

QUALITATIVE AND QUANTITATIVE PHYTOCHEMICAL ANALYSIS OF ENDOPHYTIC FUNGI (EF8; *ASPERGILLUS* SP.3) ISOLATED FROM *BOERHAVIA DIFFUSA* L., STEM.

DEEPENDRA THAKUR¹, KAMANA SAHANI^{1,2*}¹Department of Microbiology, Bangalore University, Bengaluru, Karnataka, India, ²Department of Microbiology, Andhra University, Visakhapatnam, Andhra Pradesh, India. Email: kmnsahani@gmail.com

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ABSTRACT

Objective: The objective of the present investigation was to find phytochemical constituents of endophytic fungi *Aspergillus* sp.3 isolated from *Boerhavia diffusa* L. stem.

Methods: Both qualitative and quantitative phytochemical analysis was performed.

Results: In the present investigation, endophytic fungi was isolated from the medicinal plant *B. diffusa* L. The qualitative and quantitative phytochemical analysis was performed from the endophytic fungi *Aspergillus* sp.3 which showed the presence of phenol, flavonoids, alkaloids, steroids, and terpenoids, 11.66±0.577, 17.66±0.577, 1.746±0.0057, 0.446±0.0057, and 9.746±0.0057 (µg of standard/mg of extract), respectively.

Conclusion: Hence, the endophytic fungi isolated from *B. diffusa* L. could be regarded as good sources of bioactive compounds for drug formulations.

Keywords: Phytochemical, Phenol, Flavonoids, Alkaloids, Endophytic fungi, *Boerhavia diffusa* L.

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INTRODUCTION

Boerhavia diffusa L. (Family *Nyctaginaceae*) is a herbal plant, which is common in tropics in both dry and rainy seasons. It is found in India, Nigeria, and many other countries. *B. diffusa* is used in traditional medicine for its anti-inflammatory, antibacterial, and cardiotoxic properties [1]. It is used in the treatment of elephantiasis, night blindness, and corneal ulcers [2]. Conventionally, the plant has been evaluated for its hepatoprotective, anti-diabetic, diuretic, anti-inflammatory, antibacterial, antiviral, and anticancer properties [3].

Endophytes are symbiotically associated microorganism of living plants potential sources of biologically active natural products in medical, agricultural, and industrial applications. They have been found in every plant species studied and it is approximated to be around a million or more endophytic fungi in nature. Therefore, there is an ample opportunity to unearth a novel and interesting endophytic microorganism with significant therapeutic efficacy [4]. Selection of appropriate higher plants study and isolation of microfloral components can be achieved by a superior understanding of the mechanism of endophytes existence and their interactions with the surroundings. This procedure may help in unrevealing the new natural product. However, the extent of utilization of the endophytic microorganism for food and health industries is still modest, compared to the ample number of useful microorganism [5].

Phytochemical and antioxidant activity of *B. diffusa* L. plants were studied [6] and their review by [7]. The objective of the present study was to isolate endophytic fungi from *B. diffusa* L. and to check their phytochemical constituents.

METHODS

Collection of plant material

Visakhapatnam (Location 17°40'48.32"N, 83°12'5.8"E) is situated between the Eastern Ghats and the Bay of Bengal. The annual mean temperature ranges between 24.7°C and 30.6°C (76°F-87°F), with the

maximum in the month of May and minimum in January; the minimum temperature ranges between 20°C and 27°C (68°F-81°F) and the average annual rainfall recorded is 1118.8 mm. The plant was located on the campus of Andhra University. Healthy and mature plants of *B. diffusa* L. was collected from the campus, Andhra University. The sample was tagged and placed in a sterile polyethylene bag, brought to the laboratory and processed within 24 h of collection [8,9]. Fresh plant material was used for the isolation work to reduce the chance of contamination. Sample collection was done in January 2016 and the plant used in the study was authenticated by Prof. S. B. Padal (Botanist), Department of Botany, Andhra University, Visakhapatnam and the plant material was also deposited in Botany Department herbarium (AUV), Andhra University, Visakhapatnam with Voucher specimen numbers – 22,296.

Isolation of endophytic fungi

The sample was washed thoroughly in running tap water before processing. Stem 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 cm × 1.0 cm size were excised with the help of a sterile blade. Two hundred segments of *B. diffusa* L. plant stem 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°C–27°C till the mycelia start growing from the samples [10].

Secondary metabolite production of endophytic fungal isolate

The endophytic fungus EF8, i.e., *Aspergillus* sp.3 was cultured in 1-l Erlenmeyer flasks containing 500 ml of optimized culture media (potato dextrose broth) under optimized parameters (pH:5.5–6.5, Temperature: 25°C–30°C, Incubation days:8–9 days) under static conditions. The culture broth was filtered to separate the culture filtrate and mycelium. Culture filtrate was blended thoroughly and centrifuged at 4000 rpm for 5 min. Liquid supernatant was extracted with an equal volume of solvent thrice separately and was evaporated to dryness under reduced pressure at 50°C using rotary flash evaporator [11].

Qualitative phytochemical analysis [12-14]

Alkaloids

The endophytic fungal crude extract was evaporated to dryness in the boiling water bath. The residues were dissolved in 2N HCl. The mixture was filtered and the filtrate was used for the following test:

- Wagner's test: Filtrate was treated with Wagner's reagent; formation of brown reddish precipitate indicates the presence of alkaloids
- Hager's test: Filtrate was treated with Hager's reagent; the presence of alkaloid was confirmed by the yellow color precipitate
- Flavonoids
- Alkaline reagent test: The endophytic fungal extract was treated with 10% ammonium hydroxide solution. Yellow fluorescence indicates the presence of flavonoids
- Shinoda test (Mg/HCl or Zn/HCl reduction test): In a test tube containing 0.5 ml of endophytic fungal crude extract, 5–10 drops of diluted HCl and a small piece of zinc or magnesium were added, and the solution was boiled for few minutes. In the presence of flavonoid pink or dirty brown color was produced.

Phenol

- Ferric chloride test: The endophytic fungal extract was treated with 5% ferric chloride solution a dark green color indicates the presence of phenol
- Lead acetate test: The endophytic fungal extract was treated with 10% lead acetate a bulky white color indicates the presence of phenol.

Steroid

- Salkowski test: 0.5 ml of endophytic fungal crude extract was mixed in 2 ml of chloroform and sulphuric acid was added shaken well and allow standing for some time a reddish brown color at the interface indicates the presence of the steroidal ring
- Terpenoid
- The Libermann–Burchard test: To 1 ml of the endophytic fungal crude extract with few drops of acetic acid and 1 ml concentrated sulphuric acid gives deep red at the junction of 2 layers indicates the presence of terpenoids.

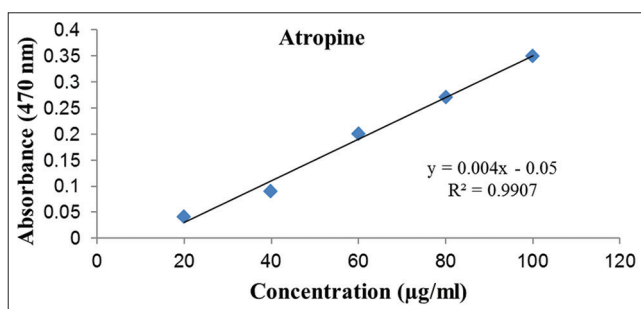


Figure 1: Calibration curve of alkaloid

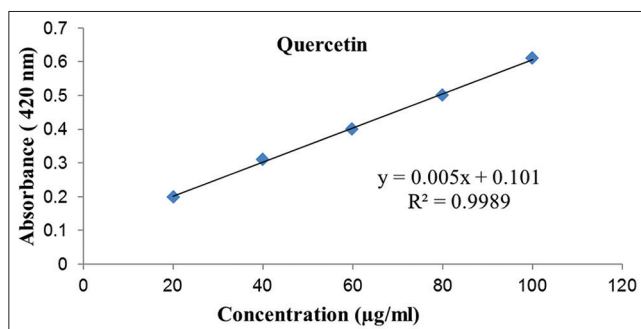


Figure 2: Calibration curve of flavonoids

Tannin

- Ferric chloride test: Endophytic fungal extract (1 ml) was taken to which few drops of 10% alcoholic ferric chloride was added bluish black color which disappears on the addition of little sulfuric acid was followed by formation of the yellowish brown precipitate.

Amino acids and protein

- Ninhydrin test: Test solution when boiled with 0.2% solution of Ninhydrin, would result in the formation of purple color suggesting the presence of free amino acids
- Biuret test: Test solution when treated with 10% sodium hydroxide solution and two drops of 0.1% copper sulfate solution and observed for the formation of violet-pink color which indicates the presence of protein.

Carbohydrate

- Molisch test: Endophytic fungal extract was treated with two drops of alcoholic α -naphthol solution in the test tube, and the mixture was shaken well and the few drops of concentrated sulfuric acid was added slowly along the sides of test tubes. A violet ring indicates the presence of carbohydrate.

Quantitative phytochemical analysis

Estimation of total alkaloids

Total alkaloid content was estimated by the method of [15,16] with minor modification.

Briefly, endophytic fungal crude extract (1 mg/ml) was dissolved in 2 N HCl and then filtered. 1 ml of this solution was transferred to separatory funnel and washed with 10 ml chloroform (three times). The pH of this solution was adjusted to neutral with 0.1N NaOH. Then, 5 ml of Bromocresol green solution and 5 ml of phosphate buffer pH 7.4 were added to this solution and the mixture was shaken properly and complex was extracted with 1, 2, 3, and 4 ml chloroform by vigorous shaking, the extract was then collected in a 10 ml volumetric flask and diluted with chloroform. A set of reference standard solutions of atropine (20, 40, 60, 80, and 100 µg/ml) was prepared in the same manner as described earlier. The absorbance for test and standard solutions were determined against the reagent blank at 470 nm with

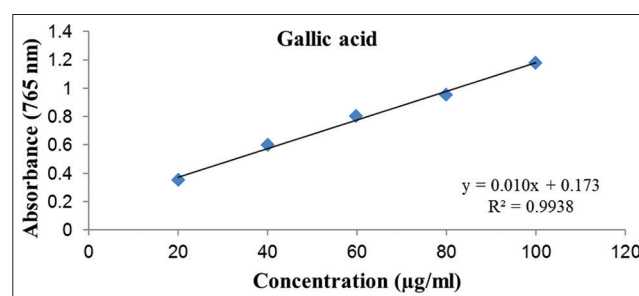


Figure 3: Calibration curve of phenol

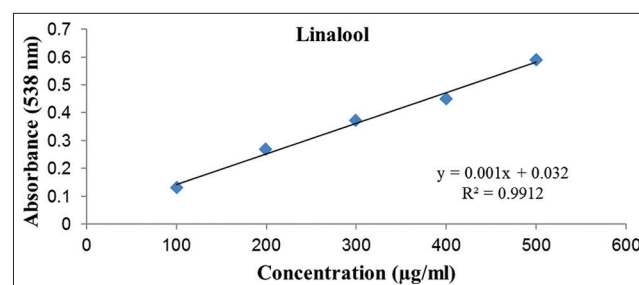


Figure 4: Calibration curve of terpenoids

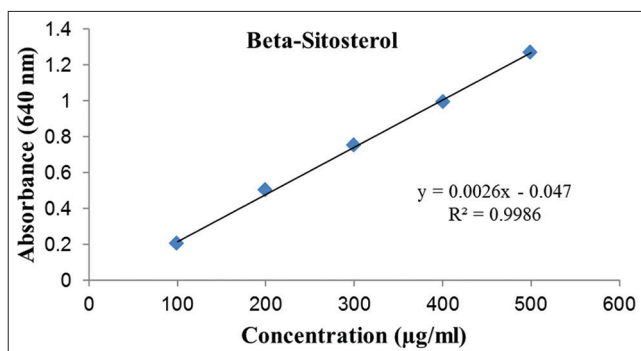


Figure 5: Calibration curve of sterols

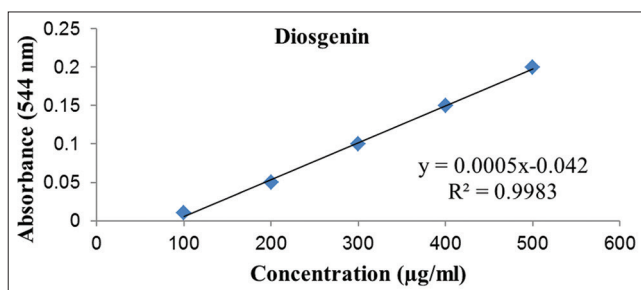


Figure 6: Calibration curve of saponin

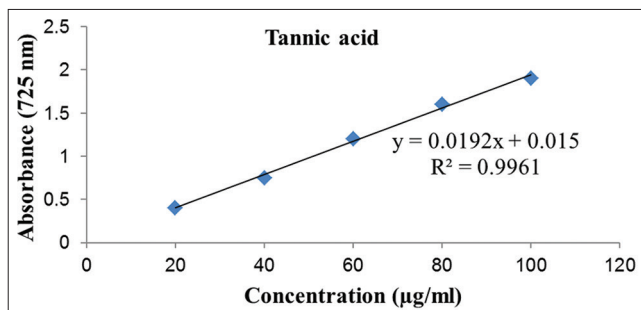


Figure 7: Calibration curve of tannins

an ultraviolet (UV)-Spectrophotometer (SHIMADZU-1800) against the blank.

Absorbance = 0.004 Atropine ($\mu\text{g/ml}$) - 0.05 ($r^2=0.9907$) (Fig. 1).

Quantification was based on the standard curve of atropine. All the tests were carried in triplicates and results were expressed atropine equivalent (μg of AE/mg extracts).

Total flavonoids content

Total flavonoid content was estimated by a colorimetric method reported by [17,18] with minor modification.

Briefly, 0.5 ml of 2% ethanolic AlCl_3 solution was added to 0.5 ml of endophytic fungal crude extract. After 1 h at room temperature, the absorbance was measured with UV-Spectrophotometers (SHIMADZU-UV1800) against the blank at 420 nm. A yellow color indicated the presence of flavonoids. Extracted samples were evaluated at a final concentration of (1 mg/ml).

Absorbance = 0.005 Quercetin ($\mu\text{g/ml}$) + 0.101 ($r^2=0.9989$) (Fig. 2).

All the tests were carried out in triplicates and the results were expressed as quercetin equivalent (μg of quercetin per mg of extract).

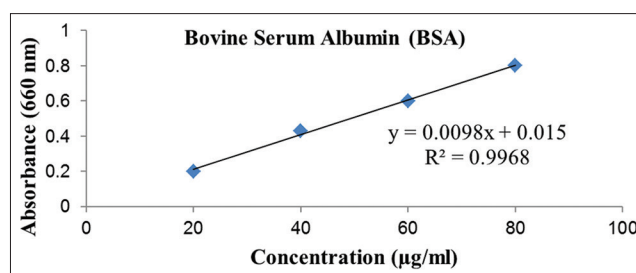


Figure 8: Calibration curve of protein

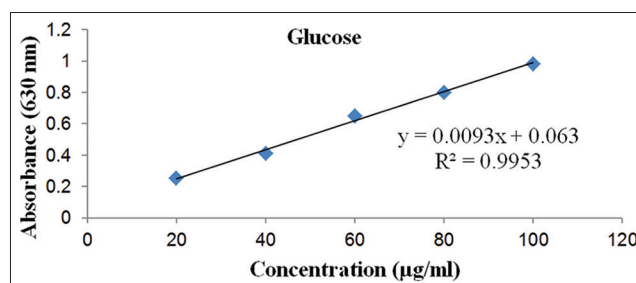


Figure 9: Calibration curve of glucose

Determination of total phenolic content

Total phenolic content was determined by using the Folin-Ciocalteu method [19,20] with minor modification.

Briefly, 0.5 ml of samples (1 mg/ml) was mixed with 1.8 ml of 10-fold diluted Folin-Ciocalteu reagent. The mixture was allowed to stand for 5 min at room temperature and then the reaction was neutralized with 1.2 ml of saturated sodium carbonate (7.5%). The absorbance of the resulting blue color was measured with a UV-vis spectrophotometer (SHIMADZU UV-1800) against the blank at 765 nm after incubation for 90 min.

Absorbance = 0.010 Gallic acid + 0.173 ($r^2=0.9938$) (Fig. 3).

Quantification was based on the standard curve of gallic acid. All the tests were carried in triplicates and results were expressed gallic acid equivalent (μg of GAE/mg extracts).

Estimation of total terpenoids

Estimation of total terpenoids in the endophytic fungal crude extract was determined by the method of [21] with minor modification.

In the test-tube containing 200 μl of endophytic fungal crude extract (1 mg/ml), 1.5 ml of chloroform was added. The sample was vortexed thoroughly and brought to rest for 3 min and then 100 μl of Conc. Sulphuric acid was added to the test-tube and was incubated in room temperature for 1.5-2 h in the dark. At the end of incubation time, reddish brown precipitation was formed in each assay tubes. All the supernatant were carefully removed from the reaction mixture without disturbing the precipitation. Then, 1.5 ml of 95% (Vol/Vol) Methanol was added and vortex thoroughly until all the precipitation dissolves in methanol completely. The sample was transferred from assay tube to colorimetric cuvette (95% [Vol/Vol] Methanol was used as blank) to read the absorbance at 538 nm by using UV-vis spectrophotometer (SHIMADZU UV-1800).

Absorbance = 0.001 linalool + 0.032 ($r^2=0.9912$) (Fig. 4).

Quantification was based on the standard curve of linalool. All the tests were carried in triplicates and results were expressed linalool equivalent (μg of linalool/mg extracts).

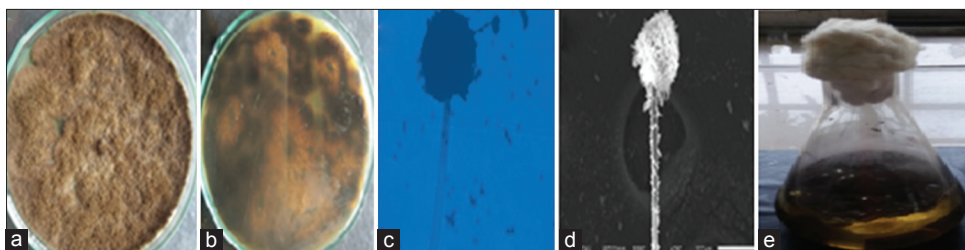


Figure 10: *Aspergillus* sp.3. (a) Front view in patent ductus arteriosus, (b) reverse view in patent ductus arteriosus, (c) microscopic image, (d) scanning electron microscope image, (e) secondary metabolite production at static condition

Estimation of total sterol

Estimation of total sterols in the endophytic fungal crude extract was done by the method of [22] with minor modification.

The Liebermann–Burchard (LB) reagent was employed for the quantitative estimation of sterol in the endophytic fungal crude extracts. It was prepared by adding 0.5 ml of concentrated sulfuric acid in 10 ml of acetic anhydride. To 1 ml of each of the extract (1 mg/ml), chloroform was added to make the volume to 5 ml in a test tube. A volume of 2 ml of LB reagent was added and mixed well. These tubes were then covered with black paper and kept in the dark for 15 min to avoid any exposure to light. The reaction mixture turned green, which was measured spectrophotometrically by using UV-vis spectrophotometer (SHIMADZU UV-1800) at 640 nm against the blank.

Absorbance = 0.0026 Beta-Sitosterol ($\mu\text{g/ml}$) – 0.047 ($r^2=0.9986$) (Fig. 5).

Beta-Sitosterol was used as the standard to prepare a calibration curve. All the tests were carried out in triplicates and the results were expressed as Beta-Sitosterol equivalent (μg of Beta-Sitosterol per mg of extract).

Estimation of total saponin

Total saponin content was analyzed spectrophotometrically following the method [23] with minor modifications.

Briefly, 1 mg of endophytic fungal crude extract was dissolved in 0.5 ml of 50% aqueous methanol. 25 μl of the aliquot was transferred to test tubes into which an equal volume of vanillin reagent (8%) was added followed by 72% (v/v) sulfuric acid. The mixture was mixed and placed in a water bath adjusted at 60°C for 10 min. The tubes were cooled on an ice-cold water bath for 3 to 4 min, and the absorbance of the yellow color reaction mixture was measured at 544 nm using a UV-Vis spectrophotometer (SHIMADZU UV-1800) against a blank containing 50% aqueous ethanol instead of sample extract.

Absorbance = 0.0005 Diosgenin ($\mu\text{g/ml}$) – 0.042 ($r^2=0.9983$) (Fig. 6).

All the tests were carried out in triplicates and the results were expressed as μg Diosgenin equivalents per mg crude extract.

Estimation of total tannins

The total tannin was determined using the method [24,25] with minor modification.

Briefly, 0.1 ml of extract (1 mg/ml), 6.5 ml of water and 0.5 ml of the Folin–Ciocalteu and 1.5 ml of 20% sodium carbonate at overnight standard solution were added and incubated at 1 h the absorbance of the sample was measured in UV-vis spectrophotometer (SHIMADZU UV-1800) against the blank at 725 nm.

Absorbance = 0.0192 tannic acid + 0.015 ($r^2=0.9961$) (Fig. 7).

All the tests were carried out in triplicates and the results were expressed as μg of tannic acid equivalents per mg of extract.

Estimation of total protein

The total protein content was estimated using the method of [26]. To 1 ml of each test sample, 4 ml of freshly prepared alkaline solution (prepared by mixing 50 ml of 2% Na_2CO_3 in 0.1 N NaOH and 1 ml of 0.5% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in 1% sodium potassium tartrate) was added at room temperature and kept undisturbed for 10 min. Subsequently, to each of these mixtures tubes 0.5 ml of Folin–Ciocalteu reagent was added and after half an hr, the OD of each sample was measured at 660 nm using UV-vis Spectrophotometer (SHIMADZU UV-1800) against the blank (without protein sample).

Absorbance = 0.0098 BSA + 0.015 ($r^2=0.9982$) (Fig. 8).

All the tests were carried out in triplicates, and the results were expressed as μg of protein equivalents per mg of extract.

Estimation of total carbohydrate

The total carbohydrate content was estimated by the method of [27]. To 1 ml of each sample 4 ml of anthrone reagent (0.2% anthrone was dissolved in ice-cold concentrated sulfuric acid, prepared fresh before use) was added, heated for 8 min in boiling water bath, cooled rapidly and the OD of each was measured at 630 nm using spectrophotometer against the blank (without glucose sample).

Absorbance = 0.0093 glucose + 0.063 ($r^2=0.9953$) (Fig. 9).

All the tests were carried out in triplicates, and the results were expressed as μg of glucose equivalents per mg of extract.

RESULTS

In the present investigation, the endophytic fungi *Aspergillus* sp. 3 (Fig. 10), isolated from the *B. diffusa* L. were analyzed for the presence of qualitative phytochemicals (Table 1) and quantitative phytochemicals (Table 2 and Figs. 1-9).

DISCUSSION

Medicinal plants are used in traditional medicine system from thousands of years and still continue to give humankind with molecules of therapeutic potentials. Fungal endophytes associated with these plants are also a rich source of novel compounds. Both the medicinal plant and the endophytic fungi are potent sources of natural products. These are also excellent sources of phytochemicals with antioxidant, anti-inflammatory, anticancer, and antimicrobial activity [28].

In the present study, total phenolic, flavonoid, alkaloids, sterols, terpenoids, tannins, etc. content from endophytic fungi isolated from *B. diffusa* L. were quantified. Total flavonoid content was more when compared to the total phenol content which is similar to the findings of [29,30].

CONCLUSION

Hence, we conclude that the endophytic fungi isolated from the *B. diffusa* L. play an important role in discovering and developing new drugs which will be more effective with no side action-like most synthetic drugs.

Table 1: Qualitative phytochemical analysis of endophytic fungi-8

Isolates	Alkaloids		Flavonoids		Phenol		Steroids		Terpenoids		Saponins		Tannins		Carbohydrate		Proteinaminoacids	
	Wagner test	Hagers tests	Alkaline reagent tests	Shinoda tests	Ferric chloride test	Lead acetate test	Salkowski test	Liebermannburchard test	Salkowski test	Liebermannburchard test	Frothing tests	Ferric chloride test	Molisch test	Biuret test	testninhydrin test	Ninhydrin test	testninhydrin test	Ninhydrin test
Preliminary phytochemical analysis of ethyl acetate crude extract of the EF																		
EF8 i.e., <i>Aspergillus</i> sp. 3	+	+	+	+	-	+	+	+	+	-	+	+	+	+	+	+	+	+
Preliminary phytochemical analysis of methanol crude extract of the EF																		
EF8 i.e., <i>Aspergillus</i> sp. 3	+	+	+	-	+	+	+	+	+	-	+	+	+	-	-	-	-	+
Preliminary phytochemical analysis of chloroform crude extract of the EF																		
EF8 i.e., <i>Aspergillus</i> sp. 3	+	+	-	-	-	+	+	+	+	-	-	-	+	-	-	-	-	-

All the experiments were performed in triplicates. EF: Endophytic fungi

Table 2: Quantitative phytochemical analysis of endophytic fungi-8

Isolate number/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-sitosterol/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, mean value\pmSD									
EF8 i.e., <i>Aspergillus</i> spp. 3	1.746 \pm 0.0057	17.666 \pm 0.577	11.666 \pm 0.577	9.746 \pm 0.0057	0.446 \pm 0.0057	2.246 \pm 0.0057	3.996 \pm 0.0057	399.666 \pm 0.577	224.666 \pm 0.577
Quantitative phytochemical analysis of methanol crude extract, mean value\pmSD									
EF8 i.e., <i>Aspergillus</i> spp. 3	1.646 \pm 0.0057	15.666 \pm 0.577	10.666 \pm 0.577	8.746 \pm 0.0057	0.296 \pm 0.0057	2.146 \pm 0.0057	3.466 \pm 0.057	389.666 \pm 0.577	219.666 \pm 0.577
Quantitative phytochemical analysis of chloroform crude extract mean, value\pmSD									
EF8 i.e., <i>Aspergillus</i> spp. 3	0.996 \pm 0.0057	7.666 \pm 0.577	4.666 \pm 0.577	3.466 \pm 0.057	0.146 \pm 0.0057	1.746 \pm 0.0057	2.966 \pm 0.0057	374.666 \pm 0.577	209.666 \pm 0.577

All the experiments are performed in triplicates and (mean value \pm SD) was calculated. 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, SD: Standard deviation, BSA: Bovine serum albumin, AE: Aqueous extract, GAE: Gallic acid equivalent, DE: Dry extract

AUTHOR'S CONTRIBUTION

All the authors contribute equally to the manuscript.

CONFLICT OF INTEREST

None.

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