90±0
	48	43.33±2.35	41.66±2.35	65±6.23	81.66±2.35	98.33±2.35
	60	55±0	61.66±2.35	68.33±2.35	83.3±2.35	100±0
EF3	12	5±2.35	38.33±2.35	60±4.08	81.66±2.35	100±0
	24	15±0	88.33±6.23	91.66±2.35	83.33±2.35	100±0
	36	15±0	93.331±2.35	95±4.08	100±0	100±0
	48	16.66±2.53	95±0	96.65±2.35	100±0	100±0
	60	30±4.08	98.33±2.35	100±0	100±0	100±0
EF4	12	0±0	6.66±2.35	28.33±2.35	35±4.08	46.66±2.35
	24	6.66±2.35	15±4.08	35±4.08	36.66±2.35	48.33±2.35
	36	41.66±2.35	35±0	43.33±2.35	80±4.08	91.66±2.35
	48	50±4.08	61.66±2.35	61.66±2.35	85±4.08	93.33±6.23
	60	63.3±2.35	63.33±2.35	66.66±2.35	93.33±2.35	95±4.08

Table 4: % Mortality of larva (*Culex Quinquefasciatus*) by EF.

Endophytic fungal extracts	Period of Treatment	LC_{50} (ppm)	LC_{90} (ppm)	Regression	R^2
EF1	12	26.74	126.976	Y=25.665x-34.331	0.95
	24	14.84	71.952	Y=25.334x-18.338	0.97
	36	4.68	38.436	Y=19.002x+20.658	0.64
	48	3.05	31.374	Y=17.168x+30.828	0.75
	60	2.66	30.814	Y=16.335x+33.993	0.56
EF2	12	42.18	500.6964	Y=16.167x-10.505	0.92
	24	12.911	161.466	Y=15.834X+9.494	0.93
	36	9.88	130.320	Y=15.506x+14.474	0.97
	48	6.91	99.504	Y=15x+20.996	0.94
	60	2.410	86.747	Y=11.164x+40.166	0.95
EF3	12	14.88	82.599	Y=23.33x-13.001	0.98
	24	4.25	47.990	Y=16.5x+26.114	0.57
	36	3.54	34.055	Y=17.667x+27.665	0.57
	48	3.17	32.622	Y=17.168x+30.158	0.55
	60	1.619	27.248	Y=14.167x+43.165	0.51
EF4	12	179.84	4817.449	Y=12.166x-13.168	0.97
	24	158.06	7138.800	Y=10.5x-3.17	0.95
	36	11.30	178.394	Y=14.5x+14.83	0.80
	48	3.16	120.073	Y=11x+37.33	0.92
	60	1.19	86.834	Y=9.34x+48.304	0.81

Mosquitoes and its associated microorganisms are responsible for various diseases in people living in tropical regions (Bhattacharya and Chandra, 2014). Anti-mosquito medicines derived from natural products were well received by the people due to easy biodegradation and less side effects (Rajasekaran and Duraikannan, 2012). In our study, the endophytic extracts showed a moderate to a significant level of larvicidal activity. All the extracts were active against larva even at 100ppm with EF4 extract showing the highest activity. The toxicity towards the larva was showing a positive relation with the time of incubation and concentration of EF.100 percent larval death was observed in all the extract when the concentration was scaled up to 500ppm. All the larvae were almost dead at 500ppm in EF1 and EF3 extract after 12h treatment (Table 3). The EF3 extract showed a significant larvicidal activity with anLC_{50} and LC_{90} values of 4.25 and 47.990 ppm (at 24h) respectively (Table 4). The extent of the larvicidal potential of EF3 was highest among the ethyl acetate extracts of the endophytes. Interestingly, Bhattacharya and Chandra (2011) have studied the larvicidal activity of *T. involucrata* extract (Bhattacharya and Chandra, 2014). However, the larvicidal potency of the endophytic extract was even better than the *T. involucrata* extract. Our study supports the view that the endophytes share the bioactive properties of the host plants as put forwarded by *Zhao et al.,* (2010). The endophytic extract in crude form or the bioactive principle present in it can be sprayed on the stagnant sewage water which is the breeding ground for mosquitoes. But understanding the mechanism of action of the leads present in the extract will help in designing the better strategy for mosquito menace. The endophytic extract of *T. involucrata* showed better activity than the extracts of endophytes of different plants (Matasyoh *et al.,* 2011) as well as some medicinal plants reported earlier (Bhattacharya and Chandra, 2014).

Conclusion

Overall the present study for the first time established the possible role of endophytes of *T. involucrata* in the development of antimicrobial and larvicidal agents. All the endophytic extracts selected in this study showed varying degree of bioactivity. Further studies on the isolation, characterization and understanding the mechanism of action of lead molecules will aid in the formulation of a novel as well as cost effective drugs against microbe-caused diseases.

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