Phytochemical analysis and antioxidant activity of endophytic fungi Curvularia aeria MTCC 12847 isolated from Tribulus terrestris L. leaf.

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Abstract

Objective: The objective of the present study is to perform phytochemical analysis and antioxidant activity of endophytic fungi isolated from Tribulus terrestris L. leaf. Background: Curvularia aeria MTCC 12847 was isolated from Tribulus terrestris L. leaf and was used for phytochemical analysis which revealed the presence of phenol, flavonoids, terpenoids, steroids, alkaloids etc from different solvents extracts. The antioxidant activity was evaluated by nine separated methods: scavenging of free radical ABTS, DPPH, Hydrogen peroxide, Hydroxyl radical, Metal cheating, Nitric oxide, Superoxide radical, Reducing power and FRAP and the isolate Curvularia aeria MTCC 12847 showed good activity at low concentration. Conclusion: Further work is needful to isolate the exact compound which is responsible for antioxidant activity and biophysical characterization will be done in the future.

Background

Endophytic fungi, by definition, are the fungi which spend the whole or part of their lifecycle colonizing inter-and/or intracellularly inside healthy tissues of the host plants, typically without causing apparent symptoms of disease [1]. Endophytes have been found in nearly all plant families examined to date [2]. Recently endophytes are viewed as outstanding source of secondary metabolites and bioactive antimicrobial natural products. Endophytic fungi are of biotechnological interest due to their ability to produce antibacterial, antiviral, anticancer, antioxidants, antidiabetic and immunosuppressive compounds [3, 4, and 5] and biological control agents [6, 7, 8, and 9].

The genus *Tribulus*, belonging to family Zygophyllaceae, comprises about 20 species in the world, of which three species, viz. *Tribulus cistoides*, *Tribulus terrestris*, and *Tribulus alatus*, are of common occurrence in India [10]. Among them, *T. terrestris* (TT) is a well-patronized medicinal herb by Ayurvedic seers as well as by modern herbalists [11]. It is an annual shrub found in Mediterranean, subtropical, and desert climate regions around the world, viz. India, China, southern USA, Mexico, Spain, and Bulgaria [12, 13]. Molecules derived from natural products, particularly those products of plants and microbes have an excellent record of providing novel chemical compounds for the development of new pharmaceutical products [14]. In the present study the endophytic fungi has been isolated from the medicinal plant *Tribulus terrestris* L. and used for the phytochemical analysis and antioxidant activity.

Methods

1. Isolation of endophytic fungi

Visakhapatnam (Location 17°40'48.32"N, 83°12'5.8"E.) is situated between the Eastern Ghats and the Bay of Bengal *Tribulus terrestris* L. was collected from the Campus, Andhra University. Leaf samples were surface sterilized by dipping in 70% ethanol (v/v) for 1 min and 3.5% NaOCl (v/v) for 3 min, rinsed thrice with sterile water and dried. Bits of 1.0 X 1.0 cm size were excised with the help of a sterile blade. Two hundred segments of *Tribulus terrestris* L. leaf segments were placed on the water agar (16%) (WA) medium supplemented with Streptomycin (100 mg/l; Sigma, St. Louis, MO, USA) were used for the isolation of endophytic fungi. The Petri dishes were sealed using parafilm and The Petri dishes were incubated at 25-27°C till the mycelia start growing from the samples [15]. The plant material was also deposited in Botany Department herbarium (AUV), Andhra University with Voucher specimen numbers – 22294.

2. Secondary metabolites extraction

The endophytic fungus EF1 i.e. *Curvularia aeria* MTCC-12847 was cultured in 1-l Erlenmeyer flasks containing 500 ml of optimized culture media (PDB) under optimized parameters (pH: 6-6.5, Temperature: 27°C-28°C, Incubation days: 8-9 day) under static conditions. The culture broth was then filtered to separate the culture filtrate and mycelium. Culture filtrate was properly blended and centrifuged at 4,000 rpm for 5 min. The supernatant was transferred to a separating funnel to which was added the same volume of ethyl acetate. An ethyl acetate solution containing the fungal metabolite was 98% concentrated in a Buchi R-300 Rotavapor (India) at 50°C and stored at 4°C until its use [16].

3. Qualitative phytochemical analysis

It was done by following methods of [17, 18, and 19].

4. Quantitative phytochemical analysis

4a. Estimation of total Alkaloids

Total alkaloid content was estimated by the method of [20, 21] with minor modification. The absorbance for test and standard solutions were determined against the reagent blank at 470 nm with an UV-Spectrophotometer (SHIMADZU-1800, Japan). Absorbance = 0.004 Atropine (µg/ml) - 0.05 (R^2 = 0.9907) (S1). The results were expressed atropine equivalents (µg of AE/mg extract).

4b. Total flavonoid content

Total flavonoid content was estimated by a colorimetric method reported [22, 23] by with minor modification.

Absorbance = 0.005 Quercetin (µg/ml) + 0.101(R^2 = 0.9989) (S2). The results were expressed as Quercetin equivalents (µg of QE/mg of extract).

4c. Determination of total phenolic content

Total phenolic content was determined by using the Folin-Ciocalteu [24, 25] method of with minor modification.

Absorbance = 0.010 Gallic acid + 0.173 (R^2 = 0.9938) (S3). The results were expressed gallic acid equivalents (µg of GAE/mg extract).

4d. Estimation of total terpenoids

Estimation of total terpenoids in the crude extract was determined by the method of [26] with minor modification.

Absorbance = 0.001 linalool + 0.032 (R^2 = 0.9912) (S4). The results were expressed linalool equivalents (µg of linalool/mg extract).

4e. Estimation of total sterol

Estimation of total sterols in the crude extract was done by the method of [27] with minor modification.

Absorbance = 0.0026 Beta-Sisterol (µg/ml) - 0.047 (R^2 = 0.9986) (S5). The results were expressed as Beta-Sisterol equivalent (µg of Beta-Sisterol/mg of extract).

4f. Estimation of Total saponins

Total saponin content was analyzed spectrophotometerically following the method of [28] with minor modifications.

Absorbance = 0.0005 Diosgenin (µg/ml) - 0.042 (R^2 = 0.9983) (S6). The results were expressed as diosgenin equivalents (µg of DE/mg crude extract).

4g. Estimation of total tannins

The total tannins were determined using the method [29, 30] with minor modification.

Absorbance = 0.0192 tannic acid + 0.015 (R^2 = 0.9961) (S7). The results were expressed as tannic acid equivalents (µg of Tannic acid/mg of extract).

4h. Estimation of total carbohydrates

The total carbohydrate content was estimated by the method of [31].

Absorbance = 0.0092 glucose + 0.063 (R^2 = 0.9953) (S8). The results were expressed as glucose equivalents (µg of Glucose/mg of extract).

4i. Estimation of total protein

The total protein content was estimated using the method of [32].

Absorbance = 0.0098 BSA + 0.015 (R^2 = 0.9982) (S9). The results were expressed as BSA equivalents (µg of BSA/mg of extract).

5. TLC (Thin layer chromatography profile)

For the separation of different phytochemical compounds in the endophytic fungal extract TLC was performed by the method of [33, 34]. After the separation of phytochemical constituents, the plates were viewed under UV light at 254 nm and 366 nm and bands were marked and R_f values were calculated by using the following formula:

$$\text{Retention factor } (R_f) = \frac{\text{Distance travelled by the solute}}{\text{Distance travelled by the}}$$

6. Antioxidant activity

6a. Free radical-scavenging ability by the use of a stable ABTS radical cation

The free radical-scavenging activity was determined by ABTS radical cation decolorization assay described by [35]. The $ABTS^{\bullet+}$ cation radical was produced by the reaction between 5 ml of 14 mM ABTS solution and 5 ml of 4.9 mM potassium persulfate ($K_2S_2O_8$) solution, stored in the dark at room temperature for 16 hr. Before use, this solution was diluted with ethanol to get an absorbance of 0.700 ± 0.020 at 734 nm. The endophytic fungal extract of different concentrations with 1 ml of ABTS solution and absorbance were recorded at 734 nm. Ethanol was run in each assay, and all measurements were taken after

at least 6 min. Similarly, the reaction mixture of standard group was obtained by mixing 950 µl of $ABTS.^+$ solution and 50 µl of BHT. As for the antiradical activity, ABTS scavenging ability was expressed as IC_{50} (µg/ml). The inhibition percentage of ABTS radical was calculated using the following formula:

$$\text{ABTS scavenging activity (\%)} = (A_0 - A_1) / A_0 \times 100$$

Where, A_0 is the absorbance of the control and A_1 is the absorbance of the sample.

6b. DPPH (1, 1-diphenyl-2-picrylhydrazyl) free radical scavenging activity

The antioxidant activity of the extract were determined using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay by the method of [36, 37] with some modifications. Freshly prepared 1ml of DPPH (0.004 % in methanol) solution was added in each of these test tubes containing extracts (100 to 500 µg/ml) and after 30 min incubation in the dark, the absorbance was taken at 517 nm. BHT was used as a positive control. The DPPH solution was used as blank. Scavenging of the DPPH free radical was measured using the following equation:

$$\text{DPPH radical scavenging (\%)} = (A_0 - A_1) / A_0 \times 100$$

6c. Hydrogen peroxide scavenging (H_2O_2) assay

The ability of the extracts to scavenge hydrogen peroxide was estimated by following the method of [38]. A solution of hydrogen peroxide (40 mmol/L) was prepared in phosphate buffer (50 mmol/L, pH 7.4). The concentration of hydrogen peroxide was determined by absorption at 230 nm using a spectrophotometer. Extract (1 mg/mL) in distilled water were added to hydrogen peroxide and absorbance at 230 nm was determined after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. Ascorbic acid was used as a positive control.

The percentage of hydrogen peroxide scavenging was calculated as follows:

$$\text{Scavenged } H_2O_2 \text{ (\%)} = (A_0 - A_1) / A_0 \times 100$$

6d. Hydroxyl Radical Scavenging Assay

Hydroxyl radicals formed from $FeSO_4$/hydrogen peroxide can be noticed by their attribution to hydroxylate salicylate thus forming hydroxylated salicylate complex which can be observed at wavelength 562 nm[39]. Several concentrations of extract were added to 6 mM hydrogen peroxide (0.7 ml), 1.5 mM FeSO4 (1 ml), 20 mM sodium salicylate (0.3 ml), were used as the reactive mixture (3 ml). Absorbance of hydroxylated salicylate complex was determined at wavelength 562 nm after 1 hr of incubation at 37°C. Again, ascorbic acid was used as positive control.

The scavenging activity was calculated by the given formula below:

$$\text{\% scavenging activity} = (A_0 - A_1) / A_0 \times 100$$

6e. Reducing power assay

The reductive potential of the extract was determined according to the method of[40] with minor modification. Different extracts and standard (1 mg/mL) in 1 mL of distilled water were mixed with phosphate buffer (2.5 mL, 0.2 mol/L, pH 6.6) and potassium ferricyanide (2.5 mL, 1% w/v) and was then centrifuged for 10 min at 3000 rpm/min. The upper layer of solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1% w/v) and the absorbance was measured at 700 nm in a spectrophotometer. High absorbance value of the reaction mixture indicates greater reductive potential.

6f. FRAP assay

The total antioxidant potential of a sample was determined using the ferric reducing ability of FRAP assay by [41] as a measure of antioxidant power.

Briefly, FRAP reagent was freshly prepared by mixing 25 mL acetate buffer (300 mM, pH 3.6), 2.5 mL 2,4,6-tris (2-pyridyl)-S-triazine (TPTZ) solution (10 mM TPTZ in 40 mM HCl) and 2.5 mL $FeCl_3$ (20 mM) at 10:1:1 (v/v/v). Extract at different concentrations (100 to 500 µg/ml) were then added to 3 ml of FRAP reagent and the reaction mixture was incubated at 37°C for 30 min. The increase in absorbance at 593 nm was measured.

6g. Nitric oxide Scavenging Activity

Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide (NO), which interacts with oxygen to produce nitrite ions, which can be estimated using Griess Illosvosy reaction by[42]. The absorbance of these solutions was measured at 540 nm against the corresponding blank solution.

Extract at different concentrations were mixed with the 100 mM sodium nitropusside (2.5 ml) and PBS (0.02% KCl, 0.88% NaCl, 0.115% Na_2HO_4 and 0.02% KH_2PO_4) to make up to maximum of 3 ml for the reaction mixture and was incubated at 25°C for 15 min, after that 0.5 ml from the reaction mixture was replaced with 0.5 ml of Griess reagent (2% phosphoric acid, 1% sulphanilamide and 0.1% naphthyl ethylenediamine dihydrochloride).

Percentage inhibition was calculated as:

$$\text{\% Scavenging of Nitric oxide} = (A_0 - A_1) / A_0 \times 100$$

6h. Metal chelating activity

It was estimated by method of [43]. Briefly, 0.1 mM $FeSO_4$ (0.2 ml) and 0.25 mM ferrozine (0.4 ml) were added subsequently into extract (0.2 ml). After leaving to stand for 10 min, the absorbance was read at wavelength 562 nm. EDTA was used as a positive control.

Chelating activity was calculated using the following formula:

$$\text{Metal chelating activity} = (A_0 - A_1) / A_0 \times 100$$

6i. Superoxide Radical Scavenging Activity (SOD)

Superoxide radicals were generated by method of Beauchamp [44, 45] with slight modification. Superoxide radicals were generated in riboflavin, methionine, illuminate and assayed by the reduction of NBT to form blue formazan (NBT^{2+}). All solutions were prepared in 0.05 M phosphate buffer (pH 7.8). The photo-induced reactions were performed using fluorescent lamps (20 W). The total volume of the reactant mixture was 3 mL and the concentrations of the riboflavin, methionine and NBT were 1.33 x $10^{-}5$, 4.46 x $10^{-}5$ and 8.15 x $10^{-}8$ M, respectively. The reactant was illuminated at 25°C for 40 min. The photochemically reduced riboflavin at different concentrations was added to the reaction mixture, in which O_2 was scavenged, thereby inhibiting the NBT reduction. Decreased absorbance of the reaction mixture indicates increased superoxide anion scavenging activity and the readings were taken at 560nm. The inhibition percentage of superoxide anion generation was calculated by using the following formula:

$$O^{\cdot -}_2 \text{ scavenging effect (\%)} = (A_0 - A_1) / A_0 \times 100$$

Results

Fig.S10 Shows the *Tribulus terrestris* L. plant from which endophytic fungi has been isolated and S11 shows the endophytic fungi *Curvularia aeria* MTCC 12847. The endophytic fungal extracts was used for the phytochemical by using different solvents i.e. ethylacetate, methanol and chloroform. The phytochemical analysis showed the presence of both primary metabolites (Carbohydrates and proteins) and secondary metabolites like (phenols, flavonoids, alkaloids, terpenoids etc). Tables 1 show the qualitative phytochemical analysis of ethyl acetate, methanol and chloroform extract. In quantitative analysis highest yield was found in ethyl acetate extract, TAC: 2.986±0.0057, TFC: 45.666±0.577, TPC: 34.666±0.577 and so on and lowest yield were found in chloroform extract. Tables 1, 2 and 3 show the quantitative phytochemical analysis of ethyl acetate, methanol and chloroform extracts respectively and the Fig. S1 and S9 show the standard graph for calculation of quantitative phytochemical analysis and Fig. S12 shows TLC image of the crude extract and the RF value. The Rf value of the Flavonoids standard (Quercentin) was found to be 0.96 and 0.88 similarly for terpenoids standard (Linalool) was found to be 0.91 similarly Phenol standard (Gallic acid) was found to be 0.45 and Alkaloids standard (Atropine) was found to be 0.57 and 0.62 while the crude extract shows the Rf value of 0.96, 0.91, 0.88, 0.74, 0.57 and 0.45. Hence the crude extract shows the presence of Flavonoids, Terpenoids, Phenol, and Alkaloids. Table 3 and Fig S13 to S19 shows ABTS, DPPH, Hydrogen peroxide, Hydroxyl radical, Nitric oxide, Metal chelating and Super oxide radical scavenging activity and S18 and S19 Contains Tables 4 and 5 for reducing power assay and FRAP assay which shows the OD values at different concentration.

Table 1 Phytochemical analysis of crude extract

Isolate	Alkaloids				Phenols		Steroids	Terpenoids	Saponins	Tannins	Carbohydrates	Protein amino acids	
	Flavonoids												
	Wagner test	Hagers tests	Alkaline reagent tests	Shinoda tests	Ferric chloride test	Lead acetate test	Salkowski test	Liebermann Burchard test	Frothing test	Ferric chloride test	Molisch test	Biuret test	Ninhy te
Preliminary Phytochemical analysis of ethyl acetate crude extract													
Curvularia aeria MTCC-12847	+	+	+	+	-	+	+	+	+	+	+	+	+
Preliminary Phytochemical analysis of methanol crude extract													
Curvularia aeria MTCC-12847	+	+	+	-	+	+	+	+	+	+	+	-	+
Preliminary Phytochemical analysis of chloroform crude extract													
Curvularia aeria MTCC-12847	+	+	-	-	-	+	+	+	-	-	+	-	-

Note: All the experiments are performed in triplicates.

Table 2 Quantitative phytochemical analysis of crude extract

Isolate no. / plants parts used	TAC (µg of AE/mg extract)	TFC (µg of QE/ mg of extract)	TPC (µg of GAE/mg of extract)	TTC (µg of Linalool/ mg of extract)	TSC (µg of Beta-Sisterol/mg of extract)	TSpC (µg of DE/ mg of extract	TTnC (µg of tannic acid/mg of extract)	TCC (µg of Glucose/mg of extract)	TPrC (µg of BSA/mg of extract)
Quantitative phytochemical analysis of ethyl acetate crude extract									
Curvularia aeria MTCC- 12847	2.986 ± 0.0057	45.666 ± 0.577	34.666 ± 0.577	38.666 ± 0.577	2.746 ± 0.0057	3.496 ± 0.0057	7.466 ± 0.0577	596.666 ± 0.577	295.866 ± 0.0577
Quantitative phytochemical analysis of methanol crude extract									
Curvularia aeria MTCC- 12847	2.886 ± 0.0057	43.666 ± 0.577	33.666 ± 0.577	36.666 ± 0.577	2.646 ± 0.0057	3.396 ± 0.0057	6.996 ± 0.0057	586.666 ± 0.577	290.866 ± 0.0577
Quantitative phytochemical analysis of Chloroform crude extract									
Curvularia aeria MTCC- 12847	2.236 ± 0.0057	35.666 ± 0.577	24.666 ± 0.577	28.666 ± 0.577	1.996 ± 0.0057	2.996 ± 0.0057	6.466 ± 0.0577	571.666 ± 0.577	280.866 ± 0.0577

Note: TAC= Total alkaloid content; TFC= Total flavonoid content; TPC= Total phenol content; TTC= Total terpenoids content; TSC= Total sterol content; TSpC=Total saponin content; TTnC= Total tannin content; TCC= Total carbohydrate content; TPrC= Total protein content). All the experiments are performed in triplicates and (Mean value ± SD) were calculated.

Table 3: Scavenging assay

	ABTS radical scavenging activity											
	Ethyl acetate extract				Methanol extract				Chloroform extract			
	IC_{50} value µg/ml	Eqn.	R^2	% Inhibition (500 µg/ml)	IC_{50} value µg/ml	Eqn.	R^2	% Inhibition (500 µg/ml)	IC_{50} value µ	Eqn.	R^2	% Inhibition (500 µg/ml)
ABTS radical scavenging assay												
aeria MTCC-	246.575	0.146X+14	0.9948	86	257.534	0.146X+12.4	0.9957	84	340.625	0.128X+6.4	0.9925	69
standard)	-				201.408	0.142X+21.4	0.9992	92				
DPPH radical scavenging activity												
aeria MTCC-	139.64	0.113X+33.7	0.9964	91	157.142	0.112X+32.4	0.9987	89	276.576	0.111X+19.3	0.9959	76
standard)	-				123.214	0.112X+36.2	0.9949	93				
Hydrogen peroxide scavenging assay												
aeria MTCC-	215.702	0.121X+ 23.9	0.9954	85	229.508	0.122X+22	0.9968	83	338.33	0.12X+9.4	0.9923	70
c acid dard)	-				192.307	0.117X+27.5	0.9969	87				
Hydroxyl radical scavenging assay												
aeria MTCC-	258.94	0.117X+ 19.7	0.9957	78	270.94	0.117X+18.3	0.9925	76	396.078	0.102X+9.6	0.9977	60
c acid dard)	-				227.272	0.11X+25	0.9967	80				
Nitric oxide scavenging assay												
aeria MTCC-	281.052	0.095X+ 23.3	0.9953	70	295.744	0.094X+22.2	0.9915	68	431.034	0.087X+12.5	0.9944	55
id	-				186.868	0.099X+31.5	0.9932	80				
Metal chelating activity												
aeria MTCC-	297.183	0.142X+ 7.8	0.9986	79	307.092	0.141X+6.7	0.997	77	423.478	0.115X+1.3	0.9908	57
andard)	-				113.207	0.106X+38	0.9936	90				
Superoxide radical Scavenging assay												
aeria MTCC-	278 .571	0.112X+ 18.8	0.9978	75	289.381	0.113X+17.3	0.9954	74	410.092	0.109X+5.3	0.9917	60
id	-				247.368	0.114X+21.8	0.9982	79				

Note: All the experiments were performed in triplicates and average values were recorded and were used for graphical representation.

Discussion

The endophytes have made greater interest in the use reservoir of natural bioactive compounds that the (host) produced [46]. Endophytic fungal species are now considered as exciting novel sources for obtaining new bioactive compounds and have been reported from several hosts [47]. Investigations on the phytochemical screening of the *Tribulus terrestris* L. revealed the presence of alkaloids, flavonoids, phenol, terpenoids etc. The presence of phytochemical within endophytes can be potential source for medicinal and industrial use. The presence of phytochemicals in the endophytes can be potential source of precursors in the development of synthetic drugs [48].

The importance of compounds bearing antioxidant activity lies in the fact that they are highly effective against damage caused by reactive oxygen species (ROS) and oxygen derived free radicals, which contribute to a variety of pathological effects such as DNA damages, carcinogenesis, and cellular degeneration [49]. Antioxidants have been considered promising therapy for prevention and treatment of ROS linked diseases as cancer, cardiovascular disease, atherosclerosis, hypertension, ischemia/reperfusion injury, diabetes mellitus, neurodegenerative diseases (Alzheimer and Parkinson diseases), rheumatoid arthritis, and ageing [50].

Conclusion

Ethyl acetate extract of endophytic fungus Curvularia aeria MTCC 12847 significantly showed good result in phytochemical analysis and antioxidant activities. This finding confirms that endophytic fungi isolated from Tribulus terrestris L. was source of the potential bioactive substances. Thus, this plant appears to be an interesting plant which harbours active fungal isolates for development as pharmaceutical agents in the future. The isolation of active metabolites which may be responsible for antioxidant activity may be investigated in future.

LIMITATIONS

The short duration of the study is an insufficiency of our work mainly related to a lack of funding.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Author's contributions

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