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Antibacterial, antioxidant and antidiabetic assays of endophytic fungi isolated from *Commelina benghalensis*

Tejaswini Halsoor Kempegowda[1,*] and Jayashankar Maddaya[1]

[1]Department of Science and Research in Microbiology, Post Graduate Centre, Mangalore University, Kodagu, India
[*]Corresponding author: tejaswinigowda1393@gmail.com

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Abstract

Seven unique fungal morphotypes were tested for antibacterial, antioxidant, and anti-diabetic activity using ethyl acetate extract. Antimicrobial activity was evaluated for crude ethyl acetate extracts against pathogenic bacteria such as *E. faecalis E. coli, S. typhi*, and *Sh. sonnei* using an agar well diffusion assay. *N. clavispora* showed a good zone of inhibition activity against pathogens with MIC (5 to 10 µg/mL) and MBC (10-15 µg/mL). A preliminary qualitative phytochemical analysis and secondary phytochemical screening was conducted on the fungal extract using standard qualitative procedures that revealed the presence of phenols and flavonoids. In which crude extract of *N. clavispora* showed maximum flavonoid and phenol content. Total phenolic and flavonoid content for *N. clavispora* was 11.93 ± 0.15 mg/mL GAE and 43.36 ± 0.44 mg/mL QE respectively. DPPH (78.88%) and FRAP (61.23 ± 0.43 mg/mL AAE) assay were studied as supportive analyses for antioxidant activity. The α-amylase inhibition assay revealed 42.18 % inhibition and showed antidiabetic efficacy. The active fungal isolate N4 was further identified using 18S rRNA sequencing analysis. The volatile metabolites in the ethyl acetate extract of *N. clavispora* were identified using gas chromatography-mass spectrometry (GC-MS) where primary components alpha- methyl 1-adamantanemethylamine, propanedioic acid, propyl, and heptyl urea have high peak area. This discovery suggests that *N. clavispora* isolated from *Commelina benghalensis* might be a new source of bioactive compounds. The data gathered has served as the foundation for its use in the pharmaceutical sector in the form of traditional and folk medicine.

Keywords: Endophyte, Sequencing, Ethyl acetate, Antimicrobial, α-amylase, Secondary metabolites

1. Introduction

Microorganisms that reside within plant tissues without causing any harm to their host are termed endophytes, and their relationship with the host fluctuates due to abiotic and biotic stress faced by the plant that serves as a host [1]. Endophytic fungi are abundant in all plants and are considered a potential reservoir for bioactive compounds that are precursors for plant protection and new drug discovery [2]. Fungal endophytes isolated from medicinal plants showed various potential and novel bioactive compounds for the effective treatment of numerous diseases [3]. Some of the naturally occurring substances that plant hosts create are also produced by the endophytes that live on those plants. One example of this is taxol, produced by numerous endophytes of *Taxus* spp., such as *Taxomyces andreannae*, *Fusarium* sp., and *Taxomyces* sp. [4,5]. It hasbeen hypothesized that this may have occurred due to genetic recombination between endophytes and their plant species [6,7]. As a result of their enormous genetic diversity and biological novelty, endophytes play a vital role in pharmacology and agriculture [8]. Since endophytes can exchange genes with their hosts, they can produce secondary metabolites that are structurally identical to those produced by the hosts [9]. Examination of the chemical composition of the secondary metabolites produced by those fungi in different bioassays reveals the wide variety of ecological roles they play [10]. Bacteria, fungi, and actinomycetes are a few of the endophytic microorganisms discovered to date; thousands of them can produce secondary metabolites comparable to those of their host plant. Alkaloids,

benzopyranones, phenolic acids, flavonoids, quinones, chinones, terpenoids, xanthones, and steroids are increasingly important because they are co-produced by hosts and their fungal endophytes [6]. Few of the biological applications of bioactive compounds produced by an endophytic fungus are useful to modern medicine, agrochemicals, Antibiotics, cancer therapies, antioxidants, insect repellents, cytotoxins, and immunosuppressants [11].

Since prehistoric times, green leafy vegetables have been used as a food source as they contain many nutrients and minerals that help in maintaining human health. *Commelina benghalensis* L. is an annual or perennial herb commonly called Benghal dayflower belongs to the *Commelinaceae* family. *C. benghalensis* leaves and stem is used in traditional medicine as an anti-inflammatory, anticancer, and anti-diarrheal substance. Other medical advantages have been reported, including therapy for aching feet, sore throat, burns, eye irritation, newborn thrush, stomach discomfort, skin problems, and sleeplessness [12]. The antibacterial and antioxidant activities of the crude extract have been linked to its wound-healing effects [13] and the phytochemicals such as tannin, alkaloid, flavonoid, sterol glycoside, and others, as well as substances such as noctacosanol, n-tricotanol, stigma-sterol, campesterol, and hydrocyanic acid, have been detected in both the vegetative and blooming sections of the plant *C. benghalensis* [14]. Although the plant has many potential applications, little is known about its biology from endophytic fungi or the substances that give its useful properties. Therefore, the current study investigated the antibacterial, antioxidant, and antidiabetic activities of endophytic fungi isolated from *C. benghalensis* to determine its secondary metabolite content appropriate for therapeutic usage.

2. Materials and methods

2.1 Isolation and identification of endophytic fungi from C. benghalensis

The edible leaves of *C. benghalensis* were collected in Chikka Aluvara, Kodagu District, Karnataka, India. On the same day, fresh, healthy leaves with no signs of infections were picked and delivered to the laboratory to isolate fungal endophytes. The leaves were surface sterilized according to the protocol [15] with minor modifications using 70% ethanol for 1 min, 4% sodium hypochlorite for 3 min, and then to remove any residues of alcohol, the leaves were thoroughly rinsed in sterile distilled water and dried on a sterile blotting paper. Using sterile lancet blades, each leaf was cut into bits from its central axis. To prevent bacterial growth, each bit was inoculated on Sabouraud dextrose agar (SDA) medium supplemented with streptomycin (1 mg/mL) and incubated in the dark for seven days at 28°C. After incubation for seven days, the growth of emerging hyphae was aseptically removed and placed on SDA plates. Under a bright-field microscope, the obtained endophytic fungal strains were stained with cotton blue for microscopic characteristics [16]. The front and reverse sides of fungal colonies were identified morphologically based on the [17] manual.

The most active endophytic fungal isolate (N4) was subjected to molecular identification. The DNA was extracted by Cetyl trimethyl ammonium bromide ((CTAB) buffer, 100 mM Tris-HCl, 20 mM Ethylene diamine tetraacetic acid (EDTA) pH. 8.0 1M NaCl) method using extra (EX) pure microbial deoxy ribonucleic acid (DNA) isolation kit. Precipitated DNA was solubilized in Tris EDTA (TE) Buffer (100 mM Ethylenediamine tetra acetic acid, 100mM Tris(hydroxymethyl)aminomethane hydrochloride (Tris HCI) pH 8) PCR analysis was performed using Taq master mix containing Taq DNA polymerase supplied in 2X Taq buffer, 0.4 mM eoxynucleotides (dNTPs), 3.2 mM MgCl 2, and 0.02% bromophenol blue in thermal cycling conditions. To amplify the specific cloned genomic DNA fragments two universal primers ITS1F (5' AGAGTTTGATCTGGCTCAG 3') and ITS4R (5'TACGGTACCTTGTTACGACTT 3') were used. 5µl of isolated DNA was mixed with 25 µL of polymerase chain reaction (PCR) solution and 5 µL of deionized water. DNA amplification was initiated with the primary denaturation process maintained at 95°C for 5 min and then 25 cycles of second-step denaturation for 30 sec.
5 µL of isolated DNA was dissolved with 25 µL of PCR solution. DNA amplification was initiated with the primary denaturation process maintained at 95°C for 5 min and then 25 cycles of second-step denaturation for 30 sec. The PCR product was detected on 1% agarose gel electrophoresis. Further PCR product was sequenced as reported in [18] using ABI PRISM Big Dye. TM terminator cycle sequencing kits with Taq DNA polymerase and analyzed by Applied Biosystem sequence scanner software.

DNA sequences of 18s ribosomal ribonucleic acid (rRNA) gene from *C. benghalensis* were submitted for the Basic local alignment search tool algorithm (BLAST) accessible through the National Centre of Biotechnology Information (NCBI) website (http://www.ncbi.nim.nih.gov) followed by multiple sequencing alignment program CLUSTAL W 3.7 version. BLAST DNA sequences are aligned and 18s rRNA gene sequences of the strain were deposited to GENBANK for accession number.

2.2 Fermentation and extraction of secondary metabolites

Endophytic fungal isolates were inoculated in Sabouraud dextrose broth (SDB) for 21 days at 27±2°C and incubated in a rotary shaker at 150 rpm. Following the incubation time, the fermented broth was filtered through

a sterile muslin cloth, and an exact volume of solvent ethyl acetate was poured into a splitting funnel and vigorously shaken. The extraction process was repeated three times, and the resulting organic phases were concentrated in a 45°C rotor evaporator. Following that, the crude secondary metabolite extracts were stored at 4°C for future use [19].

2.3 Screening of fungal metabolites produced by endophytic fungi

2.3.1 Preliminary qualitative phytochemical screening of fungal metabolites produced by endophytic fungi

According to the methodology described by [20], isolated endophytic fungal extracts were subjected to qualitative phytochemical analysis to evaluate the presence of active components such as steroids, terpenoids, phenols, flavonoids, alkaloids, saponins, tannins, and glycosides.

2.3.2 Qualitative phytochemical screening of fungal metabolites produced by endophytic fungi

2.3.2.1 Estimation of total phenolic content (TPC)

To assess the total phenolic content of endophytic fungal extracts Folin-Ciocalteu (FC) test was used, according to [21], with slight modifications. Briefly, 1 mL of fungal extract was blended with 500 µL of FC reagent, then incubated at 27±2°C for 2 h in the dark condition. After incubation, 1 mL of saturated Na_2CO_3 solution and 2 mL distilled water were added, and the absorbance was measured at 765 nm. The blank was made by replacing methanol with the same quantity of diluted extract. Gallic acid was used as a standard, and the results were represented in mg/g gallic acid equivalent. Gallic acid was employed as a standard, and the results were expressed in mg/g of gallic acid equivalent.

2.3.2.2 Estimation of TFC

A colorimetric approach was used to identify the total flavonoid according to the procedure of [22] with minor modification. 1 mL of the fungal extract mixed with 0.3 mL of sodium nitrate solution (5% w/v), 0.6 mL of aluminum chloride solution (10%), and 5 mL of distilled water followed by incubation at room temperature for 30 min. The absorbance was measured at 510 nm using a spectrophotometer. The flavonoid content was expressed as mg quercetin equivalent per gram of extract.

2.4 Antioxidant assay

2.4.1 Radical-scavenging decolorize 1,1-diphenyl-2-picryl-hydrazyl (DPPH) assay

Antioxidant activity by decolorizing DPPH is assessed based on the potential of antioxidants to DPPH. According to [23] with some modification, the ethyl acetate fungal extracts were dissolved in methanol and combined with a 60 mM methanol solution of DPPH in a 1:1 ratio. The sample mixture was stored in the dark for 30 min and then absorbance was taken at 517 nm in a spectrophotometer. Methanol was used as a blank, DPPH solution without extract served as the negative control, ascorbic acid was used as the positive control and the results are reported as mean values. According to the equation, the % inhibition was calculated.

$$\%\text{DPPH radical scavenging activity} = \frac{\text{Absorbance of blank} - \text{Absorbance of sample}}{\text{Absorbance of blank}} \times 100 \quad (1)$$

2.4.2 Ferric reducing antioxidant power (FRAP) assay

The total antioxidant activity of the endophytic fungal extracts was assessed using the FRAP assay, which was modified slightly from [24]. 2.5 mL of phosphate buffer (0.2 M) and 2.5 mL of 1 % potassium ferricyanide was added to 1 mL of fungal extract. After 20 min of incubation at 50°C, 2.5 mL of 10% trichloroacetic acid was added to each combination. The mixture was shaken vigorously, and the solution was combined with 2.5 mL distilled water and 0.5 mL 0.1 % $FeCl_3$. The absorbance was measured at 700nm after 30 min of incubation. Each assay was carried out in duplicate and used ascorbic acid as a standard reference. A stronger reducing power is shown by increased absorbance of the reaction mixture.

2.5 Antibacterial assay

2.5.1 Agar well diffusion

2.5.1.1 Test organisms

Strains such as *Enterococcus faecalis Escherichia coli, Salmonella typhi* and *Shigella sonnei* were used to evaluate the antibacterial activity obtained from Yenepoya Medical College, Mangalore, India.

2.5.1.2 Antibacterial activity

The agar well diffusion assay method was used to evaluate the antibacterial activity against human foodborne pathogenic bacteria strains [25]. The antibacterial assay was performed using the well diffusion method according to [26] with some minor modifications. Nutrient agar (NA) medium was poured aseptically and 100 mL of bacterial liquid culture in an exponential growth phase was spread onto the surface of NA plate. All the culture plates were allowed to dry for about 5 min. wells were bored on the agar surface using a 6 mm cork borer and filled with 100 mL of endophytic fungal crude extract diluted by 1mg/mL in dimethyl sulfoxide (DMSO), negative control as DMSO, and positive control as streptomycin 1 mg/mL was loaded on seeded plates and then incubated at 37°C for 24 h. The tests were all done in triplicate (n=3), and the findings are expressed as mean standard deviation values.

2.5.2 Determination of minimum inhibitory (MIC) and minimum bactericidal concentrations (MBC)

MIC and MBC were determined by a modified micro-serial dilution method for the crude ethyl acetate extracts that displayed the highest antibacterial activity. In DMSO, a stock solution of the extract was produced in several concentration ranges ranging from 0 to 100 mg/mL, dilution was carried out on nutrient broth adjusted to 0.5 Mc Farland standard. The plates were sealed and incubated overnight at 37°C. To assess cell viability, 15 µL of 0.01 percent resazurin (0.1 mg/mL) was added, then the cells were re-incubated for 4 h. The MIC was determined by sub-culturing from wells with no bacterial growth, and MBC was determined by sub-culturing from wells with no bacterial growth. The MBC was defined as the lowest concentration of extract that resulted in the full death of the specified bacterial test species [27].

2.6 Antidiabetic activity

2.6.1 Alpha-amylase inhibition (AAI) assay

The reaction mixture was made up of 50 µL, 100 µL, and 200 µL of various concentrations of the fungal extract. The reaction was started by adding 10 µL -amylase (1 mg/mL in 0.1 M sodium phosphate buffer, pH 7.0) in a 150 µL starch solution containing 1% starch and 17mM NaCl, and it was incubated at 27±2°C. The reaction was halted by adding 20 µL of NaOH solution (2M) to the reaction mixture, followed by 20 µL of dinitrosalicylic acid, which was then put in a water bath for 20 min. After cooling, the reaction mixture was diluted with 5 mL of distilled water, and the absorbance was measured at 540 nm. The enzyme solution was replaced with 40 µL of buffer to make blank tubes. The control was made without extract and the standard was made with acarbose in the same way. The studies were carried out three times, each time following the identical technique [28]. The inhibitory activity of alpha-amylase was estimated using the formula:

$$\%\ \text{Inhibitory activity} = \frac{\text{Absorbance}_{540}\ (\text{Control}) - \text{Absorbance}_{540}\ (\text{Extract})}{\text{Absorbance}_{540}\ (\text{Control})} \quad (2)$$

2.7 Gas chromatography-mass spectrometry (GC-MS) analysis

GC-MS analysis was used to discover secondary bioactive chemicals in the most active fungal extracts. The study employed a Clarus 680 gas chromatograph with a fused silica column filled with Elite-5MS (5% biphenyl 95% dimethylpolysiloxane, 30 m × 0.25 mm ID 250 µm degree of freedom (df)) to segregate the components using Helium as the carrier gas at a constant flow rate of 1 mL/min. During the chromatographic run, the injector temperature was set to 260°C. The oven temperature was as follows for the 1L of extract sample introduced into the instrument: 60°C for 2 min, then 300°C at a rate of 10°C per min for 10 min, then 300°C for 6 min. The mass detector was set to 230°C for the transfer line, 230°C for the ion source, and 70 eV for the ionization mode electron impact, with a scan time of 0.2 sec and a scanning interval of 0.1 sec. The fragments range in size from 40 to 600 Da. Component identification was carried out using computer searches in the National Institute of Standards and Technology (NIST) version 2008 [29].

2.8 Statistical analysis

All the tests were done in triplicate and the average ± standard deviations (SD) were calculated. One-way analysis of variance (ANOVA) was used to evaluate statistical significance, and then Tukey was used to determine statistical significance. *p*-value of <0.05 in the post hoc test was statistically significant.

3. Results and discussion

3.1 Isolation, characterization, and identification of fungal endophytes

Out of fifteen, a total of seven different fungal morphotypes were selected from surface-sterilized leaf segments of *C. benghalensis* (Figure 1) using SDA media. Based on morphological and microscopic features, seven fungal endophytes were identified as *Penicillium* sp., *Acromonium* sp., *Fusarium* sp., *Neopestalotiopsis* sp., *Fusarium* sp., *Penicillium* sp., and *Fusarium* sp. it was also observed that *Fusarium* sp. were most dominant, followed by *Penicillium* sp. The host specificity was observed and correlated with the findings of [30] who reported eight endophytic fungi recovered from the same plant *C. benghalensis* with maximum *Fusarium* species. These results revealed that isolated endophytic fungi are commonly present in *C. benghalensis* in different environmental conditions. One active isolate N4, *Neopestalotiopsis* sp. (Figure 2), chosen for molecular investigation in order to get precise identification. The rDNA-ITS region was amplified, sequenced, and the given accession number MW487806 were subjected to the NCBI GenBank. The sequence analyzed through Basic Local Alignment Search Tool-Nucleotide (BLASTn) program revealed the endophytic fungal isolate N4 found to be *Neopestalotiopsis clavispora* which showed 46% similarity to the type of sequence MT151848 (Table 1) and depicted 98% query coverage with 0.0 E value respectively. The *N. clavispora* has already been reported by various researchers from the medicinal plant [31,32].

Figore 1 *C. benghalensis* leaves.

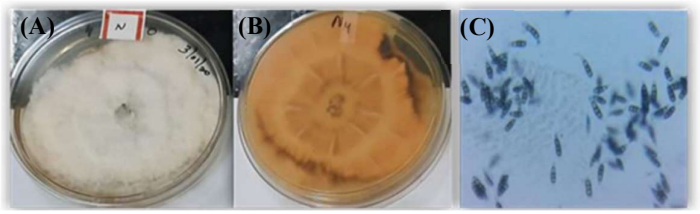


Figure 2 Colony morphology and microscopic image of *N. clavispora*; (A) top view, (B) bottom view, and (C) conidia with apical appendages.

Table 1 Blast result sequences producing significant alignments of *N. clavispora*.

Description	Max score	Total score	Query coverage	E value	Per. Ident.	Accession
N. clavispora strain IHI 201606 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence	905	905	98%	0.0	99.80%	MT151848.1
Fungal endophyte strain SV1888 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	905	905	98%	0.0	99.80%	KY765293.1

Table 1 (continued) Blast result sequences producing significant alignments of *N. clavispora*.

Description	Max score	Total score	Query coverage	E value	Per. Ident.	Accession
Fungal endophyte strain SV1887 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	905	905	98%	0.0	99.80%	KY765292.1
Fungal endophyte strain SV1886 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	905	905	98%	0.0	99.80%	KY765291.1
Fungal endophyte strain SV1885 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	905	905	98%	0.0	99.80%	KY765290.1
Fungal endophyte strain SV1882 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	905	905	98%	0.0	99.80%	KY765288.1
Fungal endophyte strain SV1841 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	905	905	98%	0.0	99.80%	KY765256.1
Fungal endophyte strain SV1822 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	905	905	98%	0.0	99.80%	KY765246.1
Neopestalotiopsis sp. strain F2186 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	905	905	98%	0.0	99.80%	KU747934.1
Neopestalotiopsis sp. strain F2155 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	905	905	98%	0.0	99.80%	KU747922.1

3.2 Screening of fungal metabolites

Numerous beneficial bioactive substances with antibacterial, antidiabetic, immune-suppressing, insecticidal, cytotoxic, and anticancer activity have been effectively isolated from endophytic fungus during the last two decades. Alkaloids, terpenoids, steroids, quinones, lignans, phenols, and lactones are some possible are some of the bioactive substances which can be classified. In recent years, endophytes have gained a reputation for being an excellent source of secondary metabolites and naturally occurring bioactive antimicrobial compounds.

3.3 Preliminary qualitative screening of fungal metabolite

Based on the findings of the qualitative analysis of fungal metabolites, it can be inferred that the isolated fungal ethyl acetate extracts contain phenols, flavonoids, alkaloids, saponins, and glycosides. However, there was no evidence of steroids in the fungal extract. Terpenoids and Tannins can only be found in a single extract. This

investigation was carried out to determine different fungal metabolites present in the seven endophytic fungal isolates that were recovered.

In the present study, the preliminary screening of fungal metabolite compounds in ethyl acetate extracts of all the isolated endophytic fungi isolated from *C. benghalensis* indicates the presence of different fungal metabolites Table 2. This shows the ability of endophytic fungi for providing antimicrobial activity. The *Penicillium* sp. showed positive for phenol, Flavonoid, alkaloid, and tannin whereas, terpenoid, steroid, saponin, and glycoside showed negative results. The *Acromonium* sp. showed positive for phenol, Flavonoid, alkaloid, terpenoid, and saponin whereas, steroids, tannins, and glycosides showed negative results. The *N. clavispora* showed positive for Flavonoid, alkaloid, and saponin whereas phenol, terpenoid, steroid, tannin, and glycoside showed negative results. The *Fusarium* sp. (N3, N5, N7) showed positive for Flavonoid, alkaloid, and glycoside whereas phenol, terpenoid, steroid, saponin, tannins showed negative results. Phytochemical analysis of crude extracts of *C. benghalensis* contains phytochemicals like phenols, alkaloids, flavonoids, triterpenoids, tannins, carbohydrates, steroids, carotenoids, amino acids, proteins [33,34] which play different roles in the therapeutics like antimicrobial, antioxidants, anti-inflammatory, anti-allergic, anticancer activity [35]. This study reveals the presence of various secondary metabolites in different fungal extracts known to produce biological action favorable to the host.

Table 2 Phytochemical analysis.

Fungal Isolate	Phytochemicals							
	Phenol	Flavonoids	Alkaloids	Terpenoids	Steroids	Saponin	Tannins	Glycosides
Penicillium sp. N1	-	+	+	-	-	-	-	-
Acromonium sp. N2	+	+	+	+	-	+	-	-
Fusarium sp. N3	-	-	+	-	-	-	-	-
N. clavispora N4	-	+	+	-	-	+	-	+
Fusarium sp. N5	-	+	-	-	-	-	-	-
Penicillium sp. N6	+	-	-	-	-	-	+	-
Fusarium sp. N7	-	-	+	-	-	-	-	+

+: present, -: Absent.

3.4 Secondary quantitative screening of fungal metabolite

3.4.1 Estimation of TPC and Flavonoid content (TFC)

The amount of total phenol was determined with the Folin-Ciocalteu reagent. Gallic acid was used as a standard compound and the total phenols were expressed as mg/g gallic acid equivalent. Whereas the amount of total flavonoid was determined using sodium nitrate and aluminum chloride solution, quercetin was used as a standard compound and the total flavonoid was expressed as mg/g Quercetin equivalent.

TPC and TFC of ethyl acetate extract from seven endophytic fungi isolated from *C. benghalensis* were presented in Figure 3. When compared to the standard gallic acid (32.14 mg/mL) and quercetin (74.04 mg/mL) the maximum phenolic and flavonoid content of ethyl acetate fungal extract was found in *N. clavispora* 11.93 ± 0.15 mg gallic acid equivalent (GAE)/mL and 38.36 ± 0.44 mg quercetin equivalent (QE)/mL respectively. However, the TPC and TFC of another six isolates ranged from 1.98 to 10.0 mg GAE/mL, and from 12.33 to 32.15 mg QE/mL respectively. Following the present results, previous studies have demonstrated that *C. benghalensis* plants have fungal metabolites [36]. According to [14] reported that the whole plant of *C. nudiflora* was extracted from different solvents and all the solvent extract showed the presence of flavonoids, Cardiac glycosides were present only in acetone extract, Chloroform extract showed the presence of sterols. Terpenoids are present in chloroform and acetone extract. Tannins were present in the ethanolic extract, whereas saponin and philobatamin were absent in all the extracts. As per our knowledge, this is the initial study reporting the TPC and TFC in ethyl acetate extract of endophytic fungi isolated from *C. benghalensis*.

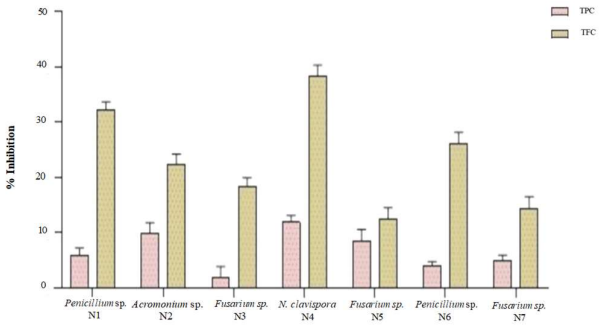


Figure 3 Total phenolic content and total flavonoid content of ethyl acetate extracts of fungal isolates.

3.5 DPPH and FRAP

The antioxidant activity of isolated fungal ethyl acetate extracts was determined by using a methanol solution of DPPH and FRAP assay (Figure 4). The ethyl acetate extract of *N. clavispora* showed the highest antioxidant activity with a DPPH of 78.88% with standard ascorbic acid (97.71%) and FRAP value of 61.23 ± 0.43 mg AAE/mL with standard ascorbic acid (92.4 mg AAE/mL) followed by *Penicillium* sp. N1 66.30% and 55.16 mg AAE/mL of DPPH and FRAP respectively. Nevertheless, the lowest antioxidant activity was recorded from the isolate of *Fusarium* sp. N3 was 21.57% and 16.51 mg AAE/mL of DPPH and FRAP respectively. When compared to the standard ascorbic acid the isolated fungal extract showed minimum activity. To the best of our knowledge, the antioxidant activity of ethyl acetate endophytic fungal extracts isolated from C. *benghalensis* has not been reported so far. According [37] examined the antioxidant activity of whole plant extract *C. benghalensis* using different solvents and found DPPH scavenging activity was 87.47% at a concentration of 250 µg/mL in ethanolic extract. Another study conducted on *C. nudiflora* by [14] investigated using three different solvents where the plant *C. nudiflora* showed significant antioxidant activity in all extracts and the IC_{50} value of ethanolic extract was 11.25 mg/mL, Acetone extract showed an IC_{50} value of 17.5 mg/mL and Chloroform extract showed a comparatively less IC_{50} value of 21.25 mg/mL and conclude that the antioxidant activity might be due to the presence of flavonoids in the extracts and states that *Commelina nudiflora* has potential antioxidant biomolecule.

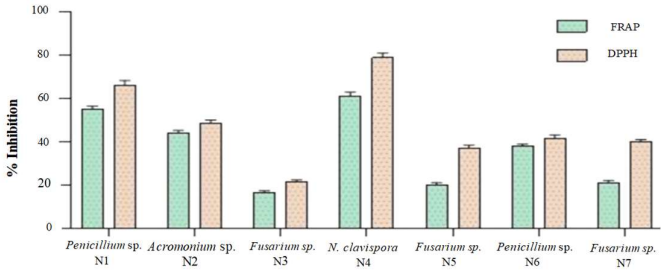


Figure 4 Antioxidant activity by DPPH and FRAP assay on isolated endophytic fungi.

3.6 Antibacterial activity

The antibacterial activity of isolated seven endophytic fungi tested against selected foodborne pathogenic Gram positive and Gram-negative bacteria by using the agar well diffusion method which has been reported in Table 3. As Table 3 shows, there are significant differences ($p< 0.05$) between the fungal isolates against tested bacterial pathogens. Three fungal isolates (*Penicillium* sp. N1, *Fusarium* sp. N3, and *N. clavispora N4*) exhibited highest activity (22 mm, 15 mm, and 24 mm) respectively against *S. typhi* in comparison with standard streptomycin (30 mm). Interestingly, *N. clavispora* showed significant inhibition activity (11 mm, 24 mm, 13 mm, and 12 mm) against the rest pathogens, *E. coli*, *S. typhi*, *Sh. sonnei*, and *E. faecalis* respectively. While other fungal isolates showed moderate inhibitory effects toward tested bacteria. The results revealed the broad-spectrum nature of fungal isolates against gram-positive and gram-negative pathogens, which is comparable to previous studies reporting the isolation of endophytic fungi from medicinal plants in the Virudhunagar region [38]. Endophytes are those organisms that colonize the living internal tissues of their hosts without causing detectable symptoms. Several fungal endophytes have been isolated from a variety of plant species which have proved to be a rich source of secondary metabolites. In this present work, a total of seven fungal endophytes were isolated from *C. benghalensi*. *Penicillium* sp.*, Acromonium* sp.*, Fusarium* sp., *N. clavispora, Fusarium* sp., *Penicillium* sp.*, and Fusarium* sp., was the main isolate and screened for the antibacterial production of secondary metabolites. Similarly, eight species of fungal endophytes were successfully isolated from *Commelina diffusa,* three fungi (CDBE 2, CDRE 1, and CDRE 2) showed potential activity against *A. niger* and two of the eight fungi (CDLE 1 and CDRE 2) showed moderate inhibitory effect towards *E. coli* with an inhibition zone of more than 12 mm, while other fungi produced inhibition less than 10 mm on *P. aeruginosa*, *B. megaterium, S. aureus* [39].

The active isolate, *N. clavispora* has a potential zone of inhibition hence MIC and MBC values of that fungus were tested against *E. coli*, *S. typhi*, *Sh. Sonnei*, and *E. faecalis* with a microdilution method which has been reported in Table 4. The MIC value of the isolate *N. clavispora* showed 5 µg/mL against *E. coli*, *S. typhi*, and *E. faecalis* where *Sh. sonnei* showed 10 µg/mL. The MBC values of the isolate *N. clavispora* ranged between 10-15 µg/mL against tested bacterial pathogens. The findings of the current study are consistent with those of [39] who studied the bi, and *Sh. sonnei* oactivity of endophytic fungi from *C. diffusa*.

Table 3 Antibacterial activity screening of isolated fungal endophytes.

Fungal name and code	Zone of inhibition (mm)*			
	E. coli	*S. typhi*	*S. sonnei*	*E. faecalis*
Penicillium sp. N1	8.0 ± 0.5^{e}	22.0 ± 0.2^{b}	11.0 ± 0.1^{b}	10.0 ± 0.0^{c}
Acromonium sp. N2	10.0 ± 0.0^{d}	8.0 ± 0.5^{a}	12.0 ± 0.1^{b}	11.0 ± 0.3^{c}
Fusarium sp. N3	9.0 ± 0.1^{a}	15.0 ± 0.0^{e}	11.0 ± 0.4^{c}	13.0 ± 0.0^{c}
N. clavispora N4	11.0 ± 0.1^{a}	24.0 ± 0.0^{d}	13.0 ± 0.1^{e}	12.0 ± 0.3^{e}
Fusarium sp. N5	13.0 ± 0.5^{e}	11.0 ± 0.1^{a}	9.0 ± 0.0^{c}	15.5 ± 0.1^{d}
Penicillium sp. N6	8.0 ± 0.0^{e}	10.0 ± 0.0^{d}	11.0 ± 0.2^{b}	12.0 ± 0.0^{d}
Fusarium sp. N7	12.0 ± 0.0^{c}	9.0 ± 0.2^{b}	8.0 ± 0.4^{a}	10.0 ± 0.1^{e}
Streptomycin (1 mg/mL)	25.0 ± 0.3^{a}	30.0 ± 0.0^{a}	22.0 ± 0.5^{a}	28.0 ± 0.3^{a}

*Values are means of three independent replicates ± standard deviation. This means in the same column with different letters are significantly ($p < 0.05$) different.

Table 4 Minimum inhibitory and minimum bactericidal concentrations (µg/mL) of *N. clavispora* extract foodborne bacteria.

N. clavispora	*E. coli*		*S. typhi*		*Sh. sonnei*		*E. faecalis*	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
	5	10	5	15	10	15	5	10

3.7 Antidiabetic activity

The antidiabetic analysis was assessed using an alpha-amylase inhibition assay. Amylase is an enzyme that hydrolyzes starch into glucose and effectually reduces glucose absorption [40]. The inhibitory activity of alpha-amylase was seen in all the fungal isolates in this investigation and is represented in Figure 5. The ethyl acetate extract of *N. clavispora* has the strongest inhibitory efficacy with 42.18 % whereas, *Fusarium* sp. showed the lowest inhibitory activity 9.09% compared to standard acarbose 68.50%. The other isolates *Penicillium* sp. (36.16%), *Acromonium* sp. (29.09%), *Fusarium* sp. (22.72%), *Penicillium* sp. (20.90%), and *Fusarium* sp. (13.63%) exhibited moderate inhibitory activity. According to [41] five endophytic fungi were isolated from *Eugenia polyantha* where each isolate of the endophytic fungi inhibited the enzyme at different rates. Among the five isolates, KSP-4 had the lowest activity. On the contrary, KSP-5 had the highest percentage of inhibitory activity, but it was lower than standard acarbose. Other findings support the ideas of [42] who reported a significant antidiabetic activity of *C. benghalensis* in the male Albino rat.

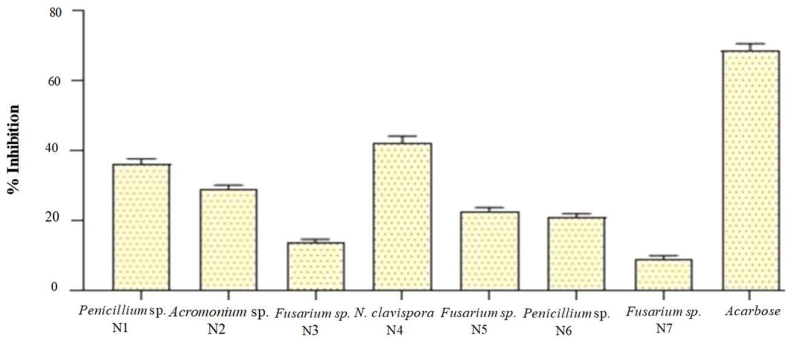
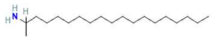
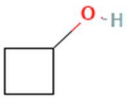
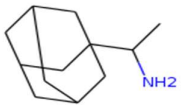
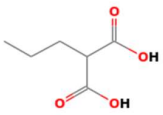
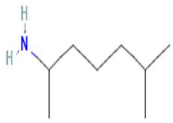
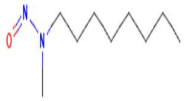
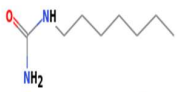
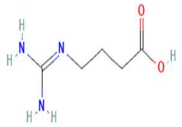


Figure 5 Alpha-amylase inhibitory activity of endophytic fungal isolates.

3.8 GC-MS analysis

The potential ethyl acetate extract *N. clavispora* was further studied to detect the bioactive compounds by using GC-MS. The active secondary metabolites from *N. clavispora* were extracted from ethyl acetate, where ten different bioactive compounds were detected based on their retention time (RT), compounds name, peak area %, Molecular formula, and Molecular weight are presented in Table 5. The result revealed the presence of 2-amininononadecane (9.24%), Cyclobutano (9.21%), 1-adamantanemethylamine, alpha-methyl (13.86%), Propanedioic acid, propyl (10.31%), 1-methyldecylamine (7.41%), Octodrine (7.73%), 1-octanamine, n-methyl-n-nitroso (6.87%), 1-methyldecylamine (8.87%), Urea, heptyl (9.98%), Gamma.-guanidinobutyric acid (6.25%). The bioactive properties of these compounds are still under research, and it is possible this compound possess biological activity such as antimicrobial, antioxidant as well as antidiabetic.

Table 5 Identified bioactive compounds from ethyl acetate fungal *N. clavispora* extract.

Peak	RT	Compound name	Structure	% of Area	Molecular formula	Molecular weight	Biological activity
1	9.494	2-aminononadecane		9.241	C19H41N	283 g/mol	Antibiofilm agents [43]
2	12.568	Cyclobutanol		9.218	C4H8O	72 g/mol	Binding affinity to beta cyclodextrin
3	18.993	1-adamantanemethylamine, alpha.-methyl		13.860	C12H21N	179 g/mol	Prevention of Asian influenza [44]
4	22.143	Propanedioic acid, propyl		10.318	C6H10O4	146 g/mol	Used as reactants and Intermediate
5	23.433	1-methyldecylamine		7.412	C11H25N	171 g/mol	Used in an anticancer drug screen
6	25.513	Octodrine		7.739	C8H19N	129 g/mol	stimulant drug [45]
7	32.543	1-octanamine, n-methyl-n-nitroso		6.872	C9H20ON2	172 g/mol	Used in Animal carcinogenicity [46]
8	37.618	1-methyldecylamine		8.873	C11H25N	171 g/mol	Used in an anticancer drug screen
9	39.783	Urea, heptyl		9.986	C8H18ON2	158 g/mol	Ptotein denature [47]
10	44.274	Gamma.-guanidinobutyric acid		6.255	C5H11O2N3	g/mol	Tumor growth inhibition and anticancer drug screen

4. Conclusion

Endophytic fungi from edible plants are gaining attention as these plants tend to produce natural components which are useful for mankind. Several edible plants have been screened for endophytes but still, the knowledge of endophytes from edible green plants is scanty. Fungal endophytes are a group of microorganisms that have not been studied thoroughly, but they are a rich and reliable source of bioactive and chemically unique substances that have the potential to be used in a broad range of pharmacological and industrial fields. Certain plants and the endophytes that are connected with host plants create the same naturally occurring compounds. Investigations have been carried out on *C. benghalensis* commonly used in Karnataka as a local herbal remedy. This study shows

that endophytes can thrive within plants and suggest that the fungal endophytes are heavily colonized, and some fungal endophytes are host specific. The isolated endophytic fungi showed promising antibacterial, antioxidant and antidiabetic activity. Endophytic fungi from *C. benghalensis* have the potential to produce bioactive molecules. However, more in-depth research on these isolated endophytes is required. It is possible that by cultivating them on a big scale, altering the culture, and providing certain stimulants, helps in improved production of specific bioactive chemicals.

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