

**Antifungal activity and phytochemical analysis of ethyl acetate extract of endophytic fungi isolated from *Citrus limon*.**

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**ABSTRACT**

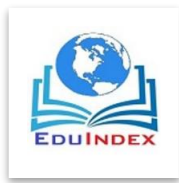
Twenty-two endophytic fungi were isolated from leaves of *Citrus limon*. The antifungal activity of endophytic fungi isolated from *Citrus limon* was tested against plant pathogenic fungi *Alternaria alternata*, *Penicillium notatum*, *Fusarium oxysporum* and *Trichoderma viride* by agar well diffusion method. Endophytic fungal extract showing highest antifungal activity was used for molecular characterization and phytochemical analysis. *Cladosporium cladosporioides* was identified after BLAST analysis which showed maximum zone of 30 mm against *Alternaria alternata* followed by *Trichoderma viride* (20 mm), *Penicillium notatum* (16 mm) and *Fusarium oxysporum* (15 mm).

**Keywords** – *Citrus limon*; antifungal activity; pathogenic fungi; phytochemical analysis.

**• INTRODUCTION**

The Citrus species belongs to family Rutaceae and is a very important commercial plant with an annual production of 123 million tons in 2010. Lemon (*Citrus limon*) occupies one-third of *Citrus* species after orange and mandarin [1]. Lemon is rich in natural compounds including citric acid, ascorbic acid, minerals, flavonoids, phenols and essential oils. Their phenolic compound and flavonoids are used in fields such as pharmacology and food technology [2]. Biological control of plant diseases has been considered using in farming system to get rid of chemical fertilizers [3].

Fungal endophytes are currently being explored for their bioactive compounds including medicines and agrochemicals like plant growth regulator substances and bio-herbicides [4]



Endophytic fungi are microorganisms which occupy micro-niches within the plant tissues without causing any apparent symptom [5, 6] and are also found to promote plant growth [7]. They are found to be ubiquitous in nature [5]. Endophytes maintain a symbiotic relationship with plants eventually helping them in growth [8] and to sustain in biotic and abiotic stress conditions [9]. Endophytes can infect plants either vertically, where fungal hyphae penetrate host seed [10] or horizontally, through asexual conidia or sexual spores [11]. Fungal endophytes possess certain exoenzymes which help them to colonize and grow in the apoplast (intercellular space) of the host tissue [12, 13].

Citrus species gets affected by fungal pathogens which include zygomycetes, ascomycetes, basidiomycetes [14, 15]. Since the extensive use of the synthetic chemical has proved unfriendly to the environment [16], efforts are being taken to minimize the use of such chemicals that create pollution of soil. The present study deals with the screening of antifungal activity of endophytic fungi isolated from *Citrus limon* against pathogenic fungi using ethyl acetate as solvent.

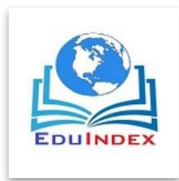
- **MATERIALS AND METHODS**

- **Collection of plant material**

Fresh and healthy leaves of *Citrus limon* were collected from Mahatma Gandhi Mission campus, Aurangabad. The samples were brought to the laboratory in sterile bags and stored at 4°C until use.

- **Isolation of endophytic fungi**

Isolation of endophytic fungi was done standardized method described by Hallmann, Berg [17] with a few modifications. The leaves were washed thoroughly under running tap water to remove any dust and unwanted adherent material. They were surface sterilized with 70% ethanol for 30 s followed by 4% sodium hypochlorite for 2 min and then rinsed in sterile distilled water for three times to remove traces of chemicals. Leaves were then blot dried on sterile Whatman paper. The leaves were later cut into small square sections of 5mm length and were placed on sterile potato dextrose agar (PDA) medium supplemented with streptomycin (100 µg/ml) to inhibit



unwanted bacterial growth. Plates were sealed with parafilm and incubated at 27°C until fungal growth from the explants was initiated.

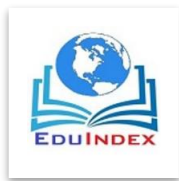
- **Molecular identification of endophytic fungi**

The endophytic fungi were grown in 300 ml Potato Dextrose Broth (PDB) for 3 weeks at 27°C. The mycelia were harvested and washed with distilled water and ground with liquid nitrogen. The nucleic acid was extracted using the Cetyl Trimethyl Ammonium Bromide (cTAB) method [18]. The polymerase chain reaction was carried out using two universal primers, ITS1 and ITS4 as mentioned by Yoo and Eom [19] to amplify the 5.8S rRNA genes. The PCR products were visualized in 0.8% agarose gel using ethidium bromide and UV transilluminator. The sequence of PCR product was edited and aligned by Codon code aligner v 3.0 (CodonCode Corporation, MA, USA). The edited sequence was aligned with the sequences in the GenBank by Basic Local Alignment Search Tool (BLAST) programs to check the sequence homology with closely related organisms [20].

- **Solvent Extraction**

Agar blocks containing fungal mycelia were used to inoculate the PDB medium. The endophytes were grown in 500 ml of the flask containing 300 ml of broth and kept in a rotatory shaker at 27°C at 120 rpm for 15-20 days. The fungal cultures were filter harvested using a muslin cloth to separate mycelia and broth. Mycelia were washed thoroughly with sterile distilled water and allowed to dry at room temperature. Dry mycelia were homogenized using ethyl acetate. Solvents were then filtered using Whatman filter paper. The solution was concentrated in vacuum evaporator at 35°C. The broth was also used for solvent extraction and filtered using Whatman filter paper and concentrated in vacuum evaporator at 35°C. Both extracts were pooled together and then dissolved in DMSO (1%) and stored at -20°C until assayed.

- **Agar well diffusion method**



Sterile PDA plates with 100 µg/ml streptomycin were prepared. These plates were spread with a 100 µl culture of *Alternaria alternata*, *Penicillium notatum*, *Fusarium oxysporum* and *Trichoderma viride* by inoculating loopful of sporulated fungal culture in 1 ml of saline. Sterile cork borer was used to prepare wells of 4mm diameter in the centre of the PDA plates. 100 µl of ethyl acetate extract was loaded into the wells. DMSO (1%) and Fluconazole (10 µg/ml) were used as negative and positive control respectively. Plates were incubated at 27°C for 48-72 hours. The zone of inhibition was measured to assess antifungal activity. Ethyl acetate extract of endophytic fungi showing maximum zone of inhibition was selected for further study.

- **Phytochemical screening of *Cladosporium cladosporioides***

Qualitative analysis of phytochemicals like Alkaloids, Phenols, Flavonoids, Carbohydrates, Saponins, Protein, Terpenoid, Steroid and Glycoside were done using ethyl acetate extract [21, 22]

### **2.6.1) Alkaloids**

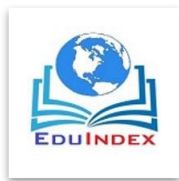
The fungal crude extract was dissolved in 2 N Hydrochloric acid. and was divided into 3 equal portions. One portion was treated with a few drops of Mayers reagent; one portion was treated with an equal amount of Hager's reagent and the other portion was treated with an equal amount of Wagner's reagent. The colour of the precipitates was observed.

### **2.6.2) Phenols**

1 ml of ethyl acetate extract was diluted in 4 mL of distilled water and 5 drops of neutral 5% ferric chloride solution was added. The change in colour of the solution was observed. 1 ml of ethyl acetate extract and 10 drops of lead acetate solution were mixed. Colour change of the solution was observed.

### **2.6.3) Flavonoids**

In 0.5 mL of ethyl acetate extract, 5 drops of diluted HCl and small pieces of magnesium of about 1 mg were added and the solution was boiled for 2 minutes. The change in colour of the solution was observed.



#### **2.6.4) Carbohydrates**

a) 1 ml Fehling's A solution and 1 ml of Fehling's B solution were mixed and boiled for 1 min and an equal volume of ethyl acetate extract was added. The solution was heated in a boiling water bath for 10 min. Change in colour of the solution was observed.

b) Benedict's reagent and ethyl acetate extract was added in 5:1 proportion and placed in a boiling water bath for 10 minutes. Change in colour of the solution was observed.

#### **2.6.5) Saponins**

The crude dry powder of fungal extract was vigorously shaken with distilled water and allowed to stand for 10 min. No froth indicates the absence of saponins and stable froth more than 1.5 cm indicated the presence of saponins.

#### **2.6.6) Terpenoids**

1ml of ethyl acetate extract, 1ml of chloroform, 2 ml of acetic anhydride and 2 drops of concentrated sulfuric acid were added in a test tube. Change in colour of the solution was observed.

#### **2.6.7) Glycosides**

1ml fungal extract, 2ml glacial acetic acid, one drop of 5% ferric chloride and 5 drops of concentrated sulfuric acid were added. Colour change in the solution was observed.

#### **2.6.8) Proteins**

1ml of ethyl acetate extract was treated with 3 drops of Biuret solution. Colour change in the solution was observed.

### **3) RESULT**

A total of twenty-two endophytic fungi were isolated from leaves of *Citrus limon*. Solvent extraction of all twenty-two isolates was carried out using ethyl acetate. Antifungal activity of

ethyl acetate extract of all isolated endophytic fungal isolates was carried out against *Alternaria alternata*, *Penicillium notatum*, *Fusarium oxysporum* and *Trichoderma viride*.

Endophyte number 19 showed maximum antifungal activity with the zone of 30 mm against *Alternaria alternata*. Molecular characterization and BLAST analysis of that endophyte showed 99% identity with *Cladosporium cladosporioides* having accession number MF196850.1.

**Table 1. Antifungal activity of ethyl acetate extract of endophytic fungi isolated from *Citrus limon*.**

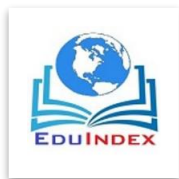
| E.F            | from | Zone of inhibition in mm |     |     |     |
|----------------|------|--------------------------|-----|-----|-----|
|                |      | A.A                      | P.N | F.O | T.V |
| <i>C.limon</i> |      |                          |     |     |     |
| 1              |      | 18                       | 19  | 12  | 17  |
| 2              |      | -                        | -   | 11  | -   |
| 3              |      | 16                       | 13  | 12  | -   |
| 4              |      | 12                       | 20  | 11  | 16  |
| 5              |      | 17                       | 22  | 11  | 12  |
| 6              |      | 17                       | 18  | 16  | 20  |
| 7              |      | 15                       | 20  | 15  | 15  |
| 8              |      | 12                       | 12  | 14  | 12  |
| 9              |      | 15                       | 14  | 12  | 21  |
| 10             |      | 15                       | 16  | 15  | 17  |

|    |    |    |    |    |
|----|----|----|----|----|
| 11 | 17 | 18 | -  | 12 |
| 12 | 15 | -  | -  | -  |
| 13 | 13 | 17 | 12 | 15 |
| 14 | 16 | 16 | -  | 20 |
| 15 | 17 | 17 | -  | 17 |
| 16 | -  | 15 | 13 | -  |
| 17 | 15 | 16 | 12 | 18 |
| 18 | 15 | -  | 16 | 19 |
| 19 | 30 | 16 | 15 | 20 |
| 20 | 21 | 20 | 16 | 17 |
| 21 | 17 | 18 | 15 | 20 |
| 22 | 15 | 16 | 12 | 15 |

**E.F-** Endophytic fungi, **A.A-Alternaria alternata**, **P.N-** *Penicillium notatum*, **F.O-Fusarium oxysporum**, **T.V- Trichoderma viride**, (-)-No activity

**Table 2. Phytochemical analysis of ethyl acetate *Cladosporium cladosporioides***

| Phytoconstituents | Test          | Observation                      | Result  |
|-------------------|---------------|----------------------------------|---------|
| Alkaloids         | Mayer's test  | Creamish precipitate in solution | Present |
|                   | Hager's test  | Orange precipitate in solution   | Present |
|                   | Wagner's test | Brown precipitate in solution    | Present |



|               |                     |                                 |         |
|---------------|---------------------|---------------------------------|---------|
| Phenols       | Ferricchloride      | Dark green colour of solution   | Absent  |
|               | Lead acetate        | Yellow precipitate in solution  | Present |
| Flavonoids    | Shinoda's test      | Pink colour of the solution     | Present |
| Carbohydrates | Fehling's reagent   | Brick red colour of solution    | Present |
|               | Benedict's reagent  | Brick red colour of solution    | Present |
| Saponins      | Foam test           | Foam formation                  | Absent  |
| Terpenoids    | Salkowski test      | Reddish precipitate in solution | Present |
| Glycosides    | Keller-Killani test | Brown ring in the solution      | Present |
| Proteins      | Biuret test         | Purple colour of solution       | Absent  |

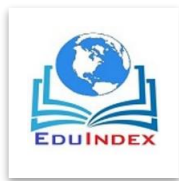
Phytochemical analysis of *Cladosporium cladosporioides* showed the presence of alkaloids, phenols, flavonoids, carbohydrates, terpenoids and glycosides.

A list of endophytic fungi isolated from a number of medicinal plants has been claimed to possess antifungal activities by some researchers [23, 24]. Most of them were anamorphs of fungi distributed in some common endophytic genera such as *Colletotrichum* species, *Alternaria* species, *Ovulariopsis* species, *Pestalotiopsis* species, *Phomopsis* species, and *Phoma* species, of which most are known to produce various bioactive products [25, 26]. The presence of endophytes from the leaves of citrus species was reported previously [27, 28].

## • DISCUSSION

*This study isolated endophytic fungi from leaves of Citrus limon showing antifungal activity which was identified as Cladosporium cladosporioides according to nuclear ribosomal RNA ITS sequence analysis. Antifungal activity of C. cladosporioides was carried out against Phomopsis*





*viticola* which showed 80% of growth inhibition at 30 $\mu$ M [29] also it showed significant antagonistic activity against chrysanthemum white rust [30]. Hulikere, Joshi [31] isolated *C. cladosporioides* from seaweed *Sargassum wightii* and its ethyl acetate extract showed significant antioxidant and angiostatic activity.

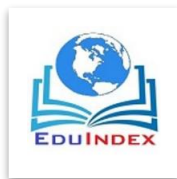
The colonization of endophytic fungi is ubiquitous in nature and have been widely known as a source of bioactive compounds [4]. Many endophytic fungal extracts have been used as a source of antifungal agents and can be used as an alternative drug for controlling plant diseases [32]. As the isolation of endophytic fungi and its extraction by different solvents can become a good source of antifungal activity as studied. It can also be of great importance in the agricultural industry which can minimize the use of chemical fertilizers.

#### • ACKNOWLEDGEMENT

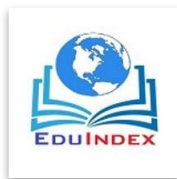
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