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# Antimicrobial and antioxidant activities of endophytes from *Citrus Limon*

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**Abstract**
*Citrus limon* is native to Asia and are used routinely by people for various purpose to make medicine. It also used to treat scurvy as it is rich in vitamin C. The antibacterial activity, antioxidant activity and extracellular hydrolytic enzymes of endophytic fungi associated with this plant were studied. Plant samples which included leaves, stems and roots were used. 24 morphotypes were isolated from the 97 segements 27 root samples, 42 leaf samples and 28 stem samples). *Fusarium* sp showed the greatest antibacterial activity against *Staphylococcus aureus* and antioxidant activity. Twelve species showed proteolytic activity, particularly *Fusarium* sp. Ten species showed positive for lipase activity and three species showed positive in cellulase and all the isolates were showed negative in amylase activity. It is clear that the endophytic fungi from *Citrus limon* have potential for the production of bioactive compounds and may be a sources of new therapeutic agents for the effective treatment of diseases in humans. To our knowledge, this is the first study of endophytic fungi from different tissues of *C.limon* and their biotechnological potential.

**Keywords:** antibacterial, antioxidant, therapeutic, vitamin C

## Introduction
Plants are naturally associated with microorganism both externally and internally in various ways. On the exterior surface of plants, diverse microbial interaction occur mostly in the root zone (rhizosphere) and on aerial parts, especially the leaves (phyllosphere) (Hilter, 1904; Yang *et al* 2001; Lindow and Brandle 2003; Gray and Smith 2005) [4, 9, 5, 3]. The term "endophytes" includes a suite of microorganism that grow intra and or intracellularly in the tissues of higher plants without causing over symptoms on the plants in which they live and have proven to be rich sources of bioactive natural products. The endophytes was coined by De Bary (1866) [2], which involves the existence of microorganism inside the infected plant tissues without having negative effects on the host plant (Schulz and Boyle 2005) [7]. Approximately 300,000 plant species growing in unexplored area on the earth are host to one or more (Strobel, 2003) [8], and the presence of diverse endophytes in huge number plays an important role on ecosystems with greatest biodiversity. Endophytes may be of algal, fungal or bacterial origins.
Fungal endophytes are reported from cryptogams to phanerogams. Endophytes have been observed from land plant to aquatic. The colonization of fungal endophytes in marine algae, bryophytes, fern and numerous gymnospermous plants have also been observed. Endophytes fungi are more diverse as well as host and tissue specific. A single plant leaf can harbor several dozens of endophytic fungi. Endophytic fungi have been reported from all parts of plant tissues such as leaves, rachis, bark, stems, buds, tubers, fruits and roots. An endophytic fungus is recorded from brown alga *Sargassum* sp. with microbial activity (yang *et al*. 2006) [9]. Fungal endophytes play a major role in determination of plant biodiversity.
In the past, many valuable bioactive compounds with antimicrobial, insecticidal, cytotoxic, and anticancer activities have been successfully discovered from the endophytic fungi. These bioactive compounds could be classifiedas alkaloids, terpenoids, steroids, quinones, lignans, phenols, and lactones (Zhang *et al*. 2006) [11]. Endophytes producing immunosuppressives, for example, subglutinols A and B are produced by *Fusarium subglutinans.* Endophytes producing Podophyllotoxin (PDT), a well-known aryltetralin lignan with potent anticancer, antiviral, antioxidant, antibacterial, immunostimulation, and anti-rheumatic properties, are obtained from endophytic fungus *Alternaria* sp. isolated from *Sinopodophyllum*, and endophytic fungus *Fusarium oxysporum* obtained from *Sabina recurva.*

***Citrus Limon***

The lemon *Citrus Limon* (L.) osbek is a species of small evergreen tree in the flowering plant family *Rutaceae*, native to Asia. The lemon tree grows to 6 m (20 ft) tall, and has stout spines. The leaves are dark green, leathery, and evergreen, oblong, elliptical, or oval and up to 14 cm (4 in) long; in contrast to several other citrus species, the petioles (leaf stems) are not winged or only narrowly winged. Flower buds are purplish but flowers open to have 5 white petals, up to 5 cm across. Fruits are globose to oblong, 7.5 to 12.5 cm long, and ripen to yellow, with smooth to bumpy rinds dotted with oil glands. The juice of the lemon is about 5% to 6% citric acid, with a $P^H$ of around 2.2, giving it a sour taste. It is a rich source of vitamin C. It contains numerous phytochemicals including polyphenols, terpenes and tannins. It contains slightly more citric acid than lime juice, nearly twice the citric acid of grapefruit and about five times the amount of citric acid found in orange juice. In 2010, commercial production of lemons (together with limes) was 14.2 million metric tons harvested from 1.0 million hectares. The leading producers are India, Mexico, Argentina, China, and Brazil. Lemon juice is widely known as a diuretic, antiscorbutic, astringent and febrifuge. Lemon peel oil consists of terpenes, particularly limonene, also gamma terpinene and beta- phellandrene. There are small amounts of sesquiterpenes and aldehydes. Among the aliphatic aldehydes are n- octyl aldehyde, n-nonyl aldehyde, and citral. *C. Limon* exhibited a broad spectrum of antibacterial activity and as a potential sources for drug development. Due to enormous medicinal properties of *C. Limon,* it is selected for the isolation and screening of endophytic fungi for their secondary metabolites and checks its biological activity. The objective of this study was to isolate, identify fungal endophytes from the leaf, roots and stem/bark of the *Citrus Limon* to explore the bioactive properties of their secondary metabolites.

## Methodology

### Collection of plant and isolation of endophytic fungi

### Sample Collection

Young and healthy plant parts were collected from disease free plant of *C. Limon* (leaf, root, stem) from nursery, Coimbatore. The plant parts was cut and placed in a polythene bag and then taken to the lab.

### Surface treatment

Collected plant parts were rinsed in running tap water for 10 to 15 minutes to remove the debris and soil particles adhered to it and then washed with double distilled water to remove microbes from thes surface of the plant sample. The surface treatment was done adopting the methodology by Petrini and coworker in 1992 with slight modifications. In this procedure at first the leaf sample was washed in 70% ethanol for 1 minute and then in 4% sodium hypochlorite (NaClO) for 30 seconds, 1 and 1.5 minutes and then washed with 70% ethanol for 10 seconds to remove the epiphytic microbes. Finally the samples was washed with autoclaved distilled water for 3 times and blotted on autoclaved blotting paper. Then the stem samples was washed in 70% ethanol for 1 minute and then in 4% sodium hypochlorite (NaClO) for 1 and 2 minutes and then washed with 70% ethanol for 10 seconds to remove the epiphytic microbes. Finally the samples was washed with autoclaved distilled water for 3 times and blotted on autoclaved blotting paper. The same process was repeated for root sample, but sample was treated for 4% sodium hypochlorite for only 2 minutes. Remaining steps were similar.

### Isolation of fungal Endophytes

Leaves were cut into small pieces of 5x1mm$^2$ with a sterile blade. The stem and root was removed carefully and dissected into small pieces (0.5x0.5cm$^2$). the pieces were placed on petriplates containing potato dextrose agar (PDA) medium supplemented with streptomycin (200mg/L) and incubated for 21 days at 26±2°C in BOD cum humidity incubator (Calton, NSW, New Delhi). Tissues were observed for fungal growth at 2 days interval for 20 days. Actively growing fungal tips immerging from plant tissues were sub-cultured on PDA petri plates for identification and enumeration.

### Purification and preservation of endophytic fungi

The collective colonization frequency of the endophytic fungi in the root, stem/bark and leaf parts of *Citrus Limon* and *Thevetia neriifolia* was calculated using the following formula:

$$\% \text{ colonization frequency} = \frac{\text{Number of isolates emerged}}{\text{Number of tissues plotted}} \text{x } 100$$

The isolates obtained from different plant parts were purified by transferring each isolate to a fresh PDA plate. A piece of agar with mycelial growth of the isolate was cut and transferred to a new PDA plate.

### Production and Extraction of Secondary metabolites from fungal endophytes

Disc of agar containing mycelial growth of the fungal endophytes was cut and was inoculated in 100ml of Potato Dextrose Broth (PDB) and was kept for incubation at 27-28°C in a BOD incubator for 21 days. The broth culture was then harvested for secondary metabolite extraction using ethyl acetate as solvent. The mycelium of the fungus was filtered from the broth by a four-fold layer of muslin cloth. The broth was taken in a new flask and equal volume of pure ethyl acetate was added to it. The mixture was poured into a separating funnel and was mixed well. The solvent layer was collected separately while the broth was extracted twice again with ethyl acetate. This is termed as triple extraction and it ensures maximum extraction of secondary metabolites from cultured broth. The ethyl acetate extract was put in the rotatory evaporator to concentrate the extracted metabolites. The concentrated metabolites were transferred in pre weighed glass vials, dried completely and their weight was calculated.

### Screening for anti-bacterial and anti-fungal activity of fungal secondary metabolites

The fungal secondary metabolites were tested for anti-bacterial activity against four gram negative bacteria: *Salmonella typhimurium*, *Escherichia coli*, *Proteus vulgaris*, *Klebsiella pneumoniae* and *Citrobacter freundii*, and one gram positive bacteria: *Staphylococcus aureus*. Anti-fungal activity was tested against two human dermatophytes: *Aspergillus flavus* and *Aspergillus terreus.*

The activity was checked by disc diffusion method. Nutrient agar (NA) was prepared for anti-bacterial assay while for anti-fungal assay; Potato Dextrose agar (PDA) was prepared. 1mg of metabolites were loaded onto 5mm sterilized filter paper discs and were placed on the agar plates swabbed with the different organisms to be tested. A disc loaded with methanol was also placed after drying as control. The plates were then incubated for 18-24 hours. Zone of inhibition so formed was

measured and recorded

**Screening for anti-oxidant activity of fungal secondary metabolites**

The metabolites of endophytic fungi were screened for free radical scavenging activity by DPPH (2, 2-di phenyl 2-picryl hydrazyl) method. DPPH, a violet colored compound, is a stable free radical that accepts an electron or hydrogen atom to become a stable diamagnetic molecule, which is yellow in color. This decolorization is a positive test for anti-oxidant activity.

The screening test was performed in micro titer plates, where 400µg of metabolite was added to 400µl of 0.2mM DPPH (i.e. at 1mg/ml concentration), incubated in dark for 30 minutes and change in colour of DPPH was observed. The EC-50 value (amount of sample required to scavenge 50% of free radicals) of good anti-oxidant metabolites was calculated by DPPH method (Alothman *et. al.*, 2009) [1] with slight modifications. The metabolite was added in increasing concentration to 3ml of 0.2mM DPPH solution and was incubated for 30 minutes in dark, after which absorbance of the solution was taken at 517nm. The % inhibition of free radicals was calculated by the following formula:

$$\% \text{ inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 10$$

## Results and discussion

**Isolation, purification and preservation of endophytic fungi**

Diversity and distribution of endophytic fungi from three different tissues (root, stem/bark, leaf) of *C. Limon* has been analysed and described from their emergence, purification and identification.

**Emergence of endophytic fungi from healthy tissue**

The incubated plates with healthy surface sterilized tissues were observed regularly at every alternate for 21 days. Emergences of endophytic fungi from some of the tissues were observed and hyphal tips of actively growing fungi were then transferred to fresh PDA plates for further processing (Fig 1)

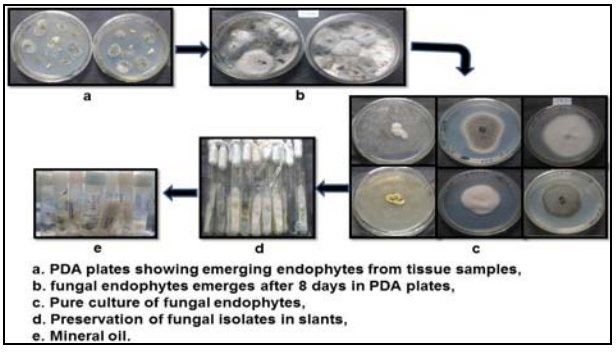


**Fig 1:** Isolation, purification and preservation of Fungal Isolates

A total 97 samples of *Citrus Limon* (27 root samples, 42 leaf samples and 28 stem samples) was plotted on PDA plates. One imprint plate was also prepared to check for the growth of epiphytes, where tissue samples were kept on the plate for 15-20 minutes and the plates were incubated to monitor any microbial growth which represents improper surface sterilization

The colonization frequency (%CF) was calculated in *C. Limon.* In *C. Limon,* a total of 24 morphotypes were isolated from the 97 segements. In which CS2 showed the maximum colonization frequency of 32.14% and CL2 and CL3 (2.22%) produced the least colonization frequency. The CF% ranged from 32.14% to 2.22%. Three of the morphotypes produced more than 10% CF (CS4, CS6 &CS10) while one morphotype produced lesser than <10% CF (CR5). Nineteen morphotypes produced<5% of the colonization frequency (Table 1).

**Table 1:** Different isolates obtained from the *Citrus Limon* along with their % colonization frequency

| S.No. | Isolates | Number of tissue plotted | Number of isolates emerged | % Colonization frequency |
|---|---|---|---|---|
| **Isolates emerges from *Citrus limon*** | | | | |
| **Root samples** | | | | |
| 1 | CR1 | 27 | 1 | 3.70 |
| 2 | CR2 | | 1 | 3.70 |
| 3 | CR3 | | 1 | 3.70 |
| 4 | CR4 | | 1 | 3.70 |
| 5 | CR5 | | 2 | 7.40 |
| 6 | CR6 | | 1 | 3.70 |
| 7 | CR7 | | 1 | 3.70 |
| **Leaf samples** | | | | |
| 8 | CL1 | 42 | 2 | 4.76 |
| 9 | CL2 | | 1 | 2.22 |
| 10 | CL3 | | 1 | 2.22 |
| **Stem samples** | | | | |
| 11 | CS1 | 28 | 1 | 3.57 |
| 12 | CS2 | | 9 | 32.14 |
| 13 | CS3 | | 1 | 3.57 |
| 14 | CS4 | | 3 | 10.71 |
| 15 | CS5 | | 1 | 3.57 |
| 16 | CS6 | | 3 | 10.71 |
| 17 | CS7 | | 1 | 3.57 |
| 18 | CS8 | | 1 | 3.57 |
| 19 | CS9 | | 1 | 3.57 |
| 20 | CS10 | | 4 | 14.28 |
| 21 | CS11 | | 1 | 3.57 |
| 22 | CS12 | | 1 | 3.57 |
| 23 | CS13 | | 1 | 3.57 |
| 24 | CS14 | | 1 | 3.57 |
| Total | | 97 | | |

**Production and extraction of fungal secondary metabolites**

The fungal isolates were incubated in PDB for 21 days, after which the secondary metabolites were extracted using ethyl acetate and were concentrated in the rotary evaporator and were collected in small glass vials with the help of methanol (Fig 2).

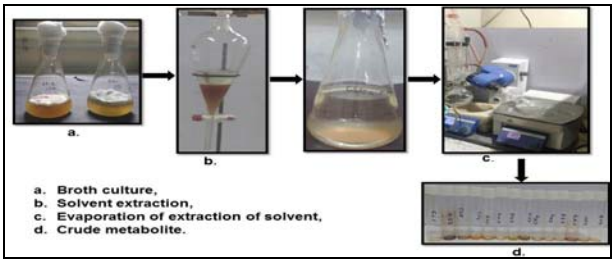


**Fig 2:** Extraction of secondary metabolites from the fungal Endophytes

**Anti-bacterial and anti-fungal activity of fungal secondary metabolites**

The secondary metabolites from different isolates were screened for anti-bacterial and anti-fungal activity against six human pathogens and two fungi. *S. aureus*, *E. coli*, *S.*

*typhimurium*, *P. vulgaris, K. pneumoniae, C. freundii, A. terreus*, *A. flavus.* The inhibition zone ranged from 7 mm to 21mm.The crude extracts of fungal secondary metabolites showed narrow degree of inhibitory activity against *S. aureus* and *C. freundii.* In *C. limon,* CR3 metabolite showing a maximum zone of inhibition (21mm) against *S. aureus*, followed by CS4 metabolite (18mm) zone against *S. aureus* and a CL2 metabolite showing 14mm zone against *S. aureus*; whereas eight metabolite showing zone ranges from (10mm to 13mm) against *S. aureus*). CL3 metabolite showing a minimum zone of inhibition (7mm). Metabolites of CS2 was active against *C. freundii.* There was no fungal metabolite activity against *E. coli*, *S. typhimurium, P. vulgaris, K. pneumoniae* (Fig 3).

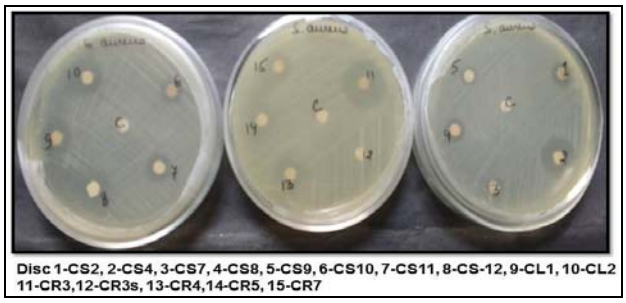


**Fig 3:** Antibacterial activity

**Anti-oxidant activity of secondary metabolites of fungal endophytes**

Antioxidant compounds can either directly scavenge reactive oxygen species or prevent the generation of reactive oxygen species in oxidative stress. The natural antioxidants isolated from plant, fungi and marine algae represent the most useful nutraceuticals and functional foods for health protection and disease prevent (Gutteridge and halliwell 1994). The free redical scavenging activity of fungal extract was carried out by using DPPH (2, 2-Diphenyl-1-picrylhydrazyl). Twenty four endophytic fungi and standard (Ascorbic acid) were assayed for antioxidant activity at 517nm in UV spectrophotometer. Among 24 isolates, eighteen isolates were emerged from *C. Limon,* two isolates from stem (CS2) and root (CR3) shown higher activity closer to std ascorbic acid. One isolate (CS11) shown moderate activity (around 50%-75%), while thirteen isolates shown below 50% activity, CR3s and CS12 showed less than 10% activity (Fig.4).

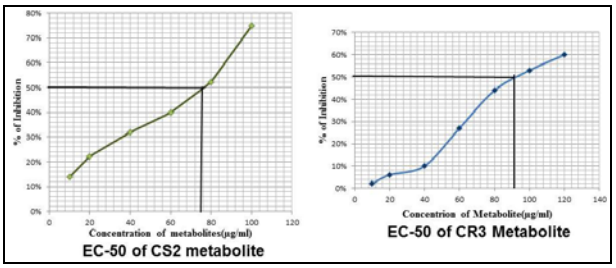


**Fig 4:** Antioxidant activity

EC-50 value was determined for CS2, CR3 and CL4 metabolites. The EC-50 value of CS2 was found to be ~79µg/ml, while that of CR3 was found to be ~90µg/ml.

**Conclusion**

As a poorly investigated store of microorganisms 'hidden' within the host plants, fungal endophytes are a rich and reliable source of bioactive and chemically novel compounds with huge medicinal potential which may be of antibacterial, antifungal, antimalarial or antioxidant nature. In the present study, we aimed at exploring the mycoflora of a medicinal plant, *C. Limon* evaluation of the biological activity by virtue of their secondary metabolites. Among all the isolates, crude metabolite of CR3 showed significant antimicrobial activity against bacterial pathogen *S. aureus*. CR3 showed a narrow spectrum anti-bacterial activity. The crude metabolite of CS2 and CR3 showed a strong antioxidant nature. Attempts can be made to characterize and purify these bioactive principles. The results of our study support the fact that endophytes are a promising source of novel bioactive compounds which can be exploited for various therapeutic applications.

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