Think India Journal
ISSN: 0971-1260 Vol-22, Special Issue-31
National Conference ETDAB-2019
Held on 23th & 24th December 2019
Organized by: Deptt. of Botany, Deogiri College, Aurangabad, M.S

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

Manisha R. Survase* and Santosh D. Taware
Mahatma Gandhi Mission, Institute of Biosciences and Technology, Aurangabad.
Email – mrsurvase1991@gmail.com

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 fungiusing 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 alternate*. 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 *C.limon*	**Zone of inhibition in mm**			
	A.A	**P.N**	**F.O**	**T.V**
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 ethyla 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 30µM [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 angiosuppressive 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**

This study was funded by Dr Babasaheb Ambedkar Research and Training Institute, Pune. I am also thankful to the Laboratory of Mahatma Gandhi Mission, Institute of Biosciences and Technology, Aurangabad for providing necessary facilities and support to carry out this work.

- **REFERENCE**

1. Makni, M., et al., *Citrus limon from Tunisia: Phytochemical and Physicochemical Properties and Biological Activities.* BioMed Research International, 2018. **2018**: p. 10.
2. Del Río, J.A., et al., *Citrus limon: a source of flavonoids of pharmaceutical interest.* Food Chemistry, 2004. **84**(3): p. 457-461.
3. Sturz, A.V., B.R. Christie, and J. Nowak, *Bacterial Endophytes: Potential Role in Developing Sustainable Systems of Crop Production.* Critical Reviews in Plant Sciences, 2000. **19**(1): p. 1-30.
4. Strobel, G., et al., *Natural products from endophytic microorganisms.* J Nat Prod, 2004. **67**(2): p. 257-68.
5. Zabalgogeazcoa, I., *Fungal endophytes and their interaction with plant pathogens: a review.* 2008, 2008. **6**: p. 9.
6. Murugaiyan, S., et al., *Endophytic Bacteria: Perspectives and Applications in Agricultural Crop Production*. 2011. 61-96.

7. Shi, Y., X. Zhang, and K. Lou, *Isolation, characterization, and insecticidal activity of an endophyte of drunken horse grass, Achnatherum inebrians.* J Insect Sci, 2013. **13**: p. 151.
8. Smith, S.E. and F.A. Smith, *Roles of arbuscular mycorrhizas in plant nutrition and growth: new paradigms from cellular to ecosystem scales.* Annu Rev Plant Biol, 2011. **62**: p. 227-50.
9. Rodriguez, R.J., et al., *Stress tolerance in plants via habitat-adapted symbiosis.* The Isme Journal, 2008. **2**: p. 404.
10. Gundel, P.E., et al., *Data on litter quality of host grass plants with and without fungal endophytes.* Data in Brief, 2016. **7**: p. 1469-1472.
11. Clay, K. and C. Schardl, *Evolutionary Origins and Ecological Consequences of Endophyte Symbiosis with Grasses*. Vol. 160 Suppl 4. 2002. S99-S127.
12. Gimenez, C., et al., *Fungal Endophytes and their Role in Plant Protection.* Current Organic Chemistry, 2007. **11**(8): p. 707-720.
13. Schulz, B., et al., *Endophytic fungi: a source of novel biologically active secondary metabolites**Paper presented at the British Mycological Society symposium on Fungal Bioactive Compounds, held at the University of Wales Swansea on 22–27 April 2001.* Mycological Research, 2002. **106**(9): p. 996-1004.
14. Wulandari, N., et al., *Phyllosticta citriasiana sp. nov., the cause of Citrus tan spot of Citrus maxima in Asia.* Fungal Diversity 34 (2009), 2009. **34**.
15. Glienke-Blanco, C., et al., *Genetic variability in the endophytic fungus Guignardia citricarpa isolated from citrus plants.* Genetics and Molecular Biology, 2002. **25**: p. 251-255.
16. Gianessi, L.P., *The increasing importance of herbicides in worldwide crop production.* Pest Manag Sci, 2013. **69**(10): p. 1099-105.
17. Hallmann, J., G. Berg, and B. Schulz, *Isolation Procedures for Endophytic Microorganisms*. Vol. 299-305. 2006. 299-319.
18. Heinig, U., S. Scholz, and S. Jennewein, *Getting to the bottom of Taxol biosynthesis by fungi.* Fungal Diversity, 2013. **60**: p. 161-170.
19. Yoo, J.-J. and A.-H. Eom, *Molecular identification of endophytic fungi isolated from needle leaves of conifers in bohyeon mountain, Korea.* Mycobiology, 2012. **40**(4): p. 231-235.
20. Khilare, V., et al., *Multiple tests on saffron find new adulterant materials and reveal that Ist grade saffron is rare in the market.* Food Chemistry, 2019. **272**: p. 635-642.
21. Vimalkumar CS, et al., *Comparative preliminary phytochemical analysis of ethanolic extracts of leaves of Olea dioica Roxb., infected with the rust fungus Zaghouania oleae (E.J. Butler) Cummins and non-infected plants.* Journal of Pharmacognosy and Phytochemistry., 2014. **3**(4): p. 69-72.
22. Solomon, C., U.I. Arukwe, and I. Onuoha, *Preliminary phytochemical screening of different solvent extracts of stems bark and roots of Dennetia tripetala.* Asian J Plant Sci Res, 2013. **3**: p. 10-13.
23. Huang, Y., et al., *Antitumor and antifungal activities in endophytic fungi isolated from pharmaceutical plants Taxus mairei, Cephalataxus fortunei and Torreya grandis.* FEMS Immunol Med Microbiol, 2001. **31**(2): p. 163-7.

24. Park, J.-H., et al., *Screening for Antifungal Endophytic Fungi Against Six Plant Pathogenic Fungi*. Vol. 31. 2003.
25. Liu, L., et al., *Isoprenylated chromone derivatives from the plant endophytic fungus Pestalotiopsis fici.* J Nat Prod, 2009. **72**(8): p. 1482-6.
26. Kharwar, R.N., et al., *A comparative study of endophytic and epiphytic fungal association with leaf of Eucalyptus citriodora Hook., and their antimicrobial activity.* World Journal of Microbiology and Biotechnology, 2010. **26**(11): p. 1941-1948.
27. Araújo, W., et al., *Variability and interactions between endophytic bacteria and fungi isolated from leaf tissues of citrus rootstocks*. Vol. 47. 2001. 229-36.
28. Glienke, C., et al., *Genetic variability in the endophytic fungus Guignardia citricarpa isolated from citrus plants*. Vol. 25. 2002.
29. Wang, X., et al., *Antifungal activity against plant pathogens of metabolites from the endophytic fungus Cladosporium cladosporioides.* J Agric Food Chem, 2013. **61**(19): p. 4551-5.
30. Torres, D.E., et al., *Cladosporium cladosporioides and Cladosporium pseudocladosporioides as potential new fungal antagonists of Puccinia horiana Henn., the causal agent of chrysanthemum white rust.* PLOS ONE, 2017. **12**(1): p. e0170782.
31. Hulikere, M.M., et al., *Antiangiogenic, wound healing and antioxidant activity of Cladosporium cladosporioides (Endophytic Fungus) isolated from seaweed (Sargassum wightii).* Mycology, 2016. **7**(4): p. 203-211.
32. Rongai, D., et al., *Antifungal activity of some botanical extracts on Fusarium oxysporum*, in *Open Life Sciences*. 2015.