JETIR.ORG ISSN: 2349-5162 | ESTD Year : 2014 | Monthly Issue
JOURNAL OF EMERGING TECHNOLOGIES AND INNOVATIVE RESEARCH (JETIR)
An International Scholarly Open Access, Peer-reviewed, Refereed Journal

# ANTIBACTERIAL ACTIVITIES OF ENDOPHYTIC XYLARIA SP. PHOMA SP STRAIN FROM CATHARANTHUS ROSEUS L AND VITEX NEGUNDO L AGAINST DRUG-RESISTANT PSEUDOMONAS SYRINGAE (MTCC 673), PROTEUS MIRABILIS (MTCC1429), BURKHOLDERIA GLUMAE (MTCC8496) AND MORAXELLA BOVIS (MTCC 1775) STRAINS

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**Abstract**

In the current work, the gymnosperm plants of *Catharanthus roseus* and *Vitex negundo* were used to extract the bioactive endophytic fungus species. Based on physical traits, the isolated fungus species was identified as *Xylaria* sp. SR2. *Phoma* sp. S2. The *Xylaria* AND *Phoma* sp. fungal isolate was cultivated in a variety of pH ranges and culture medium in order to maximise biomass output under submerged culture circumstances. The ideal temperature and pH for the highest mycelial development were discovered to be 30°C and 5.5, respectively, in PDA, where the maximum growth of *Xylaria* sp. SR2 AND *Phoma* sp. was seen. The drug-resistant *Pseudomonas syrirgae* (MTCC 673) and *Proteus mirabilis* (MTCC1429), *Burkholderi aglumae* (MTCC8496) and *Moraxella bovis* (MTCC 1775) strains were tested to antibacterial activity using the improved ethyl acetate extract of the culture filtrate.

**Key words:** Antibacterial Activity, Drug resistant Bacteria, Endophytic *Xylaria*, & *Phoma*

# 1.INTRODUCTION

## 1.1 Medicinal Plants:

Medicinal plants, also called medicinal herbs, have been discovered and used in traditional medicine practices since prehistoric times. Plants synthesise hundreds of chemical compounds for defence against insects, fungi, illnesses, and herbivorous mammals, among other things. Numerous phytochemicals have been found as having biological activity, either potential or established. The consequences of taking a complete plant as medication, however, are unknown because a single plant has a vast range of phytochemicals. The Sumerian civilisation has the first historical records of herbs, with hundreds of therapeutic plants, including opium, mentioned on clay tablets dating from around 3000 BC.

Over 50,000 medicinal plants are utilised around the world, according to the Food and Agriculture Organization.In 2016, the Royal Botanic Gardens, Kew calculated that 17,810 plant species out of 30,000 have a therapeutic value.All plants produce chemical compounds which give them an evolutionary advantage, such as defending against herbivores or, in the example of salicylic acid, as a hormone in plant defenses. These phytochemicals have the potential to be used as medications, and their content and known pharmacological action in medicinal plants provide the scientific basis for their application in modern medicine, if scientifically proven.

Many therapeutic plants having alkaloids, which are bitter-tasting compounds that are widely distributed in nature and often dangerous. As medications, there are a variety of classes with various mechanisms of action, both recreational and pharmacological. Atropine, scopolamine, and hyoscyamine (all from nightshade), berberine (from plants like Berberis and Mahonia, caffeine, cocaine, ephedrine (Ephedra), morphine (opium poppy), nicotine (tobacco), reserpine (*Rauvolfia serpentina*), quinidine and quinine (*Catharanthus roseus*).

Anthraquinone glycosides are found in medicinal plants such as rhubarb, cascara, and Alexandrian senna. *Senna alexandrina*, containing anthraquinone glycosides, has been used as a laxative for millennia. Polyphenols come in a variety of classes and play a variety of roles in plant defences against diseases and predators. Hormone-mimicking phytoestrogens and astringent tannins are among them.

Terpenes and terpenoids of various types can be found in a wide range of medicinal plants, as well as resinous species like conifers. They have a strong odour and are used to repel herbivores. Their smell makes essential oils helpful in perfumes like rose and lavender, as well as aromatherapy. Some have medical properties, such as thymol, which is an antibacterial and was previously used as a vermifuge (anti-worm medicine).

## 1.2ENDOPHYTIC FUNGI:

Endophyte is an endosymbiont, often a fungus, which lives within a plant for at least part of its life without causing any apparent disease. They form inconspicuous infections within tissues of healthy plants for all or at least a part of their life cycle. [Clay K and Schardl ]. Fungi play a significant role in every ecosystem, as they are involved in critical activities such as decomposition, recycling, and nutrient movement in many conditions. It is estimated that there are over a million different fungus species on the planet, with just a small percentage [about 5%] of them having been identified.

Many bacteria live as plant endophytes, and in most cases, they cohabit with endophytic fungus. For more than a century, endophytes have been known to exist. Most of the time, they exist as imperfect fungus and have been described as benign parasites or real symbionts. It's been proposed that they have an impact on the host plants' distribution, ecology, physiology, and biochemistry.

All organisms inhabiting plant organs that at some-time in their life, can colonize internal plant tissues without causing apparent harm to the host" or "A group that colonize living, internal tissues of plants without causing any immediate, overt negative effects "or "Endophytes are any fungi isolated from internal symptomless plant tissues" or "Fungi and bacteria which, for all or part of their life cycle, invade the tissues of living plants and cause unapparent and asymptomatic infections entirely within plant tissues, but cause no symptoms of disease".

According to previous research, the endophytes that live inside this plant have the ability to create host-specific metabolites like vincristine and vinblastine. The organic extracts of endophytic fungus that inhabit the *C. roseus* plant have recently been revealed to have substantial cytotoxicity against chosen cell lines Moreover, endophytic fungi from *C. roseus* have also been reported to produce few novel compounds with potential cytotoxic activities. The antioxidant activity of the endophytic actinomycetes that live on the *C. roseus* plant has also been noticed. Recently, more focus has been implemented for the isolation of fungal endophytes from medicinal plants with potential of synthesis of bioactive compounds.

Fungal species of the genus *Xylaria* Hill ex Scharank (Xylariaceae, Asomycetes) both macrand micro fungi, are known to produce diverseclasses of bioactive compounds including antifungal multiplolides (Boonphong*et al.* 2001), cytotoxic cytochalasins, acetylcholineesterase inhibitorxyloketals (Lin *et al.* 2001), Xanthones (Healy *etal.* 2004), XylarighanandOrthosporin (Rong Chen*et al*. 2018), Nigriterpene (Jung *et al*. 2017), Cytochalasin D (Elias Luciana 2018). The presentinvestigation has therefore been designed to studythe *in vitro* antibacterial potential of dried fungalextracts of endophytic *Xylaria*sp. SR2 against drugresistant human bacterial pathogens of clinical importance.

## 2. MATERIALS AND METHODS:

### 2.1 Collection of plant materials:

Mature individual plants *Catharanthus roseus* L. and *Vitex negundo* L. (Fig 1&2) and showing variability in leaf size, number and other morphological features are collected from Palavanattham and Chinnapareali in Virudhunagar District. The leaf segments were placed in paper bags after removal of excess moisture. Then the leaf samples were stored at 4ºC for further use.

**Fig 1 - *Catharanthus roseus* L**

**Fig 2 - *Vitex negundo* L**

**2.2 Isolation of Endophytic Fungi**:

Prior to getting processed, the leaf samples from *Catharanthus roseus* and *Vitex negundo* were properly cleaned with distilled water and allowed to air dry. According to the published methodology (Petrini 1986), which was significantly changed in light of preliminary research, endophytic fungi were isolated. All leaf samples were first surface sterilised by immersion for 1 minute in 70% (v/v) ethanol, 1 minute in sodium hypochlorite (3 percent (v/v) accessible chlorine), and 30 seconds in 70% (v/v) ethanol. They were then rinsed three times for 1 minute each in sterilised distilled water. The materials were chopped into 5-7 mm pieces after surface sterilisation and aseptically placed to Petri plates with potato dextrose agar (PDA) with streptomycin 50g/ml applied to prevent bacterial growth These Petri plates underwent regular daily light and dark cycles while being incubated at $30^0$C. For up to a month, the plates were checked daily for the growth of fungal colonies emerging from the leaf segments. Following that, the fungus emerging from the leaf tissue were transplanted onto brand-new PDA plates without antibiotics.

**2.3 Microscopic Analysis of Morphological Characterization:**

In 7-9 days, the endophytic fungus was cultured on PDA at 30°C, and the conidial development was seen under a microscope. Each specimen was inspected for asci, ascospores, paraphyses, and other features with taxonomic significance. For 50 spores, spore dimensions were calculated. As mounting material for microscopy, lacto phenol cotton blue and distilled water were employed. In 3% aqueous KOH, dried materials were rehydrated. A light microscope and a binocular microscope connected to a computer were used for photography (COSLAP). The size and morphology of hyphae and spores, colony diameter, texture, and colour, as well as the standard taxonomic key, were used to identify the isolated endophytic fungus (Ainsworth et al. 1973).

**2.4 Effect of pH on the Growth of Fungal Species:**

In potato dextrose agar plates with an initial pH range of 4.0 to 7.0, the endophytic was grown. The culture was incubated under static conditions for 7 days. The rate of radial growth was assessed following incubation. Four distinct sites were used to collect the data, and the average radial growth was noted.

**2.5 Effect of Temperature on the Growth of Fungal Species:**

In potato dextrose agar plates, the endophytic was developed with an initial temperature range of 10 to $35^0$C. The culture was incubated under static loading for 7 days. The rate of radial growth was assessed following incubation. Four distinct sites were used to collect the data, and the average radial growth was reported.

**2.6 Fermentation and Extraction of Bioactive Compounds:**

Depending on its pace of development, the endophytic fungus was cultured on potato dextrose agar (PDA) at $30^0$C for 5-7 days. Six portions of the developed culture, cut off the plate, were placed into 500 ml Erlenmeyer flasks with 300 ml potato dextrose broth (PDB), and the mixture was incubated at 30ºC for 3 s at pH 5.5. Following the incubation period, extracts from the fermented broth were prepared using our previous standard procedure (Ramesh et al. 2014, Subbulakshmi et al. 2012, Arivudainambi et al. 2011).

**2.7 Phytochemical Screening of Secondary Metabolites**:

The crude extract obtained was used to screen for four metabolites as described by Heaton and Pauley. All phytochemical screening assays were done in duplicates .Phytochemical analytical tests were performed to detect the presence of steroids, saponins, alkaloids, flavonoids, tannins, reducing compounds, terpenoids, cinnamic derivatives, and anthracene derivatives, according to the method described by Kokate (1994) and Harborne (1998).

**2.8 Test Microorganisms:**

*Pseudomonas syringae* (MTCC 673), *Proteus mirabilis* (MTCC1429), *Burkholderiaglumae* (MTCC8496) and *Moraxella bovis* (MTCC 1775) were purchased from Microbial Type Culture Collection, Chandigarh, India and were used for screening tests.

**2.9 Antibacterial activity:**

The endophytic fungi were placed through an antimicrobial assay on a solid medium, which allows for a quick and accurate screening of the bioactive microorganisms (Ichikawa et al., 1971). Each endophytic strain was grown for seven days at 30 °C on the PDA surface in Petri dishes. In order to disseminate bacteria (Müller-Hinton agar, MHA) and fungus (Sabouraud dextrose agar (SDA) and SDA enriched with 0.5 percent olive oil for M. furfur), discs were cut from the PDA plate (6 mm in diameter) and placed on the surface of Petri dishes. The Petri dishes were incubated for bacterial growth at 37°C for 24 hours and for fungal growth at 30°C for 48 hours. The assessment of any inhibitory diameter zones served as a measure of antimicrobial activity (IDZ).

**2.10 Statistical analysis**

To evaluate statistical significance, one-way analysis of variance (ANOVA) and Iqbal were used to examine the data using GraphPad Prism. Statistical significance was defined as a p-value of 0.05 or below. The Pearson coefficient () was used to construct the correlation index.

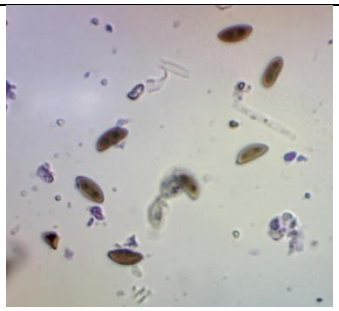
## 3. Results:

Finally, four different endophytic fungus species were identified using *Catharanthus roseus* and *Vitex negundo* leaf samples. Based on their physical traits, these species were given the labels SR1, SR2, SR3, and SR4 and s1, s2, s3, and s4 at the time of isolation (Plate 2). *Phoma* sp. and *Xylaria* sp. strains SR1 and SR2 were identified as the four species of fungal endophytes based on colony morphology and sporulating structure. The size, shape, and culture-related characteristics, such as colony colour, growth rate, and texture, have traditionally been used to discriminate between different fungal species. Due to inadequate spore formation, the remaining three fungal isolates could not be identified at the genus level. Thus, SR2, SR3, and SR4 were assigned to the sterile fungal isolates. *Xylaria* sp. and *Phoma* sp. are the only endophytic isolates among the aforementioned fungi. Strains SR1 and s2 were chosen for further growth optimization and antibacterial screening studies.

### 3.1 Morphological observations:

#### 3.1.1 *Xylaria* sp.

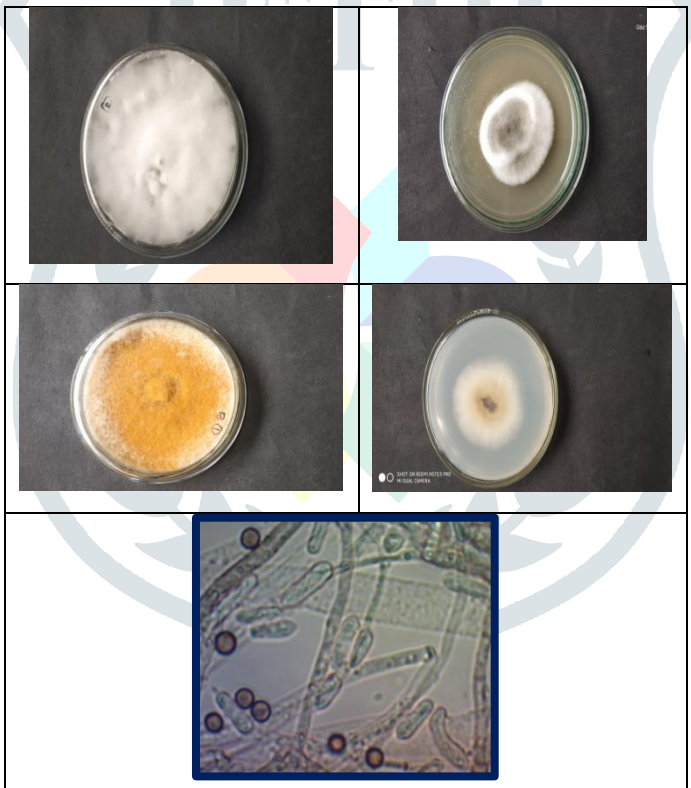
Mycelial biomass can be harvested in significant amounts and used for biological activity. The ideal conditions were established after considering how environmental variables like pH, temperature, and culture media affected the situation. The morphological characteristics of the *Xylaria* sp. strain SR1 wasobserved on PDA after 10 d of growth at 30°C. The petriplate is covered in 5–7 days thanks to the rapid growth rate of 5.4–7.2 cm/week. Early on, the mycelial mat was white; subsequently, it changed to a thick black colour. Hyphae have branching, thin-walled structures. After seven days of development at 30 0C, the endophytic fungal isolate SR2's morphological features were seen on PDA. Colonies on the PDA were circular, and the mycelium was initially raised in a whitish colour and ageing into a dark black, occasionally a light grey (Plate 2). Asci are eight-spored, cylindrical, uniseriate, and stipitate. 33.1 to 33.8 m wide. Ascospores range in length from 17.3 to 17.8 m. Each ascospore is ellipsoid, inequilateral to broad, smooth, unicellular, dark brown, with a prominent, straight, full-length germ slit ( Fig 3b). This isolate of fungus shared the same morphological characteristics as soil-growing *X. angulosa* (AB274814) (Rogers et al. 1987).



***Xylaria* sp.**

***Phoma* sp.**

The isolates' pycnidia were uniformly glabrous, globose to nsubglobose, solitary or confluent, measuring 50 to 200 75 to 250 m and having black papillate ostioles (Plate 3). The pseudoparenchymatous pycnidial wall was made up of oblong to isodiametric cells in one layer. The septate, thick mycelia were present. Conidiophores were unbranched to rarely branched, straight or flexuous, and globose as they developed from buds on the hyphae.



Phoma sp.

**Phytochemical screening**

Phytochemical test for ethyl acetate extract of *Xylaria* sp.

| Phytochemical Constituents | Ethyl acetate extracts |
|---|---|
| Phenol | + |
| Tannin | + |
| Saponin | + |
| Cardiac glycosides | + |

Phytochemical test for ethyl acetate extract of *Phoma* sp.

| Phytochemical Constituents | Ethyl acetate extracts |
|---|---|
| Phenol | + |
| Tannin | + |
| Saponin | + |
| Cardiac glycosides | - |

**3.2 Antibacterial activity:**

*Xylaria* sp.

The findings of the phytochemical investigation offer hope for testing the antibacterial action. The effectiveness of the ethyl acetate and methanol extracts of *Xylaria* sp. against 4 bacterial infections notable for creating a potent diameter of the zone of inhibition of growth around the disc was evaluated using the agar well diffusion method. All of the examined microorganisms were susceptible to the extracts' relative antibacterial activity, with inhibition zones' diameters ranging from 8 to 12 mm (Table 3 & Plate 4).

Table:3, *Xylaria* sp. SR1 Methanol and ethyl acetate extract antibacterial activity:

| Pathogens | Methanol extract (mm) | Ethyl acetate extract (mm) | Negative control (mm) | Positive control (mm) |
|---|---|---|---|---|
| *Pseudomonas syringae* | 9 | 12 | 0 | 18 |
| *Proteus mirabilis* | 8 | 8 | 0 | 17 |
| *Burkholderiaglumae* | 8 | 9 | 0 | 17 |
| *Moraxella bovis* | 11 | 10 | 0 | 18 |

*Phoma* sp.

By using the agar well diffusion technique, the antibacterial activity of the ethyl acetate extract of *Phoma* sp. against bacterial pathogens was examined. According to the findings, methanol and ethyl acetate extract were both highly efficient antibacterial agents against all of the tested pathogens. No zone of inhibition was visible against any microorganisms in the negative control. The zone of inhibition against all of the investigated microorganisms for the positive control was between 8 and 12 mm (Table 4 & Plate – 5).

Table: 4, *Phoma* sp. SR2 Methanol and ethyl acetate extract antibacterial activity:

| **Pathogens** | **Methanol extract (mm)** | **Ethyl acetate extract (mm)** | **Negative control (mm)** | **Positive control (mm)** |
|---|---|---|---|---|
| *Pseudomonas syringae* | 7 | 7 | 0 | 18 |
| *Proteus mirabilis* | 10 | 8 | 0 | 17 |
| *Burkholderiaglumae* | 8 | 8 | 0 | 17 |
| *Moraxella bovis* | 8 | 7 | 0 | 18 |

**Discussion**

The benefits of medicinal plants are widely employed in the treatment of many ailments and may have a variety of therapeutic effects. Medicinal plants include a variety of active substances. Because chemical compound synthesis depends on the kind of development, not all plants have the same amounts of phytocompunds. Secondary phytochemicals serve as the primary biological characteristics in the majority of plants. Phenol, flavonoids, terpenoids, tannins, alkaloids, and steroids are examples of secondary phytochemicals. Important phytocompunds such phenols, flavonoids, and terpenoids are directly involved in the antioxidant action.

One of the main potential sources for novel, beneficial metabolites is endophytic fungus (Dreyfuss and Chapela, 1994). Endophytic fungi have drawn a lot of attention as possible makers of new, physiologically active compounds (Schulz et al., 2002; Strobel and Daisy, 2003; Tomita, 2003; Urairaj et al., 2003; Wildman, 2003). Endophytic fungi are identified as a class of organisms that are likely to be sources of novel metabolites important in biotechnology and agriculture since they inhabit extremely unique and sometimes quite adverse settings (Bills and Polishook, 1992).

In the current study, the antibacterial activity of chosen medicinal plants including the leaves of *Catharanthus roseus* and *Vitex negundo* was assessed along with the identification of a variety of fungal species, phytochemical analysis, and phytoremediation.

Four endophytic fungal species were isolated from *Catharanthus roseus* leaf samples in order to be identified, and based on their physical traits, they were given the names SR1, SR2, SR3, and SR4 (Plate 2). SR1 was recognised as *Xylaria* sp. strain SR1 out of the four species of fungal endophytes. *Catharanthus roseus* colonisation was similar in another plant, *Vitex negundo* leaf portions. Four endophytes were isolated from leaves and given the names S1, S2, S3, and S4 based on their physical traits (Plate 3). S2 was recognised as *Phoma* sp. strain S2 out of the four species of fungal endophytes.

To understand the biological action, basic phytochemical research on phenols, terpenoids, saponins, and cardiac glycosides components was crucial. In order to screen the ethyl acetate extract of *Xylaria* sp. for

qualitative phytochemicals, this study was conducted. *Phoma* sp. has three phytocomponents that were found in the ethyl acetate extract, including phenol, saponin, and terpenoid. The ethyl acetate extracts produced positive findings for all investigated phytochemicals.

The antibacterial activity of the fungal extracts of *Xylaria* sp. and *Phoma* sp. in several solvents, including ethyl acetate and methanol, was tested. The antibacterial activity of *Xylaria* sp methanol .'s and ethyl acetate was better against all of the studied microbes, with inhibition zones of 8 to 12 mm in diameter.

*Pseudomonas syringae, Proteus mirabilis, Burkholderi agluma,* and *Moraxella bovis* are among the bacterial pathogens that the ethyl acetate extract of *Phoma* sp. has strong antibacterial action against.

According to this study's findings about the antibacterial activity against drug-resistant *Pseudomonas syringae, Proteus mirabilis, Burkholderi agluma,* and *Moraxella bovis,* endophytic *Xylaria* sp. strain SR2 and *Phoma* sp. strain S2 have medical value. As a result, the extract of *Xylaria* sp. strain SR2 and *Phoma* sp. strain S2 may one day be used as a strong antibiotic therapy for infectious disorders caused by *Pseudomonas syringae, Proteus mirabilis, Burkholderi agluma,* and *Moraxella bovis*. To demonstrate the veracity of its bacterial activity and its possible usage, more findings and chemical nature research are needed.

## 4. ACKNOWLEDGEMENTS:

The authors thank the Managing Board of Vivekananda College as well as Virudhunagar Hindu Nadar's Senthikumara Nadar College, Virudhunagar-626 001,Tamilnadu, India, for providing research facilities.

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# Antibacterial activities of endophytic *Xylaria* sp. strain SR2 from *Araucaria heterophylla* against drug resistant *Staphylococcus aureus* strains

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*J. Mycopathol. Res.* 59(4) : *435-441, 2021;*
ISSN 0971-3719

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# Antibacterial activities of endophytic *Xylaria* sp. strain SR2 from *Araucaria heterophylla* against drug resistant *Staphylococcus aureus* strains

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Received : 10.8.2021 Accepted : 23.10.2021 Published : 27.12.2021

In the present study, the bioactive endophytic fungal species was isolated from the gymnosperm plants of *Araucaria heterophylla*. The isolated fungal species was identified as *Xylaria* sp. SR2. based on morphological characteristics. The fungal isolate of Xylaria was grown in various range of pH, temperature and culture media for optimizing maximum biomass production in submerged culture conditions. The maximum growth of Xylaria sp. SR2 was observed in PDA and the optimal temperature and pH for the maximum mycelial growth was found to be 30°C and 5.5 respectively. The optimized ethyl acetate extract of cultural filtrate were subjected to antibacterial activities against *Pseudomonas syrirgae* (MTCC 673), *Proteus mirabilis* (MTCC 1429), *Burkholderia glumae* (MTCC 8496), *Moraxella bovis* (MTCC 1775) and drug resistant *Staphylococcus aureus* strains (1-10). The maximum inhibition zone of 23.2 mm and 20.4 mm against *S. aureus* strain 6 and 5 respectively.

**Key words:** Antibacterial Activity, Drug resistant Bacteria, Endophytic *Xylaria*,

## INTRODUCTION

In recent past, emerging of new infectious diseases such as Covid-19, drug resistant microbial diseases, Influenza, SARS, and H1N1 has become the greatest challenges for researchers to safeguard the human health against pathogenic microorganisms. Most of these newly emerged diseases are caused by microorganisms and the causative organisms are increasingly becoming drug resistant over the recent past times (Tauhidur *et al*. 2020, Bhatia and Narain 2010). For treating such infectious diseases, novel bioactive natural compounds from plants as well as microbes could provide the best and significant alternatives as source of potential and promising drugs (Morens *et al.* 2004). Although, plant is the major source of bioactive natural compounds, nevertheless, endophytic fungal species could play a vital role in the search of new novel bioactive natural products (Jalgaonwala *et al*. 2011).

In the last couple of decades, endophytic fungal species are emerged as significant natural resources of bioactive natural products against drug resistant microorganisms like bacteria and fungi, because most of them are occupying millions of peculiar biological niches growing in so many unusual stresses and environmental conditions. There is enormous scope for the discovery and recovery of novel fungal species, genera and biotypes from these environments.

Bioactive natural compounds produced by the endophyticfungal species are promising potential use in safety and human health concerns, eventhough there is still a significant demand of drug industry for synthetic products due to economic and time consuming reasons (Strobel *et al.* 2004).

Endophytic fungal species are to be found in virtually every tissue of plant parts on the earth. They reside in the living tissues of the host plant and do so in a variety of relationships ranging from symbiotic to pathogenic microorganisms (Strobel *et al*. 2004). Endophytic fungal species have been reported from all groups of plants starting from algae to angiosperms and various research groups reported including algae (Zuccaro *et al*. 2008,

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Suryanarayanan *et al.* 2010) lichens (Suryanarayanan *et al*. 2005) , mosses and angiosperms (Tejesvi *et al*. 2010), and may persist even in aseptically cultured plants also (Lucero *et al*. 2008). Moreover, endophytic fungi are also reported from plants that grow in various environments including tropic (Mohali *et al*. 2005), temperate (Ganley *et al.* 2004), xerophytic (Suryanarayanan *et al.* 2005) and coastal mangroves (Kumaresan and Suryanarayanan 2002). Environment plays an important role on endophyte biodiversity, while the species diversity is dependent upon the nature of the host plant and their ecological location. For example, endophytic fungi in woody plants are highly abundant and diverse, particularly in the tropical areas (Arnold *et al.*2001).

Fungal species of the genus *Xylaria* Hill ex Scharank (Xylariaceae, Asomycetes) both macro and micro fungi, are known to produce diverse classes of bioactive compounds including antifungal multiplolides (Boonphong *et al.* 2001), cytotoxic cytochalasins, acetylcholineesterase inhibitor xyloketals (Lin *et al.* 2001), Xanthones (Healy *et al.* 2004), Xylarighanand Orthosporin (Rong Chen *et al*. 2018), Nigriterpene (Jung *et al*. 2017), Cytochalasin D (Elias Luciana 2018). The present investigation has therefore been designed to study the *in vitro* antibacterial potential of dried fungal extracts of endophytic *Xylaria* sp. SR2 against drug resistant human bacterial pathogens of clinical importance.

## MATERIALS AND METHODS

### *Collection of plant materials*

The healthy stem segments of *Araucaria heterophylla* were collected from our holy Gurukula Institute of Vivekananda College, Tiruvedakam west, Madurai, Tamil Nadu during the month of August 2019 (Fig. 1). The stem segments were placed in paper bags after removal of excess moisture. Then the stem samples were stored at 4oC for further use.

### *Isolation of endophytic fungi*

The stem samples collected from *A. heterophylla*were washed thoroughly with distilled water and air dried before they were processed. Endophytic fungi were isolated according to the reported protocol (Petrini 1986) which was slightly modified based on preliminary research. All the stem samples were washed twice in distilled water and then surface sterilized by immersion for 1 min in 70% (v/v) ethanol, 1min in sodium hypochlorite (3% (v/v) available chlorine) and 30 s in 70% (v/v) ethanol and further washed three times in sterilized distilled water for 1 min each time. After surface sterilization, the samples were cut into 5-7 mm pieces and aseptically transferred to Petri plates containing potato dextrose agar (PDA) with 50 ?g/ml of streptomycin to suppress the bacterial growth. These Petri plates were incubated at $30^0$C with normal daily light and dark periods. The plates were examined daily for up to one month for the development of fungal colonies growing out from the stem segments. The fungi growing out from the stem tissue were subsequently transferred onto fresh PDA plates without antibiotics.

### *Microscopic analysis of morphological characterization*

The endophytic fungus was grown on PDA at 30°C for 7–9 days, and the formation of conidia was examined under a microscope. Each specimen was examined for morphological characteristics of asci, ascospores, paraphyses and other structures of taxonomic value. Spore dimensions were determined for 50 spores. Lacto phenol cotton blue and distilled water were used as mounting media for microscopy. Dried materials were rehydrated in 3% aqueous KOH. Photography was carried out with a light microscope and binocular microscope attached with computer (COSLAP). The isolated endophytic fungus was identified based on morphological characteristics and standard taxonomic key included colony diameter, texture, color and the dimensions and morphology of hyphae and spore (Ainsworth *et al.* 1973).

### *Effect of pH on the growth of fungal species*

The endophytic was grown in potato dextrose agar plates with the initial pH of 4.0 to 7.0. The culture was incubated for 7 days under static condition. After the incubation, radial growth was measured. The measurements were taken from four different points and the average radial growth was recorded.

### *Effect of temperature on the growth of fungal species*

The endophytic was grown in potato dextrose agar

plates with the initial temperature of 10 to 35$^0$C. The culture was incubated for 7 days under static condition. After the incubation, radial growth was measured. The measurements were taken from four different points and the average radial growth was recorded.

### *Fermentation and extraction of bioactive compounds*

The endophytic fungus was grown on potato dextrose agar (PDA) at 30ºC for 5-7 days depending on growth rate. Six pieces of the grown culture cut from the plate were inoculated into 500 ml Erlenmeyer flasks containing 300 ml potato dextrose broth (PDB) and incubated at 30ºC for 3 s at pH 5.5. After the incubation period, the preparation of extracts from fermented broth was based on our earlier standard protocol (Ramesh *et al.* 2014, Subbulakshmi *et al*. 2012, Arivudainambi *et al*. 2011)

### *Test Microorganisms*

*Pseudomonas syringae* (MTCC 673), *Proteus mirabilis* (MTCC1429), *Burkholderiaglumae*(MTCC8496), *Moraxella bovis* (MTCC 1775) were purchased from Microbial Type Culture Collection, Chandigarh, India and were used for screening tests. The clinical strains of *Staphylococcus aureus* (1–10) were obtained from Bose Clinical Laboratory and X-ray (Madurai, Tamilnadu, India). *S. aureus* strains were identified by standard biochemical methods (Essers andRadebold 1980, Pourshadi and Klaas 1984).

### *Antibiotic Susceptibility Test*

The Kirby–Bauer disk diffusion test was used to determine the antibiotic resistance of *S. aureus* strains (1–10). The nutrient broth was prepared and well-isolated colonies of the same type from a culture agar plate were inoculated into it. The broth was incubated at 37ºC until the culture equalled 0.5 McFarland standards. A McFarland 0.5 turbidity standard corresponded to an inoculum of 1×108 CFUml-1 (Acarand Goldstein 1996). The remaining procedure was done based on our earlier standard protocol (Ramesh *et al.* 2015, Essers & Radebold 1980)

### *Antibacterial activity*

For preliminary screening, the antibacterial activity was done based on our earlier standard protocol (Ramesh *et al*. 2015, Arivudainambi *et al*. 2011).

### *Statistical analysis*

The triplicate data are expressed as the mean value ± standard error and presented in the form of figures. The error bars are depicted at 5% limit. The overlapping and non-overlapping bars show no significant and significant respectively differences among different treatments.

## RESULTS AND DISCUSSION

Totally six endophytic fungal species were isolated from the stem samples of Araucaria sp. at the time of isolation, they were named as SR1, SR2, SR3, SR4, SR5 and SR6 based on morphological characteristics (Fig. 2). Among the six species of fungal endophytes, SR2 was identified as *Xylaria* sp. strain SR2 based on the colony morphology and sporulating structure. Traditionally, identification of fungal species has been based on size, shape of conidia and culture characteristics such as colony colour, growth rate and texture. The remaining five fungal isolates were not able to identify at the genus level due to insufficient spore production. So, they named as fungal isolates of sterilia SR1, SR3, SR4, SR5 & SR6. Among these above fungal isolates, only one endophytic fungal isolates *Xylaria* sp. strain SR2 was selected to further growth optimization and antibacterial screening studies.

In order to obtain large quantities of mycelial biomass, then this could be used for biological activity. The effect of environmental factors such as pH, temperature and culture media were examined and the optimal conditions were determined. The morphological characteristics of the *Xylaria* sp. strain SR2 was observed on PDA after 10 d of growth at 30°C. Growth rate is high, 5.4 - 7.2 cm/week, covering petriplate in 5 - 7 days. Mycelial mat was white at early stage, later it was brown to thick black coloured. Hyphae were thin walled and branched. The morphological characteristics of the endophytic fungal isolate SR2 was observed on PDA after 7 day of growth at 30 $^0$C. Colonies on PDA was circular, mycelium is raised at first at whitish colour and becoming black with age, sometimes pale grey (Fig. 3a).Asci are 8 spored, uniseriate, cylindrical and stipitate. Ascospores are 17.3 – 17.8 μm broad, 33.1 – 33.8 μm in length. Each ascospore is ellipsoid,

**Table 1:** Antibacterial activities of ethyl acetate extract of *Xylaria* sp. SR2 against bacterial pathogens

| Microorganisms Used | Zone of Inhibition (mm) |
|---|---|
| *Pseudomonas fluroescus* (MTCC103) | 12.2 ±0.25 |
| *Pseudomonas syrirgae* (MTCC 673) | 11.3 ±0.02 |
| *Proteus mirabilis* (MTCC 1429) | 11.2 ±0.15 |
| *Burkholderiaglumae* (MTCC 8496) | 10.2 ±0.11 |
| *Moraxella bovis* (MTCC 1775) | 10.4 ±0.23 |

**MTCC:** Microbial Type Culture Collection

moderate radial growth was observed at pH 5 and 6.0. At pH 8.0, there was no fungal growth (Fig. 4). Earlier reports revealed that many kinds of fungi grow at acidic pH optima (Ramesh *et al.* 2014, Kim *et al.* 2005, Shu and Lung 2004). The radial growth of *Xylaria* sp. Strain SR2 was investigated at various temperatures (10°C to 35°C) in PDA plates at pH 5.5. The radial growth of the mycelium was measured after 7 days of incubation. The influences of temperature on the radial growth of fungal isolates were presented in the Fig 5. The maximum radial growth of 3.5 cm was observed at 30°C in *Xylaria* sp. Strain SR2. Here the influence of the days are directly proportional the radial growth of fungal isolates. The results indicated that the optimum temperature for maximal radial growth was 30°C. This observation was comparable to the growth of many other kinds of fungal species in various regions (Bae *et al.* 2000, Kim *et al*. 2003). Similarly, this present results seem to be consistent with other reports in which the optimal temperature for fungal growth was 20ºC to 30ºC ( Lee *et al*., 2004, Lai *et al.* 2014).

**Table 2:** Antibacterial activities of ethyl acetate extract of *Xylaria*sp. SR2 against drug resistant *Staphylococcus aureus* strains

| *S. aureus* | Zone of inhibition (mm) | | | |
|---|---|---|---|---|
| | Penicillin (10 uits/mL) | Methicillin (10 µg/mL) | Vancomycin (30 µg/mL) | Ethyl acetate extract of *Xylaria*sp. SR2 |
| Strain 1 | 12.8 ± 0.15 (R) | 10.8 ± 0.14 (R) | 16.5 ± 0.08 (S) | 18.3±0.21 |
| Strain 2 | 12.8 ± 0.14 (R) | 11.2 ± 0.31 R) | 15.4 ± 0.28 S) | 19.6±0.11 |
| Strain 3 | 14.2 ± 0.28 (R) | 12.1 ± 0.16 (R) | 16.0 ± 0.22 (S) | 15.2±0.12 |
| Strain 4 | 11.5 ± 0.35 (R) | 10.2 ± 0.31 (R) | 17.2 ± 0.14 (S) | 16.1±0.11 |
| Strain 5 | 10.8 ± 0.14 (R) | 8.9 ± 0.22 (R) | 18.5 ± 0.28 (S) | 20.4±0.06 |
| Strain 6 | 10.3 ± 0.15 (R) | 9.5 ± 0.35 (R) | 12.4 ± 0.35 (R) | 23.0±0.13 |
| Strain 7 | 13.8 ± 0.16 (R) | 15.5 ± 0.07 (S) | 16.9 ± 0.14 (S) | 18.4±0.11 |
| Strain 8 | 10.5 ± 0.23 (R) | 8.8 ± 0.14 (R) | 13.5 ± 0.21 (R) | 17.7±0.13 |
| Strain 9 | 14.0 ± 0.21 (R) | 12.2 ± 0.10 (R) | 14.8 ± 0.22 (R) | 18.4±0.12 |
| Strain 10 | 14.9 ± 0.1 (R) | 8.9 ± 0.18 (R) | 17.4 ± 0.35 (S) | 16.6±0.32 |

**R:** Resistant, **S:** Sensitive

inequilateral to broad, dark brown, unicellular, smooth, germ slit conspicuous, straight and running full length of spore( Fig 3b). The morphological characters of this fungal isolates was identical with *X. angulosa* (AB274814) growing in soil (Rogers *et al*. 1987). On the other hand, many species of *Xylaria* are actively growing in decaying wood of angiosperms and are known to be saprobic (Rogers *et al*. 2005). The genus *Xylaria* was classified in the family xylariaceae of the class of Pyrenomycetes. The Xylariaceae is a large and relatively well known family, which is the representative of Ascomycete in most countries.

The effect of initial pH on the radial growth of *Xylaria* isolates was studied at pH ranging from 4.0 to 8.0 in PDA medium for 7 d. the maximum radial growths of 4.8. cm was obtained at pH 5.5, whereas, the

To study the effect of culture media on biomass production, three different culture media were used. The fungal isolates were grown in MEB, PDYEB, PDB and basal growth medium of pH 5.5 at 30°C for a period of 10, 20, 30 and 40 day. The maximum biomass production of 1.8 g/l was observed on PDB over a period of 40 d at pH 5.5 & temperatures 30°C (Fig. 6). Likewise, Arivudainambi*et al.* (2011) reported that the maximum biom-

ass of 1.9 g/l produced by the endophytic fungus Collectotrichum gloeos porioides VN1 was studied in PDB at pH 5.5 for a period 28 d. On the contrary, Bilay *et al*. (2000) reported that Ganoderma lucidum had a slow growth rate in PDB medium, whereas there was a significant fungal biomass production in basal medium. Similarly, the maximum fungal biomass production of *Ganoderma* species was observed in basal medium (Roberts 2004).

For the preparation of extract, the endophytic fungus *Xylaria* sp. strain SR2 was grown in PDB at pH

**Fig. 1 :** *Araucaria heterophylla*.

5.5 for 35 d. After that, the culture filtrate was extracted with various solvents such as hexane, ethyl acetate and methanol. The mycelium was dried and extracted with various solvents. The mycelium extract was also combined with the culture filtrate extract. After extraction the solvents were concentrated by using rotary vacuum evaporator. The dried extracts were dissolved in DMSO and stored at 4$^0$C for studying antibacterial activity.

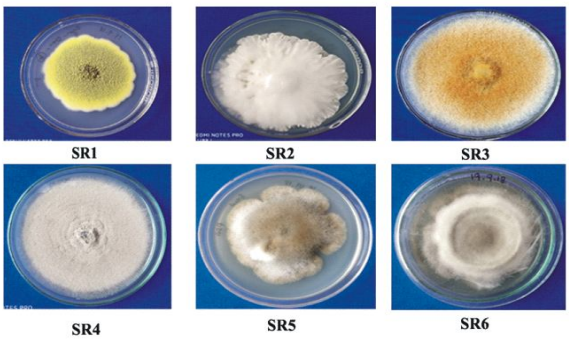


**Fig. 2 :** Colony morphology of endophytic fungal isolates from *Araucaria heterophylla*

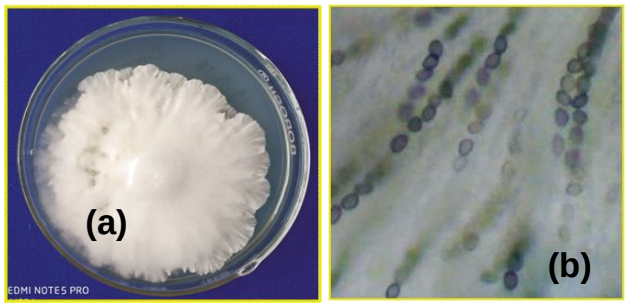


**Fig. 3 :(a)** Cultural morphology on PDA plate (a) and ascospores (b) of *Xylaria* sp. strain SR2

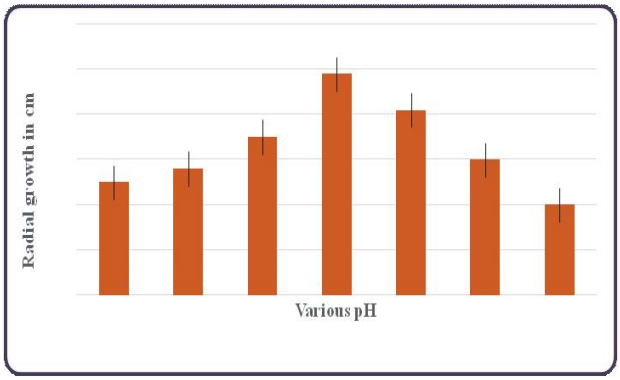


**Fig. 4 :** Effect of various pH (ranging from4 to 8) on radial growth *Xylaria* sp.Strain SR2

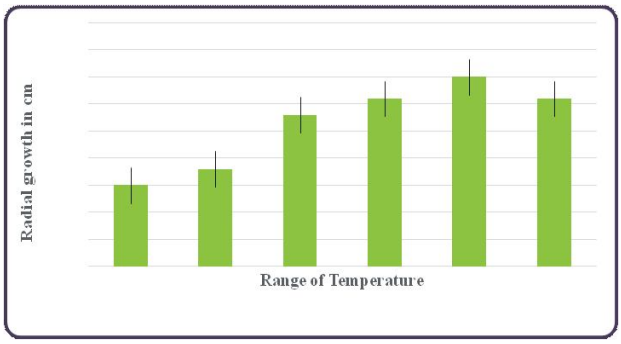


**Fig. 5 :** Effect of various temperatures ( ranging from 10$^0$C to 35$^0$C) radial growth *Xylaria* sp. strain SR2

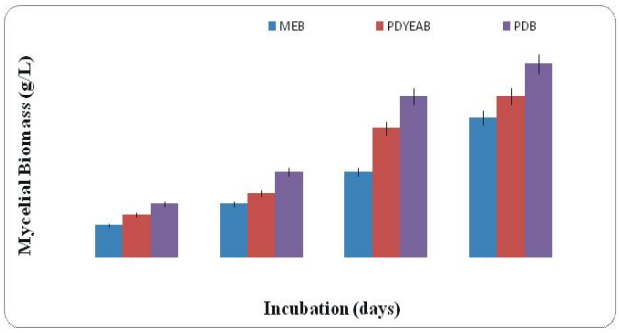


**Fig. 6 :** Effect of various culture media on fungal biomass production of *Xylaria* sp. strain SR2

The accelerating haunt for new antimicrobial drugs to provide assistance in medical community to combat drug resistance microorganism, the appearance of life-threatening bacteria, and the tremendous increase in the incidence of fungal infections in the world's population. So far, many pharmaceutical, novel and bioactive natural products with unique health enhancing properties have been identified from fungal species and distributed worldwide (Cairney 1999). The antimicrobial activity of the extract of *Xylaria* sp. strain SR2 against five bacterial strains was investigated by agar well method. The results showed that ethyl acetate extract showed significant antibacterial activity against all the tested bacterial pathogens. The Ethyl acetate extract of *Xylaria* sp. strain SR2 produced the maximum inhibition zone of 11.3 mm against *Pseudomonas syrirgae*, 11.2 mm against *Proteus mirabilis*, 10.4 mm against Moraxella bovis and 10.2 mm against *Burkholderia glumae* (Table 1). However, the methanol extract had low inhibitory effect against all the tested organisms. The observed inhibitory effect is in line with the report of Phongpaichit *et al.* (2006) who worked on the antimicrobial metabolites in *Xylaria* sp. Similarly, Ramesh et al. (2012, 2012a, 2015) reported that the crude extract of *Xylaria*spp. exhibited significant antimicrobial activities against human bacterial and fungal pathogens as well as drug resistant bacterial pathogens. Moreover, recently Devaraju *et al.* (2020) studied the antimicrobial potential of culture broth of *Xylaria* sp. FPL-25 exhibited broad-spectrum antimicrobial activity against human bacterial and fungal pathogens by bioactivity guided fractionation using bioautography and chromatography.

Further the ethyl acetate extract was only taken for antibacterial activity against multidrug resistant bacterial strains based on the results of earlier preliminary antimicrobial screening. Among the drug resistant bacteria, methicillin resistant *S. aureus* (MRSA) gained much attention in the last couple of decades (Ramesh *et al*. 2015). Hence, the emergence of the MRSA strain possesses a substantial threat to public health. So we have chosen this multidrug resistant *S. aureus* for further antibacterial bioactivity and obtained from Bose Clinical Laboratory and X-ray (Madurai, Tamilnadu, India). The identification and antibiotic resistant profile of clinical strains of *S. aureus* (1–10) were done and reported earlier (Phongpaichit *et al*. 2006, Ramesh *et al*. 2015, Arivudainambi *et al.* 2011). The ethyl acetate extract showed significant inhibition zone of 23.0 mm and 20.4 mm against *S. aureus* strains 6 and 5, respectively (Table 2). Whereas the moderate inhibition zone of 19.6 mm and 18.4 mm were observed against *S. aureus* strain 2 and 9, respectively. This kind of observation was lined with the activity of macro fungal extract of *Xylaria* spp. (Ramesh *et al*. 2012, 2015). Similarly, Elias Luciana *et al*. (2018) isolated endophytic fungal isolates of *Xylaria* spp. from leaves the of guarana plant. They also reported that the fungistatic activity of that endophytic *Xylaria* spp. against the plant pathogen *Colletotrichum gloeosporioides*. Moreover, Mohd Adnan (2018) also reported that the antibacterial activity of *Xylaria* sp. aginst *Staphylococcus aureus*.

In conclusion, this is the study which is revealing the medicinal importance of endophytic *Xylaria* sp. strain SR2 in terms of antibacterial activity against drug resistant *S. aures*. Therefore, the extract of *Xylaria* sp. strain SR2 may have potential use in the future as an effective antimicrobial solution, in the form of the treatment for *S. aureus* associated infectious diseases. Further results are required, the active chemical nature studies to prove the authenticity of its bacterial activity and its potential use.

## ACKNOWLEDGEMENTS

The authors thank the Managing Board of Vivekananda College as well as Virudhunagar Hindu Nadar's Senthikumara Nadar College, Virudhunagar-626 001, Tamilnadu, India, for providing research facilities.

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E-ISSN: 2278-3229

IJGHC, March 2023–May 2023;Sec. A; Vol.12, No.2, 120-127.
DOI: 10.24214/IJGHC/GC/12/2/12027.

International Journal of Green and Herbal Chemistry

An International Peer Review E-3 Journal of Sciences

*Available online at* www.ijghc.com

Section A: Green Chemistry

Research Article

CODEN (USA): IJGHAY

# Characterization and study of antimicrobial activity of silver nanoparticles synthesized from *Escherichia coli*

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**Received:**21 March 2023; **Revised:** 02 April2023; **Accepted:**12 April 2023

**Abstract**: The usage of prokaryotic microorganisms within the synthesis of nanoparticles emerges as an eco-pleasant the existing method. On this have a look at, Sliver nanoparticles were correctly synthesized by *Escherichia coli*, in which exposure of supernatant to silver ions resulted in the extracellular reduction of the metallic ions and formation of silver nanoparticles. The check bacterium was isolated from an infected water sample on Eosin- methylene blue (EMB) agar medium and diagnosed as an Escherichia coli microorganism. The silver nanoparticles had been characterized by several strategies. The nanoparticles show absorbance at 400 nm on UV-Visible spectroscopy. The presence of proteins turned into recognition by way of Fourier Transform Infra-Red (FTIR) analysis. Silver nanoparticles' size become studied using Transmission electron microscopy (TEM). The antimicrobial activity of these nanoparticles changed into studied towards microorganisms such as *Klebsiella pneumonia, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, and Methicillin-resistant staphylococcus aureus*.

**Keywords**: Silver nanoparticles, *Escherichia coli*, UV-Visible spectroscopy, FTIR, Antimicrobial activity.

## 1.INTRODUCTION

The is envisaged that nanotechnology would change both science and society. To create usable materials and technologies, nanotechnology entails manipulating materials that are 100,000 times smaller than a human hair strand at the atomic level. One billionth of a meter-scale technology is involved. The word "NANO" comes from the Greek word for "Dwarf" [1]. Nanotechnology is anticipated to have a profound impact on research, and the economy and the 21st century's way of life may serve as one of the causes behind the coming industrial revolution. Spectrally selective coating for solar energy absorption, intercalation material for electrical batteries, visual receptors, biolabeling, and antibacterial agents are only a few of the several significant uses for silver nanoparticles [2].

One of the most active fields of research in contemporary material science is the topic of nanotechnology. One of the nanoparticles with an inhibitory impact on bacteria found in industrial and medicinal processes is silver, and this has long been known [3]. Due to their numerous uses in fields including biotechnology, electronics, coatings, cosmetics, and medicine, metal nanoparticles made of silver and gold are attracting a lot of attention. The more significant area of research in nanotechnology is the creation of metal nanoparticles. The most popular approach for creating metallic nanoparticles is chemical synthesis [4].

The potential of several plants for the manufacture of silver nanoparticles (SNPs) has recently been investigated. Prokaryotes and eukaryotes, in particular, are used by microbes to synthesize nanomaterials. Microbes are involved directly or indirectly in a variety of biological processes. Earth's metals and nonmetals are frequently found in contact with biological elements. Bacteria are the most prevalent species in our biosphere. The synthesis of nanoparticles may be greatly benefited from little climatic changes, which have the potential to be terrible for the life processes of bacteria. many bacterial cultures were employed for a variety of nanoparticles, including gold nanoparticles produced by the marine bacteria Shewell algae and silver nanoparticles produced by the fungus Verticillium [5].

It has been claimed that eukaryotic organisms, such as fungi, may produce gold and silver nanoparticles. For the effective generation of metal nanoparticles, many strains of Fusarium, including *Fusarium oxysporum*[6], Aspergillus fumigatus, and Aspergillus flavus, were utilized. Recently, stable silver nanoparticles have also been made using the white rot fungus Coriolus Versicolor. Biosensors, bio-labeling, cancer treatments, and coating of medical devices are only a few uses for biologically created nanoparticles [7]. Silver (Ag) is the preferred noble metal for use in biological systems, living things, and medicine.

A new area of nanotechnology is the green production of nanoparticles. As silver nanoparticles are quick and precise in their goal towards the applications where they are utilized [8], an effort was made in the current work to synthesize silver nanoparticles of varied concentrations and assess their antibacterial activity. The medical business, including common ointments to prevent infection against burns and open wounds, is where silver and silver nanoparticles are most commonly used [9]. In both in vitro and in vivo settings, silver nanoparticles have a wide range of uses. Several methods, including chemical, photochemical, and solution reduction the creation of silver nanoparticles is possible through reverse micelle reactions[10], thermal degradation of silver compounds, radiation assistance, electrochemical[11], Nano chemical[12], microwave-aided method, and most recently via green chemistry approach.

Since ancient times, people have employed the non-toxic, secure inorganic antibacterial chemical known as silver to destroy a variety of disease-causing germs [13]. Silver has long been recognized to have strong antibacterial, antifungal, and antiviral properties, but in recent years, there has been a

major resurgence in the use of silver as a biocide in suspension, solution, and notably in nanoparticulate form. The usage of Nanosilver in consumer and medical products is now on the rise because of the characteristics of silver at the nanoscale.

The development of goods using Nanosilver has recently increased, in large part due to its extraordinarily potent antibacterial properties. Controlling the physical attributes of nanomaterials, such as attaining uniform particle size distribution, same shape, morphology, chemical content, and crystal structure, is the major problem in their creation.

The microbial generation of silver nanoparticles was examined in the current work. This research involves the manufacture of silver nanoparticles and their characterization by UV-visible spectrometer, TEM, and to analyze their inhibitory activity against bacterial growth using the bacterial strain *Escherichia coli* as a reducing agent. Species *Staphylococcus aureus, methicillin-resistant Staphylococcus aureus, Klebsiella pneumonia, Escherichia coli, and Pseudomonas aeruginosa* against antimicrobial activity.

## 2.MATERIALS AND METHODS

**2.1 Material:**India Merck (Mumbai) Silver Nitrate. Products from HiMedia, India were utilized for the bacterial growth investigation including EMB medium, Nutrient agar, LB broth, PDA, and PDB. The PG&Research department of microbiology, V. H. N. SenthikumaraNadarCollege, Virudhunagar. provided cultures of the bacteria such as*Klebsiella pneumonia, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, and methicillin-resistant Staphylococcus aureus.*

**2.2 Sample gathering:** The soil samples used for the *E. coli* bacterium isolation were chosen at random. The obtained soil sample was kept in sterilebagsand kept at 4°C before being quickly transferred to the lab.

**2.3 Isolation of bacteria:**Bacterial isolation was done using the "*Serial Dilution Method*." A Soli sample of 10 ml was obtained and documented as a 10:1 dilution in a 100 ml beaker. Using a sterile pipette, 1 ml of water from a $10^{-1}$ dilution was collected and added to the 9 ml of distillate water, and the result was reported as a $10^{-2}$ dilution. 1mm of the $10^{-2}$ dilution sample was collected, combined with 9 mm of sterile distilled water, and labeled as the $10^{-3}$ dilution. The procedure kept on until the dilution reached $10^{-6}$. Then, to isolate the bacterium *E. coli*, 1 ml of sample from $10^{-3}$ and $10^{-5}$ dilution was placed on a plate (EMB medium).The plate was incubated in the incubator overnight at $37^{0}$C. It was the best way for isolating each colony that was growing on the plate from a single cell. utilizing EMB Agar medium for the isolation of E. coli bacteria. This medium was created by dissolving 15g of agar, 2g of dipotassium hydrogen phosphate, 4g of eosin Y, 10g of lactose, 0.65g of methylene blue, and 10g of peptone in 1000 ml of distilled water. The isolation of *E. coli* bacteria can only be done using this medium.

**2.4 Identification:**The culture was identified and described using morphological (colony color, shape, and size) and biochemical testing. The strain was described as gram-negative, motile, facultatively anaerobic, and non-spore-producing bacteria. The Catalase test, Indole production, Methyl Red, Macconkey growth, D-glucose acid/gas, and D-mannitol fermentation test all yielded positive results for this strain. With the Hydrogen Sulfide ($H_2O_2$), Oxidase, Voges Proskauer, Urea hydrolysis, and Citrate (Simmons) tests, they had negative results. For accurate identification of the organisms, the observed traits were matched to Bergey's Manual of Determinative Bacteriology.

**2.5 Production of biomass:** *Escherichia coli* bacteria were grown in an LB medium to create the biomass needed for biosynthesis. The culture flask was heated to 370°C and stirred at 200 rpm while

being incubated on an orbital shaker. After 24 hours of growth, the biomass was collected and centrifuged for 10 minutes at 10,000 rpm. For a subsequent reaction, the supernatant was collected.

**2.6 Synthesis of silver nanoparticles:**A different reaction mixture without silver nitrate was employed as a reference. For the biogenesis of silver nanoparticles, 10 ml of supernatant was combined with 5 ml of silver nitrate ($AgNO_3$) solution (10 mM). For 24 hours, the produced solutions were incubated at 30°C. To prevent any photochemical reactions throughout the experiment, all liquids were maintained in the dark. The solution changed from yellow to brown after 24 hours. The silver nanoparticles (AgNPs) were collected for further analysis after being purified by centrifugation twice for 5 minutes at 10,000 rpm.

**2.7 Characterization of nanoparticles:**The presence of a brown hue indicates that the reaction mixture has formed silver nanoparticles and that the Ag ions have been reduced effectively. Although it is yet unclear how silver ions are reduced and how silver nanoparticles are created, protein molecules and enzymes, including nitrate reductase, are thought to play an important role in controlling the production of silver nanoparticles. The developed color solution enabled the conformation of the production of silver nanoparticles by measuring the absorbance against certain wavelengths. Transmission electron microscopy was used to examine the size of the silver nanoparticles and their lattice structure (TEM)

**2.7.1 UV-Vis Spectroscopy:** Due to the colored nanoparticle solution's 400 nm peak, spectroscopy can identify the formation of silver nanoparticles. In this work, the optical density of the solution was determined using a Shimadzu spectrophotometer.

**2.7.2 Transmission Electron Microscopy:**The JEOL JSM 1oocx apparatus was used for transmission electron microscopy. The particle size and form, together with any crystal structure present, are visible using TEM. A drop of the nanoparticle solution was applied to a carbon-coated copper grid to prepare the grid for TEM investigation, and the water was then allowed to evaporate within a vacuum drier. A transmission electron microscope was used to scan the grid of silver nanoparticles.

**2.8 Antibacterial activity:**Using the Kirby-Bauer well Diffusion Susceptibility Test technique, the antibacterial activity of AgNPs against the *chosen Klebsiella pneumonia, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, and methicillin-resistant staphylococcus aureus*, was assessed (Bauer et al., 1966). Using a sterile cotton swab, the bacterium strains were dispersed on Mueller-Hinton agar (MHA) (Merck, Germany). The test was conducted using a sterile blank antimicrobial susceptibility well. The well on the agar plate was filled with 10 L of culture extracts, 1 mM silver nitrate solution, and a solution containing *E.coli* extract mediated produced AgNPs separately. It was then incubated at 37°C for 24 hours. After 24 hours of incubation, the zone of inhibition was visible.

## 3.RESULT

**3.1 Isolation of bacteria from the soil sample:** Soil samples were taken from Virudhunagar. These samples were serially diluted and plated for the screening of efficient *E.coli* bacteria **(Fig.1 & 2).** After 24 hours of incubation, the plates were kept at 37ºC. Totally 15 strains (N.N1 to N.N15) were isolated and among these strains four strains such as N.N1. N.N3, N.N5, and N.N7 exhibited zone formation in a nutrient agar medium.


**Figure 1:** *Screening* of efficient *E.coli* bacteria

**3.2 Identification of efficient bacteria:**The selected strain was identified by various morphological and biochemical characteristics. Strain N.N7 shows a maximum dye decolorizing zone in nutrient agar medium than other strains.

3.3**Morphological and biochemical characteristics:** This strain was identified as Gram Negative, rod-shaped and motile. According to Bergey's Manual of Determinative Bacteriology, the selected strain was identified as *E.coli* Biochemical studies were performed and results were presented.

**3.4 Synthesis of silver nanoparticles:** After 24 hours of incubation, the reaction mixture's color changed from light yellow to brown, indicating the conformation of nanoparticle creation in the medium. When Ag+ ions were added to the supernatant, brown color developed as a result **(Fig. 2).** The intensity of the color grew with time due to a decrease in $Ag^0$. When incubated under the same conditions and for the same amount of time, the control (without silver nitrate) exhibited no color production in the culture.

**Fig 2:** Synthesis of silver nanoparticles

**3.5 UV-Vis Spectroscopy:** Displays the comparable UV-Vis absorption spectra. The control solution, which does not include silver nitrate solution, exhibits no signs of absorption between 300 and 900 nm. After being subjected to a silver nitrate solution, the samples display a broad-spectrum range of about 400 nm. The wide resonance's existence suggests that the silver nanoparticles in the fluid have aggregated.

**3.6 Transmission Electron Microscopy:** The Carbon-coated copper TEM grid with silver nanoparticles on it was examined using TEM. This image demonstrated their uniform distribution and size range of 20–50 nm. The majority of nanoparticles have a spherical form.

**3.7 Antibacterial activity:**By employing the well diffusion technique, researchers investigated the antibacterial effectiveness of silver nanoparticles against pathogenic bacterial strains of *Klebsiella pneumonia, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, and Methicillin-resistant Staphylococcus aureus* growth (Fig.3a-3e). The control group included culture extracts, AgNO3, distilled water, common antibiotics such as Penicillin G and Chloramphenicol, and these substances.

According to the antibacterial activity data, both gram-positive and gram-negative bacterial strains were effectively inhibited by all synthetic silver nanoparticles. AgNP had inhibitory zones of 6.5 0.3, 6 0.2, 5.5 0.2, and 7.5 0.3, 7 0.2, and 7.7 0.4 mm against *Klebsiella pneumonia, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, and methicillin-resistant staphylococcus aureus*, respectively. Both gram-negative and gram-positive bacterial strains were not susceptible to the culture extract or $AgNO_3$'s antibacterial effects.

This suggests that AgNP is the only factor in the antibacterial action. AgNPs' method of action against bacteria is still not well understood. Nonetheless, several theories are put out to account for silver nanoparticles' antibacterial properties: Reactive oxygen species are produced, followed by the release of Ag + ions from AgNPs, which denaturize proteins by interacting with sulfhydryl groups, and the attachment of AgNPs to bacteria, which causes harm to the bacteria.

Numerous published studies on the antibacterial effects of silver nanoparticles against gram-positive and gram-negative bacteria revealed that gram-positive bacteria were only mildly inhibited by silver nanoparticles. It's worth noting that the silver nanoparticles (AgNP) produced by the *E. coli* culture extract effectively inhibited both gram-positive and gram-negative bacteria, indicating that they had antibacterial properties are not affected by the difference in the bacterial wall.

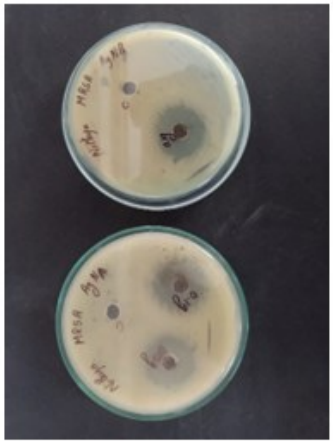

**Figure 3: (a)**

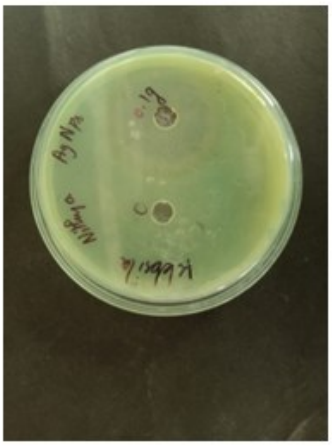

**Figure 3: (b)**

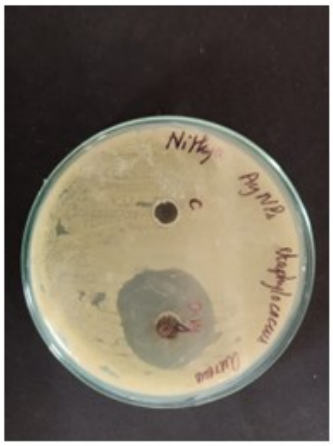

**Figure 3:(c)**

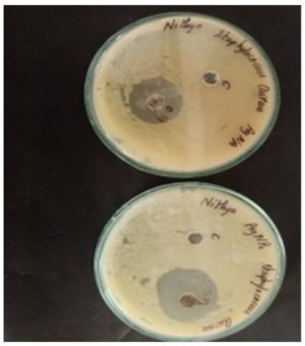

**Figure 3:(d)** **Figure 3:(e)**

**Fig:3(a-e):** Antibacterial activity of AgNPsagainst both **(a).** *Klebsiella pneumonia,* **(b).** *Escherichia coli,* **(c).***Pseudomonas aeruginosa,* **(d).***Staphylococcus aureus,* **(e).***Methicillin-resistant staphylococcus aureus growth*

## 4.CONCLUSION

Metal nanoparticle production by microbes is a dependable and environmentally benign method. The conversion of silver nitrate to silver nanoparticles and the characterization of silver ions subjected to microbial strains were both validated by UV-Vis Spectrophotometer. The size of the Ag nanoparticles' particles ranges from 20 to 50 nm. Uncertainty surrounds the method by which bacteria produce metal nanoparticles. However, it has been taken into account in this research to comprehend the potential mechanisms of metal and microbial interaction, which may be caused by the structural specialization of microbial cells, as well as how metal availability affects microbial resistance.

In this research, we describe the *E. coli* bacteria that were used to create silver nanoparticles from culture filtrate. UV-VIS and other methods have been used to characterize the created nanoparticles. readings from a TEM. The antibacterial activity of the nanoparticles was outstanding. Therefore, the biological technique seems to be a more affordable alternative to traditional physical and chemical methods of silver nanoparticle synthesis and would be appropriate for creating a microbiological process for large-scale commercial production.

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Online publication Date: 14.04.2023

Studies in Fungi 2 (1): 119–129 (2017) www.studiesinfungi.org ISSN 2465-4973
Article

Doi 10.5943/sif/ 2/1/14


# Endophytic Mycodiversity of Sacred Tree – *Couroupita guianensis* Aubl.

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Velmurugan R, Ramesh V, Meenatchi A, Bagyalakshmi, Rajendran A 2017 – Endophytic Mycodiversity of Sacred Tree – *Couroupita guianensis* Aubl. Studies in Fungi 2(1), 119–129, Doi 10.5943/sif/2/1/14

**Abstract**

In the present study, a total of 142 fungal strains were isolated from 180 samples of the leaf, stem and bark tissues of sacred tree *Couroupita guianensis* from an unusual semi-arid tropical region. The tissues of the samples were grown in potato dextrose agar (PDA) medium and the endophytic mycoflora were identified based on the morphological characteristics. Among all the strains, 130 were fertile, which belong to 11 species and 12 sterile morpho species. The relative frequency of isolated individual endophytic fungal group consists of hyphomycetes (41.5%), coelomycetes (32.4%), ascomycetes (13.4%), zygomycetes (4.2%) and sterile fungi (8.5%). Among the fungal isolates, *Scytalidium acidophilum* and *Mycosphaerella* sp. were recorded as the most dominant fungal isolates in the leaf tissues, *Cladosporium cladosporioides* and *Colletotrichum falcatum* were observed as the dominant endophytic fungal isolates in the stem tissues and *Botryodiplodia theobromae* was found to be predominant species in bark tissues of *C. guianensis.* The species of *Colletotrichum* were found only in stem tissues. Therefore, the endophytic mycodiversity were high and abundant and they were distinctly associated with host plant. All statistical analyses confirmed that leaf tissues contained more endophytes than the stem and bark while *Colletotrichum* exhibited organ specificity. The present study revealed that the sacred tree *C. guianensis* is one of the ecological niches for sheltering endophytic mycoflora eventhough in harsh climatic conditions.

**Key words** – *Colletotrichum* – PDA organ specificity – semi-arid region – tropical region

## Introduction

*Couroupita guianensis* is an ever green tropical forest tree that belonging to the family Lecythidaceae, commonly known as cannonball tree. It is native to the Amazon rainforests of Central and South America and also seen in India, especially in the southern states of Western Ghats (Savinaya et al. 2016). Leaves are simple and clustered; flowers are showy with a ring of stamens like hood. In India this tree is sacred to Hindus, who believe its hooded flowers look like the naga (Snake), and it is grown at Shiva temples. Plants associated with temples are sacred trees that possess supernatural power and are preserved and protected by the local people on religious basis over a period of time in Sacred Groves or "Gardens of Gods" and also in their region. The plants growing on arid land contain some functional components that protect them from their stress

Submitted 21 June 2017, Accepted 14 July 2017, Published 7 November 2017
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habitat in which the endophytes may also possess novel strategies for their survivability. The sacred tree has several medicinal properties like antimicrobial, antimycobacterial and antibiofilm and used to treat the common cold, malaria, stomachache, toothache, hypertension, skin infections, wounds, tumors, pain and inflammation (Jayapal et al. 2014)

Endophytes are the part of the microbial community found in all species of plant (Arnold et al. 2000) that include bacteria, fungi, actinomycetes and mycoplasma (Bandara et al. 2006) which live inside plant tissue for at least a part of their life cycle without causing any disease symptoms in the host (Petrini 1991). The most frequently encountered endophytes are fungi (Staniek et al. 2008). Plants may serve as a reservoir of large number of endophytic fungi (Bacon & White 2000). Research of biodiversity of endophytic fungi has a long history and their diversity among plants has been found to be considerably large with reliable source of genetic diversity and novel undescribed species. Endophytic fungi are known to harbour compounds beneficial for plant health as well as human health which can produce various bioactive chemicals (Tejesvi et al. 2011), that promote host growth and resistance to environmental stress (Saikkonen et al. 2010) and also serve as potential sources of novel natural products for exploitation in medicine, agriculture, and industry (Strobel & Daisy 2003). Since natural products are adapted to a specific function in nature, the search for novel secondary metabolites should concentrate on organisms that inhabit novel biotopes. An endophytic fungus inhabits such a biotope. The isolation of novel secondary metabolites from the endophytes is an important aspect of present day research. The present study was carried out to determine the endophytic mycoflora associated with *Couroupita guianensis*, a rarely occurring sacred tree in semi-arid region.

## Materials & Methods

### Collection of plant material

A total of 180 segments of leaf stems and bark tissues of 60 each from the *Couroupita guianensis* Aubl. (Lecythidaceae) were collected from the semi-arid tropical region of Virudhunagar district, Tamil Nadu, India. Those were used for the investigation of endophytic fungal communities. The samples were taken 3–4 feet above the ground level of tree and brought to the laboratory in sterile polythene bags and then inoculated within 3 h of collection.

### Isolation and identification of endophytic fungi

Isolation of endophytic fungi was standardized and modified based on the method described by Hallman et al. (2007). The samples were washed with running tap water to remove dust, soil and debris adhering to them and surface sterilized with 70% ethyl alcohol and 4% sodium hypochlorite to remove adhering microorganisms. Finally the samples were rinsed with deionized double distilled sterile water to remove the surface sterilization agents and dried on sterile tissue papers in a laminar air flow chamber. The leaves and the inner bark tissue were cut in to segments in 3 x 3 $mm^2$ size and 3–5 mm thickened respectively. Stem was also cut in to segments of 1 mm thickness under aseptic conditions using a sterile knife and scalpel. Five sterilized leaf, stem or bark segments were placed in a petridish containing Potato Dextrose Agar medium (PDA) supplemented with the antibiotic streptomycin sulfate 0.4 mg/mL to arrest bacterial growth. The petridishes were sealed with parafilm and incubated at 25±2°C for 15 days under dark conditions and monitored the growth of endophytic fungal mycelium in every day. After 15 days, individual fungal colonies growing out of the explants were sub-cultured on separate PDA petri plates for pure cultures at room temperature and identified in their sporulation stage from 8–15 days. The fungi which failed to sporulate were designated as "mycelia sterilia". The morphological characters of the fungal isolates were observed and described according to the method of Photita et al. (2004). Morphological identification was done according to the standard taxonomic key included colony diameter, texture, color, morphology of hyphae and conidia (Anisworth et al. 1973). Finally, the endophytic fungal isolates were transferred separately to PDA slants and maintained at 4°C for further study.

**Statistical analysis of endophytic fungi**

The Relative Frequency (RF) of isolation, used to represent fungal density, was calculated as the number of isolates of one fungal group divided by the total number isolates, and also expressed as percentage (Photita et al. 2001). The Colonization Frequency (CF %) was used to compare diversity and it was calculated by using the method of Gond et al. (2007). The Colonization Frequency (CF %) of a single endophytic species was calculated using Equation, CF % = (Ncol / Nt) X 100 where, Ncol = number of segments colonized by each fungus and Nt = total number of segments studied. Similarly the percentage frequency of dominant endophytes was calculated as the number of endophytic fungal colonies divided by total number of all endophytic fungal colonies (Mahesh et al. 2005). The Colonization Rate (CR %) was used for the comparison of endophytic fungi in different tissues of given sample. The colonization rate was calculated as the total number of segments colonized by endophytic fungi divided by the total number of segments incubated for that plant sample, and expressed as percentage (Petrini et al. 1982). The Isolation Rate (IR) of endophytic fungi was used to measure the fungal richness in different tissues. The isolation rate was calculated as the number of isolates obtained from segments divided by the total number segments, but not expressed as percentage (Petrini & Fisher 1988). Different diversity parameters were calculated using PAST – Paleontological Statistics Software Packages, ver. 3.05. The Simpson's diversity (1-D) was used to estimate the abundance of endophytes and Shannon-Wiener Diversity index (H') and Fisher's alpha index were analyzed to determine the species diversity of fungal endophytes colonized in leaf, stem and bark and the Evenness index that used to expresses the distribution of individual among the other endophytic fungi in every part of plant.

**Results**

Altogether 142 endophytic fungal isolates were obtained from 180 samples of leaf, stem and bark tissue of *Couroupita guianensis.* Among the isolates, 130 were fertile which belong to 11 species, and they consisted of 4 hyphomycetes, 5 coelomycetes, 1 ascomycetes, 1 zygomycetes and 12 non sporulating sterile morphospecies. The endophytic fungi were identified based on the colony morphology and sporulating structures. The fertile fungal isolates were *Botryodiplodia theobromae, Cladosprium cladosporioides, Colletotrichum dematium, C. falcatum, C. truncatum, Fusarium moniliforme, F. oxysporum, Mycosphaerella* sp., *Mucor racemosus, Phyllosticta hymanaeae* and *Scytalidium acidophilum,*

In the present study, the distribution of endophytic fungi in leaf, stem and bark tissues were investigated for the assemblage, tissue specificity, diversity and abundance. Endophytic fungi may inhabit all available tissues, some endophytic fungi preferred to colonize in the leaves and other endophytic fungi may colonize the tissue of other organs of the plant. Among 142 endophytic fungal isolates, 54 isolates were recovered from the leaves, 48 from stem and 40 from bark tissue of *C. guianensis.*The isolates of *Botryodiplodia, Fusarium, Mycosphaerella* and *Scytalidium* were reported to be higher in leaf than stem and bark. The isolates of *Cladosporium* isolated from stem were found to be higher than in the leaf and bark. Endophytic fungal strains of *Colletotrichum* showed tissue specificity that was only isolated from the stem (Table 3). The Relative Frequency (RF) of isolated individual endophytic fungal group belonged to hyphomycetes (41.5%), coelomycetes (32.4%), ascomycetes (13.4%), zygomycetes (4.2%) and non sporulating sterile form (8.5%) from leaf, stem and bark tissues of *C. guianensis* (Table 1 & Fig. 1).

In this study, the overall colonization frequency of endophytic fungal isolates in leaf was found to be 90% as highest when compared to stem (79.8%) and bark (66.6%) tissue. The colonization frequency of *Cladosporium cladosporioides* was 10 % in stem and 8.3% in leaf and bark respectively. Among the species of *Fusarium*, the colonization frequency of *Fusarium*

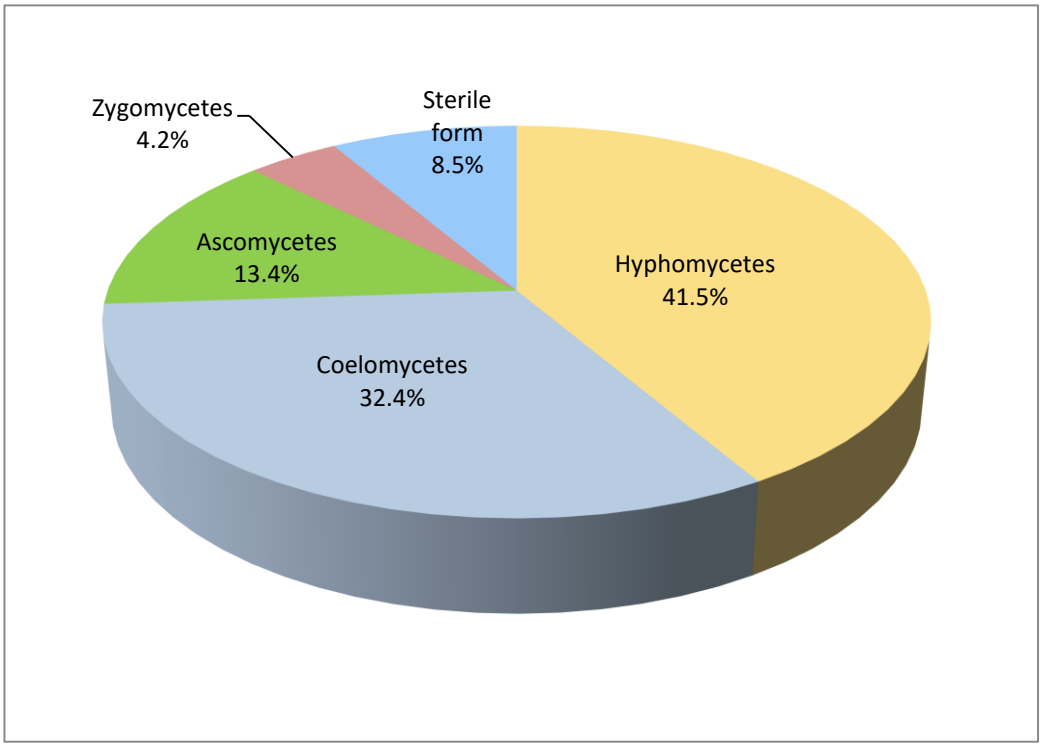


**Fig. 1** – Relative Frequency of different groups of endophytic fungal isolates from *Couroupita guianensis*

*moniliformae* was 10% in leaves that gradually decreased in to 8.3% and 5% in stem and bark respectively whereas the colonization frequency of *Fusarium oxysporum* was 5% alike in leaf stem and bark tissues. In *Scytalidium acidophilum*, the highest colonization frequency was reported in leaf (15%) followed by bark (10%) and stem (8.3%). The colonization frequency of *Botryodiplodia theobromae* was 13.3% in leaf, 11.7% in bark and 8.3% in stem.

The isolates of *Colletotrichum* spp. were reported only in stem, among them the colonization frequency of *Colletotrichum dematium* was 3.3%, *Colletotrichum falcatum* was 10% and *Colletotrichum truncatum* was 5%. In *Phyllosticta hymanaeae*, the colonization frequency was 11.7% in leaf, 8.3% in stem and 5% in bark tissues. The colonization frequency of *Mycosphaerella* sp. was 15% as maximum in leaf and 8.3% in stem and bark respectively whereas the colonization frequency of *Mucor racemosus* was found to be 5% in leaf and bark respectively and absent in stem. In sterile form, the colonization frequency was 8.3% in stem, 6.79% in leaf and 5% in bark tissues of *C. guianensis* (Table 2 & Fig. 2).

Among the fungal isolates, *Botryodiplodia theobromae* was identified to be dominant species (17.5%) in bark tissue, *Scytalidium acidophilum* and *Mycosphaerella* sp. were found to be the dominant endophytic fungi inthe tissue of leaf (15%) and *Cladosporium cladosporioides* and *Colletotrichum falcatum* were observed as the dominant endophytic fungal isolates (12.5%) in stemof *C. guianensis* (Table 2 & Fig. 3).

The colonization and isolation rates of endophytic fungi in leaf were higher followed by stem and bark tissues in the present study. The high range of colonization rates (55%–80%) of endophytic fungi were obtained in the present study. The highest colonization rate of 80% was found in leaf and the lowest colonization rate of 55% was acquired in bark tissues (Table 3 & Fig. 4). The isolation rates of endophytic fungi were recorded as 0.7–0.9 in which the low rate of isolation (0.7) was found in bark and high rate of isolation (0.9) was recorded in leaf (Table 3 & Fig. 5).

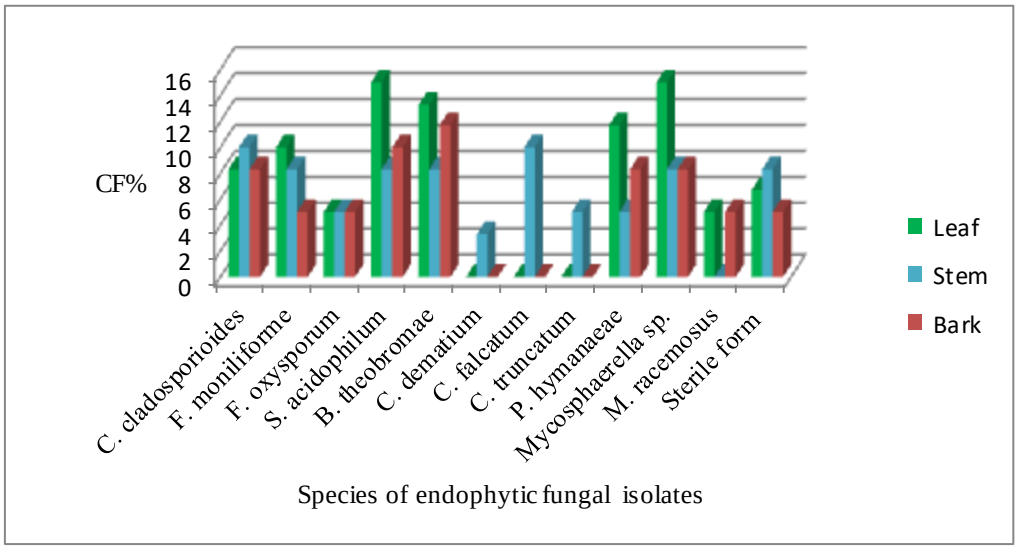


**Fig. 2** – Colonization Frequency (CF %) of Endophytic Mycoflora in different parts of *Couroupita guianensis*

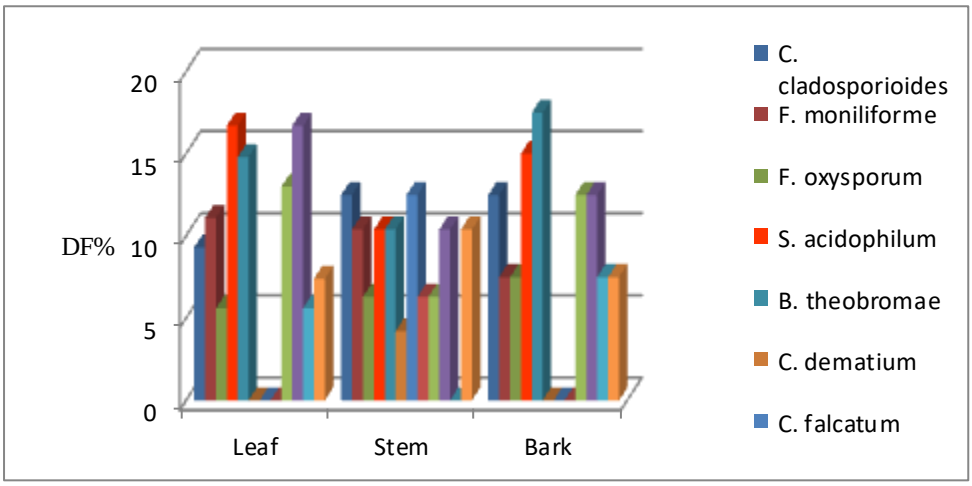


**Fig. 3** – Dominant Endophytic Fungi (DF %) isolated from leaf, stem and bark tissues of *Couroupita guianensis*

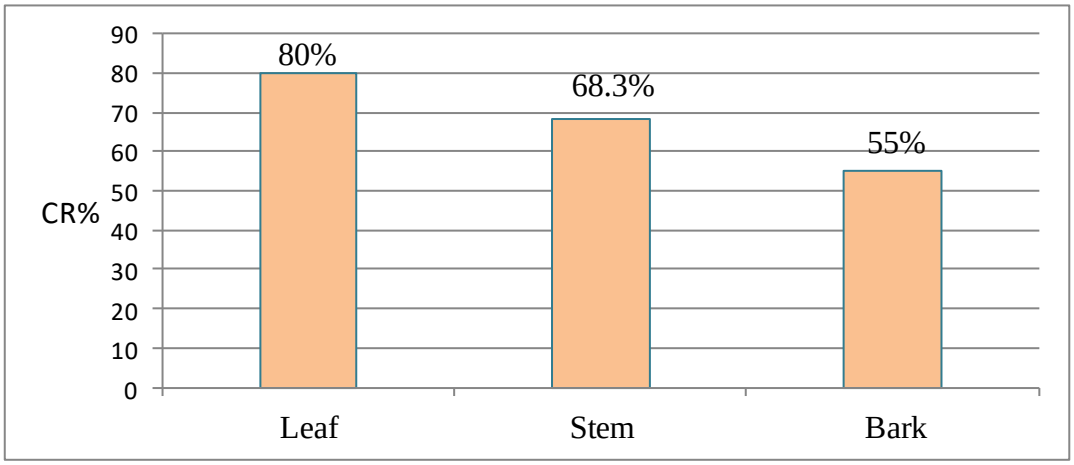


**Fig. 4** – Colonization Rate (CR %) of endophytic fungal isolates from leaf, stem and bark tissues of *Couroupita guianensis*

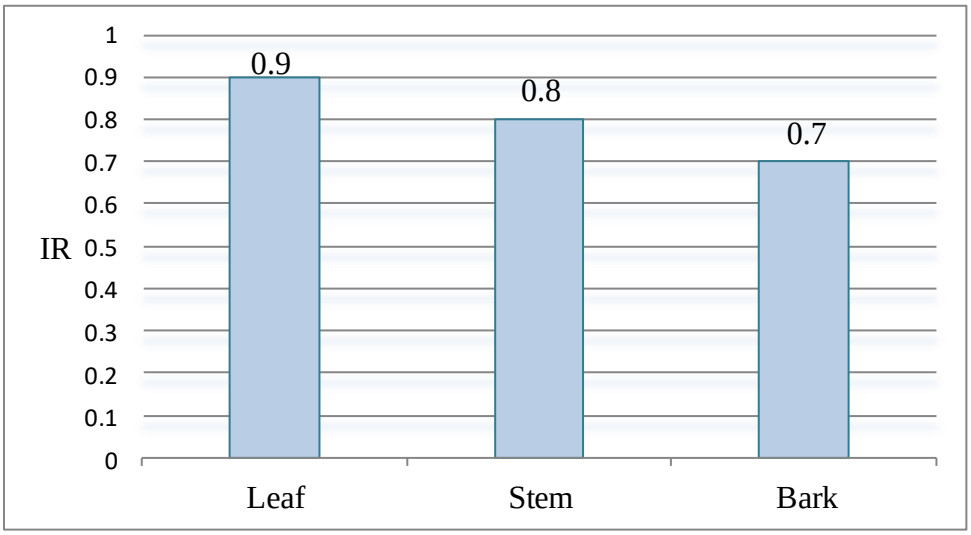


**Fig. 5** – Isolation Rate (IR) of endophytic fungal isolates from leaf, stem and bark tissues of *Couroupita guianensis*

**Table 1** – Isolates and Relative Frequency (RF %) of endophytic fungi from *Couroupita guianensis*

| **SN** | **Fungal groups** | **Endophytic fungi** | **Number of isolates** | | | **RF (%)** |
|---|---|---|---|---|---|---|
| | | | **Leaf** | **Stem** | **Bark** | |
| 1 | **Hyphomycetes** | *Cladosprium cladosporioides* | 5 | 6 | 5 | 41.5 |
| 2 | | *Fusarium moniliforme* | 6 | 5 | 3 | |
| 3 | | *Fusarium oxysporum* | 3 | 3 | 3 | |
| 4 | | *Scytalidium acidophilum* | 9 | 5 | 6 | |
| 5 | **Coelomycetes** | *Botryodiplodia theobromae* | 8 | 5 | 7 | 32.4 |
| 6 | | *Colletotrichum dematium* | -- | 2 | -- | |
| 7 | | *Colletotrichum falcatum* | -- | 6 | -- | |
| 8 | | *Colletotrichum truncatum* | -- | 3 | -- | |
| 9 | | *Phyllosticta hymanaeae* | 7 | 3 | 5 | |
| 10 | **Ascomycetes** | *Mycosphaerella* sp. | 9 | 5 | 5 | 13.4 |
| 11 | **Zygomycetes** | *Mucor racemosus* | 3 | -- | 3 | 4.2 |
| 12 | **Sterile form** | Sterile form | 4 | 5 | 3 | 8.5 |
| Total | | | 54 | 48 | 40 | 100 |

**Table 2** – The Colonization frequency (CF %) and Dominant Endophytic Fungi (DF %) isolated from leaf, stem and bark tissues of *Couroupita guianensis*

| Sl No | Endophytic Fungi | Leaf (%) | | Stem (%) | | Bark (%) | |
|---|---|---|---|---|---|---|---|
| | | CF | DF | CF | DF | CF | DF |
| 1 | *Cladosprium cladosporioides* | 8.3 | 9.3 | 10 | 12.5 | 8.3 | 12.5 |
| 2 | *Fusarium moniliforme* | 10 | 11.1 | 8.3 | 10.4 | 5 | 7.5 |
| 3 | *Fusarium oxysporum* | 5 | 5.6 | 5 | 6.3 | 5 | 7.5 |
| 4 | *Scytalidium acidophilum* | 15 | 16.7 | 8.3 | 10.4 | 10 | 15 |
| 5 | *Botryodiplodia theobromae* | 13.3 | 14.8 | 8.3 | 10.4 | 11.7 | 17.5 |
| 6 | *Colletotrichum dematium* | -- | -- | 3.3 | 4.2 | -- | -- |
| 7 | *Colletotrichum falcatum* | -- | -- | 10 | 12.5 | -- | -- |
| 8 | *Colletotrichum truncatum* | -- | -- | 5 | 6.3 | -- | -- |
| 9 | *Phyllosticta hymanaeae* | 11.7 | 13 | 5 | 6.3 | 8.3 | 12.5 |
| 10 | *Mycosphaerella* sp. | 15 | 16.7 | 8.3 | 10.4 | 8.3 | 12.5 |
| 11 | *Mucor racemosus* | 5 | 5.6 | -- | -- | 5 | 7.5 |
| 12 | Sterile form | 6.7 | 7.4 | 8.3 | 10.4 | 5 | 7.5 |
| Total | | 90 | | 79.8 | | 66.6 | |

Among the parts of plant, the stem possessed the maximum diversity of endophytic fungi (Shannon –Wiener Diversity (H): 2.35) followed by bark (H: 2.15) and leaf (H: 2.12). The Evenness index was highest and similar in stem and bark (0.95) and lowest in leaf (0.93). The result of Simpson Diversity Index (1 - D) revealed that the stem exhibited the maximum abundance of endophytic fungi (0.90) followed by bark (0.88) and leaf (0.87) of *C. guianensis* (Table 3).

**Table 3** – The Colonization Rate (CR %), Isolation Rate (IR) and Diversity Indices of Endophytic Mycoflora in *Couroupita guianensis*

| Source | No. of segments colonized by fungi | No. of Fungal Isolates | CR (%) | IR | Shannon - Wiener Diversity Index (H) | Evenness Index | Simpson Diversity Index (1-D) |
|---|---|---|---|---|---|---|---|
| Leaf | 48 | 54 | 80 | 0.9 | 2.12 | 0.93 | 0.87 |
| Stem | 41 | 48 | 68.3 | 0.8 | 2.35 | 0.95 | 0.90 |
| Bark | 33 | 40 | 55 | 0.7 | 2.15 | 0.95 | 0.88 |

## Discussion

Endophytes may increase host fitness in harsh environments (Redman et al. 2002). This is especially true of plants in arid environments (Faeth & Hammon 1997). The sacred trees are medicinally important and they are preserved and protected by the local people on religious basis. Medicinal plants are reported to harbour endophytes (Strobel 2002) and have a capacity to protect their host from infectious agents and also provide adaptability to survive in adverse conditions. Many studies have shown that some medicinal properties of plants may be due to endophytic fungi living inside the plants (Azevedo et al. 2002). Endophytic fungi from medicinal plants could be a rich source of functional metabolites (Huang et al. 2008). Endophytes are now considered as an outstanding source of bioactive natural products, because they occupy unique biological niches as they grow in so many unusual environments (Strobel & Daisy 2003).

In this study, a tropical forest sacred tree *Couroupita guianensis* from an unusual semi-arid region, yielded 142 endophytic fungal isolates from 180 samples of leaf, stem and bark tissue segments. Most of the fungal isolates belonged to hyphomycetes and coelomycetes in which hyphomycetes were predominant (41.5%) over other fungal classes. Such dominance of hyphomycetes as endophytes has also been reported from several plants such as *Azadirachta indica* and *Terminalia arjuna* (Mahesh et al. 2005) and also both in leaf and bark tissues of several plants species (Maheswari & Rajagopal 2013). The dominance nature of hyphomycetes may be attributed to their ability to colonize the host rapidly by producing abundant asexualspores and most fungi of this class occur as phylloplane flora but they are capable of penetrating the superficial layers of leaf and grow as endophyte, suggesting that phylloplane fungi might have adapted to endophytic mode of life to overcome adverse environmental conditions (Cabral et al. 1993).

The isolated two genera *Colletotrichum* and *Phyllosticta* are ubiquitous endophytes and have been reported from several plant hosts (Suryanarayanan et al. 2002) in which *Colletotrichum* sp. are the most frequently encountered endophytic fungi (Photita et al. 2005) that may be either host specific or generalist (Farr et al. 1989) and the remaining genera such as *Cladosporium* in *Azadirachta indica* (Tejesvi et al. 2006), *Coffea arabica* (Oliveira et al. 2014), *Fusarium* in *Murraya koenigii* (Suradkar et al. 2014), *Ocimum sanctum* (Banerjee et al. 2009), *Botryo diplodia* in *Terminali aarjuna* (Tejesvi et al. 2005), *Mucor* in *Vitex negundo* (Desale & Bodhankar 2013), *Mycosphaerella* in *Achyranthes bidentata* (Bing-Da Sun et al. 2013) and *Scytalidium* in the leaves of *Plumeria rubra* (Suryanarayanan & Thennarasan 2004) were also reported as cosmopolitan endophytes.

Endophytic fungi may inhabit all available tissues, but the leaves of tropical plants are densely colonized by endophytes (Suryanarayanan et al. 2002). All the isolated endophytic fungi showed their preference for specific tissue. Among 142 endophytic fungal isolates, 54 isolated from leaves, 48 isolated from stem and 40 isolated from bark tissue of *C. guianensis.* This results shown that more endophytic fungi could be isolated from leaves than stem and bark and also allied with Gangadevi & Muthumary (2007). The colonization rate (80%) and isolation rate (0.9) of endophytic fungi in leaf were higher in the present study this result was correlated with Kumar & Hyde (2004) in *Tripterygium wilfordii*. Maheswari & Rajagopal (2013) were of the opinion that high colonization of endophytes in leaf tissue may be due to their anatomical structure and supply of nutrient elements on which the endophyte depends.

Although, endophytic fungi have been recovered from almost all aerial tissues, some of them show organ and tissue specificity. In this study, more endophytic isolates of *Fusarium, Scytalidium, Botryodiplodia* and *Mycosphaerella* were obtained from leaves than in the stem and bark; *Cladosporium* was isolated more from stem than in the leaves and bark. Endophytic *Colletotrichum* exhibited organ and tissue specificity that was only isolated from the stem. The isolates of *Mucor* were reported in the tissues of leaf and bark and not in stem. Petrini et al. (1992) reported that organ specificity of endophytic fungi is due to adaptation to particularmicro-ecological and physiological conditions present in a particular organ.

The colonization of the endophytic fungi is ubiquitous yet selective in nature. Okane et al. (1998) reported that the composition and frequency of colonization related with the place and the

host condition and also differs within the tissue or organs of a host plant (Kumar & Hyde 2004). However, usually one or a few fungal species dominate in a host plant (Rajagopal et al. 2010). In this study, the overall colonization frequency of endophytic fungal isolates in leaf was found to be 90% as highest when compared to stem and bark tissue. The fungal isolates *Scytalidium acidophilum* and *Mycosphaerella* sp. showed the highest colonization frequency of 15% and also they found to be the dominant endophytic fungi (16.7%) in leaf. The endophytic fungi *Cladosporium cladosporioides* and *Colletotrichum falcatum* were exposed the highest colonization frequency of 10% and also observed as the dominant isolates (12.5%) in the tissues of stem. In bark tissues of *C. guianensis*, *Botryodiplodia theobromae* was identified to be the dominant species (17.5%) and the colonization frequency was 11.7% as reported as highest.

The Shannon-Weiner diversity index was employed to evaluate and compare the diversity of fungi community between different parts in which the stem possessed the maximum diversity of endophytic fungi (Shannon – Wiener Diversity (H): 2.35) followed by bark (H: 2.15) and leaf (H: 2.12). The Evenness index was similar (0.95) in stem and bark and that was closely related with leaf (0.93). The results of Simpson Diversity Index (1-D) revealed that the differences of the abundances of endophytes in leaf, stem and bark tissues were not significant (stem (0.90)>bark (0.88)>leaf (0.87).

A rich diversity of endophytic fungal genera in *C. guianensis* was observed in the present study. The endophytic mycodiversity in the study area was high and they were distinctly associated with host plant parts (leaf > stem > bark). The hyphomycetes were predominant over other fungal classes. In Coelomycetes, *Colletotrichum* exhibited organ specificity. This study demonstrates that the sacred tree *C. guianensis* is one of the rich ecological niches for endophytic fungi in leaf, stem and bark tissues Eventhough the sacred tree grew in a semi-arid region of tropics.

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ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 10, Issue 3, 2017

Online - 2455-3891
Print - 0974-2441

Research Article

# LARVICIDAL AND ENZYME INHIBITORY EFFECTS OF *ACALYPHA FRUTICOSA* (F.) AND *CATHARANTHUS ROSEUS* L (G) DON. LEAF EXTRACTS AGAINST *CULEX QUINQUEFASCIATUS* (SAY.) (DIPTERA: CULICIDAE)

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*Received: 04 November 2016, Revised and Accepted: 30 November 2016*

**ABSTRACT**

**Objective:** The prime objective of this study is to evaluate the larvicidal and enzyme inhibitory effects of *Acalypha fruticosa* and *Catharanthus roseus* leaf extracts against *Culex quinquefasciatus*.

**Methods:** Insecticidal susceptibility tests were carried out using the World Health Organization standard method, and the mortality was observed after 24 hrs exposure.

**Results:** The tested extracts showed a significant larvicidal activity depending on the dose of the plant extracts. However, maximum larval mortality was detected in ethyl acetate extract of *A. fruticosa* with a lethal concentration ($LC_{50}$) value of 253.08 ppm and ($LC_{90}$) value of 455.40 ppm followed by hexane extract of *C. roseus* with $LC_{50}$ value of 645.33 ppm and $LC_{90}$ value of 1452.88 ppm against the larvae of *C. quinquefasciatus.* In control, there were five different fractions of α-carboxylesterases resolved in the gel with the mobilities ranging between 0.14 and 0.66. β-carboxylesterase isozyme profile of larvae treated with hexane extract of *A. fruticosa* showed higher enzyme activities by way of intense staining of fractions both at 1/4 and 1/10 $LC_{50}$ value of 48 hrs treatments. *A. fruticosa* ethyl acetate extract, and *C. roseus* hexane extract exposed larvae indicated increased quantities of β-Est4/5 isozymes in the concentration of 1/4 and 1/10 of $LC_{50}$ value of 24 hrs treatment when compared to control. The protein quantity in the majority of treatments decreased compared to control.

**Conclusion:** The results revealed that the organic leaf extract of *A. fruticosa* and *C. roseus* had significant larvicidal and enzyme inhibitory effects against *C. quinquefasciatus*.

**Keywords:** *Acalypha fruticosa*, *Catharanthus roseus* leaves extract, β-carboxylesterase, *Culex quinquefasciatus,* Lethal concentration$_{50}$.

 DOI: http://dx.doi.org/10.22159/ajpcr.2017.v10i3.16029

## INTRODUCTION

Mosquitoes are medically most important and responsible for transmitting the vector-borne diseases, parasites, and pathogens which continue to have a devastating impact on human beings, public hygiene and ecological perspectives [1]. Mosquitoes under the genus *Culex* are the vectors of encephalitis and filariasis [2]. Frequent outbreaks of these diseases result in restricting the socioeconomic status in developing countries in the tropical and subtropical belts. Considerable economic, ecological, and public health impacts of vector-borne diseases are expected to continue, given limited domestic and international capabilities for detecting, identifying, and addressing likely epidemics. It is estimated that every year at least 500 million people in the world suffer from one or the other tropical diseases that include malaria, lymphatic filariasis, schistosomiasis, dengue, trypanosomiasis, and leishmaniasis. 1-2 million deaths are reported annually due to malaria worldwide. Lymphatic filariasis affects at least 120 million people in 73 countries in Africa, India, Southeast Asia, and Pacific Islands. Synthetic insecticides are effective in controlling mosquitoes, but their environmental consequences are unpredictable. In the context of ever-increasing trend to use more powerful synthetic insecticides to achieve immediate results in the control of mosquitoes, an alarming increase of physiological resistance in the vectors, its increased toxicity to non-target organism and high costs are noteworthy [3]. Most of the synthetic chemicals are expensive and destructive to the environment and also toxic to humans, animals, and other beneficial organisms. Besides, they are non-selective and harmful to other beneficial organisms. Therefore, effective and low-cost alternate vector control strategies, especially are extremely imperative [4,5]. Use of different parts of locally available plants and their various products in the control of mosquitoes have been accepted globally by numerous researchers.

Phytochemicals derived from plant sources can act as larvicides, insect growth regulators, repellents, and ovipositional attractants and also have deterrent actions as observed by many researchers [6-8]. The larvicidal properties of indigenous plants have also been documented in many parts of India along with the repellent and anti-juvenile hormones activities [9]. Traditionally, plants and their derivatives were used to kill mosquitoes and other household and agricultural pests. In all probability, these plants used to control insects contained insecticidal phytochemicals that were predominantly secondary compounds produced by plants to protect themselves against herbivorous insects [10,11].

Esterase is an enzyme that played a significant role both physiologically and biochemically during any stress on insects. They are excellent key enzymes involved in the metabolism of hormones [12,13], digestion [14], and resistance to insecticides. Esterases are considered an important tool for analysis of genetic differentiation and evolutionary relationship of insects [12]. These enzymes are tissue specific in insects [15] and are closely linked with morphological, physiological, or biochemical ontogenetic alterations [16]. Ffrench-Constant *et al.* [17] described an electrophoretic technique, based on patterns of esterase bands on polyacrylamide gel (PAGE) for

distinguishing large samples of *Myzus antirrhinic* from susceptible or resistant populations of the closely related species of *Myzus persicae.* In addition to the insecticidal activity, most of toxicological approaches on botanical insecticides have been focused on inhibition or induction of detoxifying enzymes such as carboxylesterase, glutathione-S-transferase and cytochrome P450. Rachokarn *et al*. [18] reported similar results on different enzymes, that is, carboxylesterase activity had a tendency to be induced after treated with *Amaranthus viridis* extracts. These inductances of detoxifying enzyme suggest a possibility on the development of resistance against the extracts as shown in many reports on increased induction in detoxifying enzyme activity and discussed on development of resistance of many insect pests [19-22]. In the present work, both quantitative and qualitative analyses of esterases were undertaken to assess the efficacy of hexane, ethyl acetate, and methanol extracts of *Acalypha fruticosa* and *Catharanthus roseus* leaves against esterase induction/activity in the fourth-instar larvae of *Culex quinquefasciatus*. With this background, the present investigation was undertaken to assess the efficacy of various solvent extracts of the leaves of very well-known Indian medicinal plants *A. fruticosa* and *C. roseus* against the fourth-instar larvae of *C. quinquefasciatus*.

## MATERIALS AND METHODS

### Materials

#### *Plant collection and preparation of leaf extracts*

The healthy leaves of the selected plant species of *A. fruticosa* (Euphorbiaceae) and *C. roseus* (Apocynaceae) were collected during 2012 from in and around Chennai, Tamil Nadu, India. Plant specimens were identified by plant taxonomists and standard flora. The leaves were shade dried at room temperature and coarsely powdered in a powdering machine. A total of 200 g powder of the plant was extracted sequentially with increasing polarity of hexane, ethyl acetate, and methanol at room temperature for 1 week with occasional shaking in an aspirator bottle. The extract was filtered through Watmann No.1 filter paper and evaporated in vacuum evaporator.

### Rearing of mosquitoes

The test was carried out against laboratory reared *C. quinquefasciatus* mosquitoes free of exposure to insecticides and pathogens. Cyclic generations of *C. quinquefasciatus* were maintained at 25-29°C and 80-90% R.H in the insectarium. Larvae were fed on larval food (powdered dog biscuits and yeast in the ratio of 3:1) and adult mosquitoes on ten percent glucose solution. Adult female mosquitoes were periodically blood-fed on restrained albino mice for egg production.

### Larval toxicity test

A laboratory reared colony of *C. quinquefasciatus* larvae was used for the larvicidal activity. Twenty individuals of fourth-instar larvae were kept in a 500 ml glass beaker containing 249 ml of decholorinated water and desired concentration of *C. roseus* and *A. fruticosa* leaf extracts in 1 ml of acetone with Tween-20 (1%). Larval food was given for the test larvae. At each tested concentration, two to five trials were made, and each trial includes five replicates. The negative control was setup by mixing 1 ml of acetone with 249 ml of dechlorinated water. The larvae exposed to dechlorinated water without acetone served as positive control. The control mortalities were corrected by using Abbott's formula [23].

$$\text{Percentage mortality} = \frac{\text{Number of dead larvae}}{\text{Number of larvae introduced}} \times 100$$

### Statistical analysis

All data were subjected to analysis of variance. The means were separated using Duncan's multiple range tests by Alder and Rossler [24]. The average larval mortality data were subjected to probit analysis for calculating lethal concentration ($LC_{50}$) and $LC_{90.}$ Values were calculated using the Finney [25] method. Statistical software package 9.0 version was used. Results with $p<0.05$ were considered statistically significant.

### Preparation of whole body homogenates of larvae and pupae for esterase analyses

The level of esterase enzymes (against both alpha and beta naphthyl acetates as substrates) were estimated in the fourth-instar larvae of *C. quinquefasciatus* subjected to hexane, ethyl acetate, and methanol extracts of *A. fruticosa* and *C. roseus* leaves. A suitable control was also maintained. After treatment, the larvae were removed from the treatment tray, washed with double distilled water, and the adhering water was completely removed from the body by blotting with tissue paper. The larvae (25 individuals) from each of the treatments were transferred separately to eppendorf tubes and homogenized using a teflon hand homogenizer in 150 µl of ice-cold phosphate buffer (20 mM, pH 7.0) for extraction of esterases. The whole body homogenates were centrifuged at 10000 rpm at 4°C for 20 minutes, and the clear supernatants were collected for further esterase assays.

### Quantitative analyses of biochemical constituents

The impact of exposure of plant extracts on fourth-instar larvae of *C. quinquefasciatus* was quantitatively analyzed in response to the level of protein and expressed as unit enzyme activity per mg protein.

### Determination of protein concentration

Protein concentrations in the larval homogenates were estimated following the method of Bradford [26]. To 5 µl of protein or blank solution, 195 µl of Bradford reagent was added and mixed well. The optical density was read between 5 and 20 minutes after addition of protein reagent at 595 nm. Protein concentration per ml of the test sample was calculated as follows.

$$\text{Protein concentration in the sample}\left(\frac{\text{mg}}{\text{ml}}\right) = \frac{\text{OD of sample}}{\text{OD of standard}} \times \text{Concentration of standard}\,(40\ \mu\text{g})$$

### Estimation of carboxylesterase activity

The carboxylesterase activity in the whole body homogenates was measured by the modified method of Van Asperen [27] as described by Argentine and James [28].

### Estimation of α-carboxylesterase activity

A volume of 10 µl of whole body homogenate of larvae was incubated with 198 µl of 20 mM sodium phosphate buffer (pH 7.0) containing 250 µM of α-naphthyl acetate for 10 minutes at RT. After incubation, 50 µl of freshly prepared 0.3% fast blue B salt in 3.3% SDS were added to stop the enzymatic reaction and allowed to develop color for 15 minutes at RT. The optical density of samples was read at 430 nm against the blank consisting of same reagents and buffer substituted for the homogenate. The level of α-carboxylesterase activity was calculated and expressed as µM/minute/mg protein.

### Estimation of β-carboxylesterase activity

The whole body homogenate of larvae was first diluted 3 times with sodium phosphate buffer (20 mM, pH 7.0), and 100 µl of diluted homogenate was incubated with 1 ml of sodium phosphate buffer (20 mM, pH 7.0) containing 250 µM of β-naphthyl acetate for 10 minutes at RT. After incubation, 400 µl of freshly prepared 0.3% fast blue B salt in 3.3% SDS were added to stop the enzymatic reaction and allowed to develop color for 15 minutes at RT. Blank consisted of same reagents and buffer instead of homogenate. The optical density of samples was read at 580 nm against the blank. The level of β-carboxylesterase activity was calculated and expressed as µM/minute/mg protein.

### Qualitative analyses of esterase enzymes using native PAGE

The effect of exposure of fourth-instar larvae to various solvent extracts of plants was qualitatively analyzed by native-PAGE with esterase enzymes.

### Native-PAGE

The profiles of esterase enzymes in the whole body homogenates of fourth-instar larvae were analyzed in discontinuous PAGE under non-

denaturing conditions following Maurer [29]. This was performed using 3% stacking gel (pH 6.7) and 7% separating gel (pH 8.9) in Tris-glycine buffer (pH 8.3). Samples of the whole body homogenates (each 80 µl) of control and experimental larvae were electrophoresed at a constant current of 3 mA per sample at 10°C on a slab gel (170 × 150 × 1.5 mm). After electrophoresis, the gels were suitably stained for detection of esterase activity.

**Detection of carboxylesterase activity**

The electrophoretically separated bands with esterase activity were detected in the gel following the method of Argentine and James [28]. Accordingly, the gel was first incubated with phosphate buffer (20 mM, pH 7.0) for 15 minutes at RT. After decanting the buffer, the gel was then re-incubated for 30 minutes at RT with freshly prepared α-naphthyl acetate + fast blue B solution for detection of α-carboxylesterase or β-naphthyl acetate + fast blue B solution for detection of β-carboxylesterase. The gels were washed with distilled water and stored in 7% acetic acid. After the electrophoresis, the destained gel was measured for relative mobility of protein bands. Relative mobility is the distance migrated by a band divided by the distance migrated by the dye (i.e., dye front).

**Densitometry analysis of esterase isozyme**

All the native PAGE gels performed for α-carboxylesterase or β-carboxylesterase banding pattern were subjected to densitometry analysis using Image J 1.46r software developed by National Institutes of Health (NIH), U.S. Department of Health and Human Service.

## RESULTS

The results of larvicidal activity of hexane, ethyl acetate and methanol extracts of *A. fruticosa* and *C. roseus* are summarized in Table 1. The effect on larval mortality was concentration dependent. It has observed that the ethyl acetate extracts of *A. fruticosa* possess maximum larvicidal activity against 4$^{th}$ instar larvae of *C. quinquefasciatus*. The percentage mortality of larvae treated with *A. fruticosa* recorded with 87.25% at 5% concentration followed by hexane (65.0%) and methanol (57.50) extracts compared with untreated control. The results of the larvicidal activities of different crude extracts of *C. roseus* in hexane against *C. quinquefasciatus* was 62.50% at 5% concentration followed by extracts of methanol (58.75%) and ethyl acetate (43.75%) when compared with untreated control. No mortality was recorded in various replicates of control.

The results of the $LC_{50}$ and $LC_{90}$ values of the plant extracts after 24 hrs against the larvae of *C. quinquefasciatus* are shown in Table 2.

The ethyl acetate leaf extract of *A. fruticosa* was found to be effective with a $LC_{50}$ value of 253.08 ppm and $LC_{90}$ value of 455.40 ppm followed by extracts of hexane with $LC_{50}$ value of 429.99 ppm and $LC_{90}$ value of 774.45 ppm and methanol with $LC_{50}$ value of 574.82 ppm and $LC_{90}$ value of 1035.00 ppm against 4$^{th}$ instar larvae of *C. quinquefasciatus.* The efficacy of hexane extract of *C. roseus* was most active with $LC_{50}$ value of 645.33 ppm and $LC_{90}$ value of 1452.88 ppm followed by extracts of methanol with $LC_{50}$ value of 715.39 ppm and $LC_{90}$ value of 1287.54 ppm and ethyl acetate with $LC_{50}$ value of 1370.06 ppm and $LC_{90}$ value of 2089.43 ppm against the larvae of *C. quinquefasciatus.* This study showed the larvicidal potency of various solvent extracts of leaves of these two plants against *C. quinquefasciatus*. Thus *A. fruticosa* and *C. roseus* could be exploited further for identification of specific larvicidal agents.

Esterase enzyme gel electrophoresis was carried out using 7% PAGE (Native-PAGE) with α- and β-naphthyl acetates as staining substrates for all the plant extracts treated mosquito larvae of *C. quinquefasciatus.* In control, there were five different fractions of α-carboxylesterases resolved in the gel with the mobilities ranging between 0.14 and 0.66. On exposure of the larvae to different plant extracts, intensity of staining of various enzyme fractions was decreased with disappearance of at least two or three α-carboxylesterase enzyme fractions (Fig. 1). On the contrary, the staining intensity of one slow moving fraction α-Est1 with a mobility of 0.49 was increased significantly or maintains the same level in all experimental groups when compared to control. A similar trend was observed in the larvae of *C. quinquefasciatus* treated against ethyl acetate and hexane extracts of *A. fruticosa* and hexane and methanol extracts of C. *roseus* (Fig. 1). The relative mobility of β-carboxylesterase isozyme profile of larvae of *C. quinquefasciatus* treated against ethyl acetate extract of *A. fruticosa* revealed four different enzyme fractions in control and three fractions in 24 hrs treatment and four fractions in 48 hrs treatment (Fig. 2).

Among various treatments, the staining intensities of various enzyme fractions were significantly higher in 24 hrs and 48 hrs treatments of ethyl acetate at 1/4 of $LC_{50}$ value at 24 hrs treatment of 1/10 of $LC_{50}$ value at 24 hrs treatment. Likewise, β-carboxylesterase isozyme

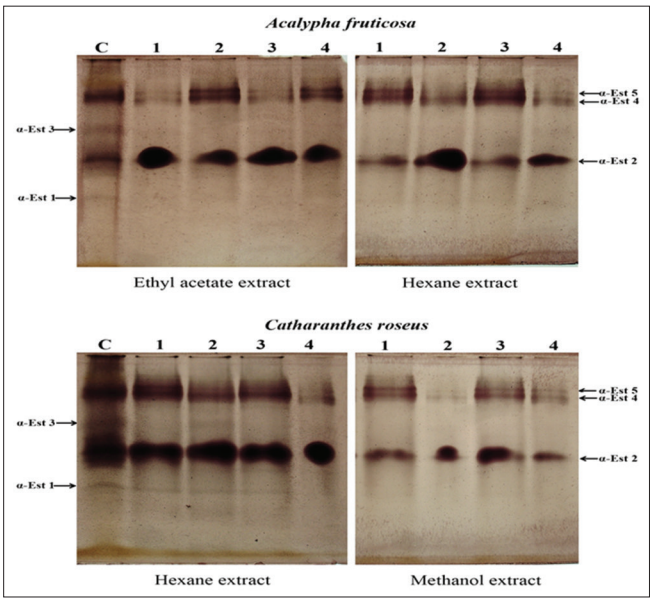


**Fig. 1: α-Carboxylesterase isozyme pattern of *Culex quinquefasciatus* upon exposure to different medicinal plant extracts. C: Control, 1: 1/4 dilution 24 hrs, 2: 1/4 dilution 48 hrs, 3: 1/10 dilution 24 hrs, 4: 1/10 dilution 48 hrs**

**Table 1: Percent larvicidal activity of leaf extracts of *Acalypha fruticosa* and *Catharanthus roseus* against fourth instar larvae of *Culex quinquefasciatus***

| Name of the plants | Solvent | Control | Concentration in ppm | | | | |
|---|---|---|---|---|---|---|---|
| | | | 100 | 125 | 250 | 500 | 1000 |
| *A. fruticosa* | Hexane | 0±0$^{a}$ | 13.75±0.95$^{bc}$ | 21.25±1.01$^{bc}$ | 35.00±1.91$^{c}$ | 48.11±1.15$^{d}$ | 65.00±0.22$^{b}$ |
| | Ethyl acetate | 0±0$^{a}$ | 25.00±0.12$^{c}$ | 33.75±1.36$^{d}$ | 53.75±1.20$^{c}$ | 65.75±1.33$^{c}$ | 87.25±1.52$^{c}$ |
| | Methanol | 0±0$^{a}$ | 10.00±1.41$^{b}$ | 20.00±1.41$^{b}$ | 16.25±0.83$^{a}$ | 46.00±0.56$^{b}$ | 57.50±0.51$^{ab}$ |
| *C. roseus* | Hexane | 0±0$^{a}$ | 12.25±0.77$^{b}$ | 18.50±1.77$^{a}$ | 24.75±1.65$^{b}$ | 44.50±1.02$^{b}$ | 62.50±0.44$^{a}$ |
| | Ethyl acetate | 0±0$^{a}$ | 11.25±1.59$^{b}$ | 16.25±1.30$^{a}$ | 23.75±0.36$^{b}$ | 28.75±1.07$^{a}$ | 43.75±1.20$^{a}$ |
| | Methanol | 0±0$^{a}$ | 10.75±1.41$^{b}$ | 27.5±1.54$^{c}$ | 42.50±0.96$^{b}$ | 52.50±0.63$^{d}$ | 58.75±1.38$^{a}$ |

Values carrying same alphabets in a column are not statistically significant by Turkey's Test at p= 0.05% level

**Table 2: Probit analysis on larvicidal efficacy of *Acalypha fruticosa* and *Atharanthus roseus* against fourth instar larvae of *Culex quinquefasciatus***

| Name of the plants | Solvent extract | $LC_{50}$ | $LC_{90}$ | Chi-square Value | Regression value |
|---|---|---|---|---|---|
| *A. fruticosa* | Hexane | 429.99 | 774.45 | 0.82* | 2.72 |
| | Ethyl acetate | 253.08 | 455.40 | 1.52* | 2.54 |
| | Methanol | 574.82 | 1035.00 | 8.19 | 2.88 |
| *C. roseus* | Hexane | 645.33 | 1452.88 | 2.40* | 2.92 |
| | Ethyl acetate | 1370.06 | 2089.43 | 0.96* | 3.22 |
| | Methanol | 715.39 | 1287.54 | 5.82* | 2.96 |

*Significant at P<0.05 level, C. *quinquefasciatus: Culex quinquefasciatus, A. fruticosa: Acalypha fruticosa*

profile of larvae treated with hexane extract of *A. fruticosa* showed higher enzyme activities by way of intense staining of fractions both at 1/4 and 1/10 $LC_{50}$ value of 48 hrs treatments (Fig. 2).

In the case of relative mobilities of β-carboxylesterase enzyme profile of larvae of *C. quinquefasciatus* treated against hexane and methanol extracts of *C. roseus* indicated higher enzyme activities in the gel, especially 24 hrs treatment in both the concentrations of 1/4 and 1/10 of $LC_{50}$ value (Fig. 2).

Individual band intensities of α- or β-carboxylesterase isozyme from the profile obtained from native PAGE gel were quantified using ImageJ (NIH). Values in the linear range of enzyme fraction/band intensities were selected for quantifications. Samples of control and treatments (control and plant extract exposed larval samples) were run side by side on the same gel. The densitometric analysis revealed that the α- Est2 isozyme expression level was increased in *A. fruticosa* ethyl acetate extract, *C. roseus* hexane and methanol extract exposed larval samples, especially in the concentrations of 1/4 and 1/10 of $LC_{50}$ value of 24 hrs treatment except hexane extract of *A. fruticosa*. Likewise, α- Est4/5 isozymes were found to be intensely stained in *A. fruticosa* ethyl acetate extract *C. roseus* hexane and methanol extract exposed larval samples (especially 24 hrs treatment in the concentrations of 1/4 and 1/10 of $LC_{50}$ value) except *A. fruticosa* ethyl acetate extract exposed larvae (Fig. 3).

*A. fruticosa* ethyl acetate extract, and *C. roseus* hexane and methonal extract exposed larval samples, especially in the concentrations of 1/4 and 1/10 of $LC_{50}$ value of 48 hrs treatment were intensely stained in β-Est2 isozyme when compared to control (Fig. 4). Furthermore, *A. fruticosa* ethyl acetate extract and *C. roseus* hexane extract exposed larvae indicated increased quantities of β-Est4/5 isozymes in the concentration of 1/4 and 1/10 of $LC_{50}$ value of 24 hrs treatment when compared to control.

Total protein concentrations of larvae exposed to various plant extract treatments revealed that there were decreased levels of protein in the majority of treatments when compared to control. These values of protein were also used to express the quantity of enzymes (Fig. 5).

The α-carboxylesterase and β-carboxylesterase in the fourth-instar larvae treated with various solvent extracts of *A. fruticosa* and *C. roseus* exhibited significantly high enzyme quantities in all the treatments when compared to control. In control, the quantity of α-carboxylesterase was 0.89 μM.minute$^{-1}$.mg protein$^{-1}$. It was observed to be much lower against all the treatments except 1/4 of $LC_{50}$ value of hexane extracts of *C. roseus* and in which it was recorded as 0.92 μM.minute$^{-1}$.mg protein$^{-1}$ (Table 3).

The quantity of β-carboxylesterases in control was observed to be 1.87 μM.minute$^{-1}$.mg protein$^{-1}$. Among various treatments, the results revealed that except a few, all the other solvent extracts of plants yielded higher quantities of β-carboxylesterases (Table 4).

**Table 3: Quantity of α-carboxylesterase in the fourth instar larvae treated with various solvent extracts of plants**

| Name of the plants | Solvent | Esterase activity (μM.minute$^{-1}$.mg protein$^{-1}$) | | | |
|---|---|---|---|---|---|
| | | 1/4th dilution | | 1/10th dilution | |
| | | 24 hrs | 48 hrs | 24 hrs | 48 hrs |
| *A. fruticosa* | Ethyl acetate | 3.92 | 3.68 | 4.42 | 3.64 |
| | Hexane | 5.70 | 4.62 | 5.08 | 3.69 |
| *C. roseus* | Hexane | 1.17 | 0.92 | 1.21 | 1.27 |
| | Methanol | 2.96 | 1.48 | 2.24 | 2.11 |

Control - 0.89 μM.minute$^{-1}$.mg protein$^{-1}$, *C. roseus: Catharanthus roseus, A. fruticosa: Acalypha fruticosa*

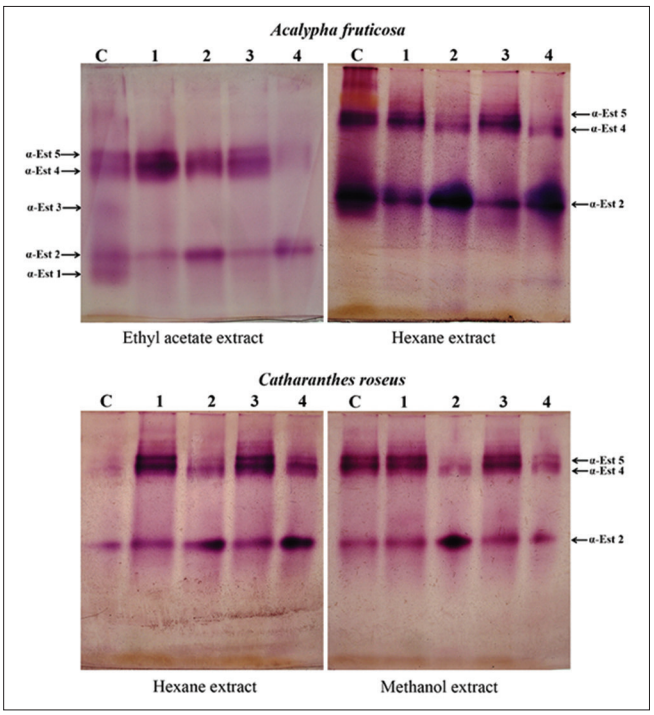


**Fig. 2: β-Carboxylesterase isozyme pattern of *Culex quinquefaeciatus* upon exposure to different medicinal plant extracts C: Control, 1: 1/4 dilution 24 hrs, 2: 1/4 dilution 48 hrs, 3: 1/10 dilution 24 hrs, 4: 1/4 dilution 48 hrs**

## DISCUSSION

Plant-based medicine, which uses medicinal plants as the first medicine is a general phenomenon. Every civilization on earth, through written or oral tradition, has relied on the vast variety of healing plants for their healing attributes. The majority of medicinal plant products available today, originated from the same traditional [30] traditional medicine has spread throughout the world and has gained popularity not only in the developing countries but also to industrialized countries which have access to alternative medicine [31]. The control of mosquitoes at larval stage is focused with plant extracts. The advantage of targeting mosquito at the larval stage is that they cannot escape from their breeding habitat

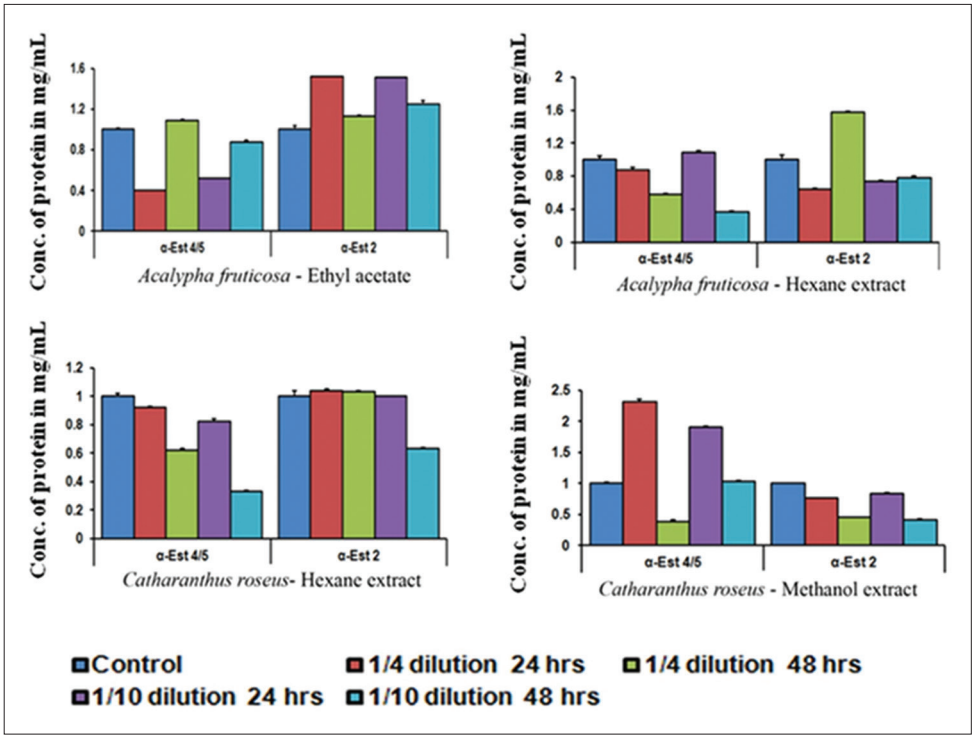


**Fig. 3: Densitometry analysis of α-carboxylesterase isozyme banding pattern by using image 1.46r software**

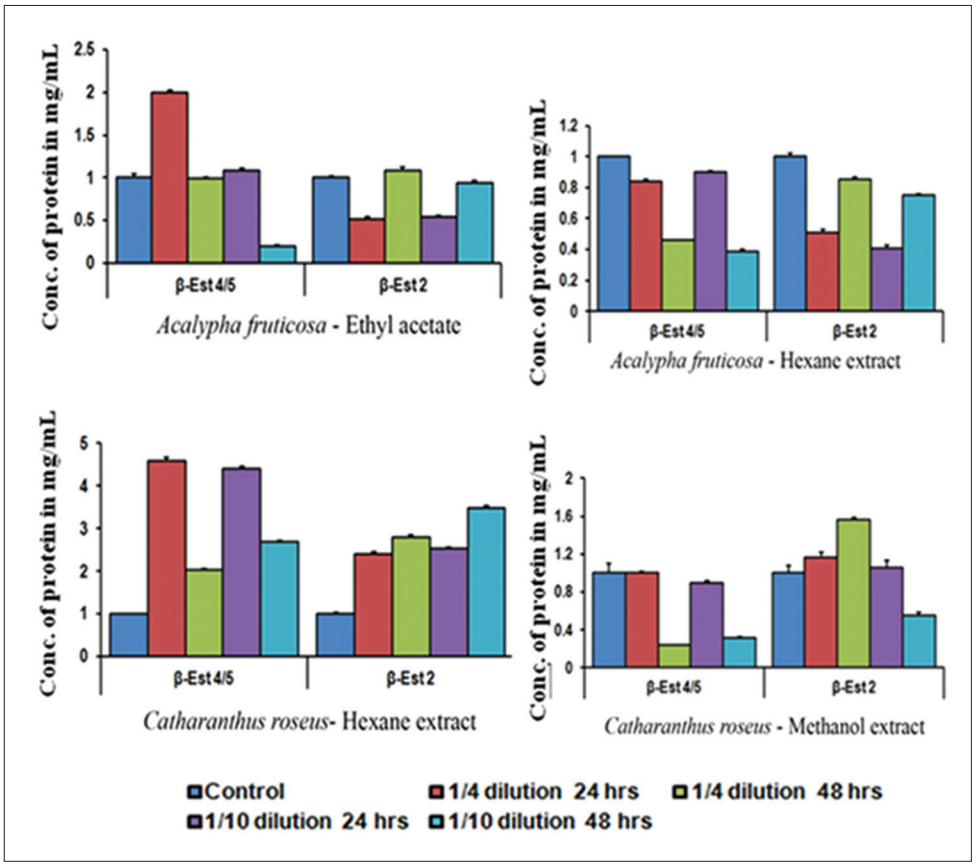


**Fig. 4: Densitometry analysis of β-carboxylesterase isozyme banding pattern by using image 1.46r software**

until the adult emergencies and also to reduce the overall pesticide use to control of adults by aerial application of adulticidal chemicals. The application of synthetic insecticides could be thus reduced by botanical pesticides through natural control mechanism. These botanicals are often active against a number of insect pest species, less expensive, easily biodegradable to non-toxic products and potentially suitable for use in mosquito control program [32]. Plant allelochemicals may be quite useful in increasing the efficacy of biological control agents because plants produce a large variety of compounds that increase plant resistance to insect attack [33]. Recently, bio-pesticides with plant

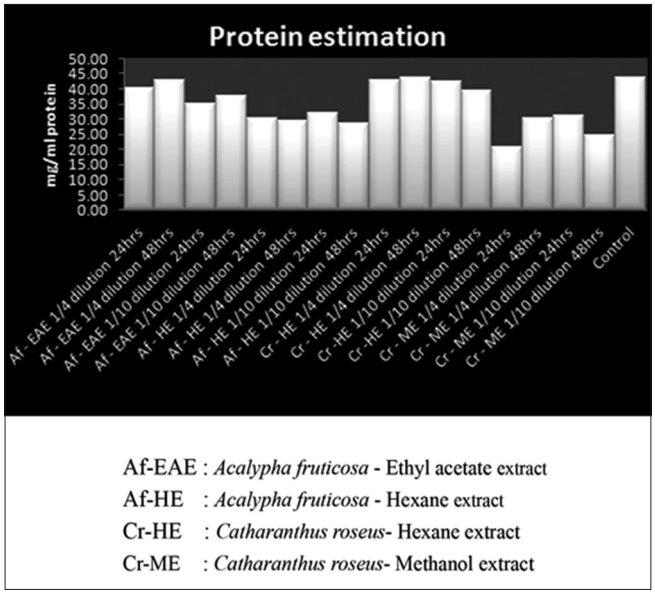


**Fig. 5: Determination of protein concentration from whole larval homogenate**

**Table 4: Quantity of β- carboxylesterase in the fourth instar larvae treated with various solvent extracts of plants**

| Name of the plants | Solvent | Esterase activity ($\mu M.minute^{-1}.mg\ protein^{-1}$) | | | |
|---|---|---|---|---|---|
| | | 1/4th dilution | | 1/10th dilution | |
| | | 24 hrs | 48 hrs | 24 hrs | 48 hrs |
| *A. fruticosa* | Ethyl acetate | 1.37 | 1.90 | 3.02 | 3.13 |
| | Hexane | 3.50 | 3.35 | 4.14 | 1.55 |
| *C. roseus* | Hexane | 1.40 | 1.97 | 2.64 | 3.11 |
| | Methanol | 5.53 | 3.35 | 4.70 | 2.00 |

Control: 1.87 $\mu M.minute^{-1}.mg\ protein^{-1}$, *C. roseus: Catharanthus roseus, A. fruticosa: Acalypha fruticosa*

origins are used against several insect species especially for the control of insect vectors [34,35]. Natural pesticides, derived from plants are more promising in this aspect. It is evident from our results that these two plants would be useful for further exploration of active compounds for the control of *C. quinquefasciatus* larvae.

The present study showed clearly that the ethyl acetate and hexane extracts of *C. roseus* and *A. fruticosa* had a prominent larvicidal effect on *C. quinquefasciatus*. As reported earlier in similar such studies it may be due to the presence of the phytochemicals such as alkaloids, carboxylic acids, flavanoids, phenols, proteins, quinones, resins, steroids, and saponins in the extracts of the plants [36]. In a related study conducted by Maheswaran *et al*. [37] on hexane extracts of leaves of *Leucas aspera* against the fourth-instar larvae of *C. quinquefasciatus* with $LC_{50}$ values of 122.50 ppm was reported.

The findings of the present study are quite comparable with previous reports of Maheswaran *et al*. [1] who have reported that the maximum larvicidal activities of different solvent leaf extracts of *L. aspera* in which hexane, chloroform and ethanol extracts showed 230.71, 518.88, and 1059.13 ppm against *Aedes aegypti.* Likewise, there were reports of hexane, ethyl acetate, and methanol extracts of *Momordica charantia, Moringa oleifera, Ocimum gratissimum, Ocimum tenuiflorum, Punica granatum* and *Tribulus terrestris* with promising larvicidal activity against *C. gelidus* and *C. quinquefasciatus* [38]. Zahir *et al*. [39] studied the inhibition of adult emergence and adulticidal activities of hexane, chloroform, ethyl acetate, and acetone leaves extracts of *Anisomeles malabarica*, *Euphorbia hirta*, *Ocimum basilicum*, *Ricinus communis*, *Solanum trilobatum*, *Tridax procumbens* and seeds of *Gloriosa superba* against *Anopheles stephensi*. These studies provided a clue that there are probabilities for the presence of active principles in these plants with several solvent extracts. The larvicidal and adult emergence inhibition activities of castor (*R. communis*) seed extract against three potential mosquito vectors *A. stephensi*, *C. uinquefasciatus, A. albopictus* in India studied by Mandal [8] (2010) suggest that the seed extract provided an excellent potential property for the control of mosquito vectors.

Many similar experiments on the efficacy of plant extracts on the larvicidal action on several species mosquitoes were conducted previously. The findings of the presence results, report of 50% larval mortality with some of the solvent extracts of these two plants also corroborate with earlier findings of Macedo *et al*. [40] who reported that the ethanol extract of *Tagetes patula* was able to cause only 50% larval mortality at 100 ppm concentration.

Similarly, methanolic leaf extract of *Cassia fistula* was tested for larvicidal activity against *C. quinquefasciatus* and *A. stephensi* with $LC_{50}$ values of 17.97 and 20.57 mg/l, respectively [41]. The petroleum ether fraction of *Acacia nolotica* and *Citrullus colocynthis* showed 100% mortality at 100, 250, and 500 ppm and 60% and 50% mortality at 125 and 62.5 ppm, respectively, against *C. quinquefasciatus* [42]. The leaf extract of *Acalypha indica* with different solvents *viz*, benzene, chloroform, ethyl acetate and methanol were tested for larvicidal and ovicidal activity against *A. stephensi.* The larval mortality was observed after 24 hrs exposure. The $LC_{50}$ values are 19.25, 27.76, 23.26, and 15.03 ppm, respectively [41]. The leaf extract of *C. fistula* with different solvents such as methanol, benzene, and acetone were studied for the larvicidal, ovicidal and repellent activity against *A. aegypti*. The 24 hrs $LC_{50}$ concentration of the extract against *A. aegypti* were observed at 10.69, 18.27, and 23.95 mg/l, respectively [43]. Cheng *et al.* [44] reported that the leaf and bark essential oil of *Cryptomeria japonica* showed larvicidal activity against *A. aegypti*. Singh *et al.* [45] reported the mosquito larvicidal properties of the leaf extract of *Ocinum canum* against *A. aegypti*. The $LC_{50}$ values for 2nd, 3rd and 4th instar larvae were 177.82, 229.08, and 331.13 ppm, respectively. Gusmao *et al*. [46] reported that the extract of *Derris urucu* (Lonchocarpus) showed larvicidal activity against *A. aegypti* with $LC_{50}$ value of 17.6 µg/ml which supports the present results were comparably good. Singh *et al*. [45] demonstrated that the larvicidal activity of *O. canum* oil tested against *A. aegypti* and *C. quinquefasciatus* (LC50 301 ppm) and *A. stephensi* (234 ppm).

The increased detoxification is a common mechanism of resistance to pesticides (Openoorth, 1985) [47]. In *Culex pipiens*, such a mechanism is often involved in resistance to organophosphates. However, the low levels of organophosphate and pyrethroid resistance could be conferred by either the elevated esterase enzymes [48]. Elevation in the α-carboxylesterases and β-carboxylesterases are commonly found in the organophosphate resistant strains [49]. In some cases, overproduction is seen only in one type of esterase, that is, α - esterase or β-esterase, in others both the types are overproduced simultaneously [50]. In the present study, we have also observed an increased α - and β-carboxylesterase activities in *C. quinquefasciatus* to various solvent extracts of *A. fruticosa* and *C. roseus*. *C. quinquefasciatus* being an urban vector that breeds in the water stagnations might not have been exposed to various agricultural pesticides, before the introduction of plant products. This might have triggered the overproduction of both esterases through genetic selection/gene amplification. Therefore, increased tolerance to the extracts of these plants in *C. quinquefasciatus* might be a result of gene amplification of esterases through genetic selection, resulting in elevated levels of esterase activities.

## CONCLUSION

The present findings on various solvent extracts of leaves of *A. fruticosa* leaves are promising sources for the control of *C. quinquefasciatus* larvae. It is an indigenous medicinal plant of India and is easily available to local people. It may be a safe alternative to synthetic chemicals.

Further studies on isolation of bioactive fraction/constituent as well as application on larger scales in the field may provide promising lead products for effective mosquito control. The results of the present investigations are more or less in agreement with aforementioned findings.

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Studies in Fungi 2 (1): 26–37 (2017) www.studiesinfungi.org ISSN 2465-4973
Article

Doi 10.5943/stif/ 2/1/4

# The molecular phylogeny and taxonomy of endophytic fungal species from the leaves of *Vitex negundo* L.

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Ramesh V, Arivudainambi USE, Rajendran A 2017 – The molecular phylogeny and taxonomy of endophytic fungal species from the leaves of *Vitex negundo* L. Studies in Fungi 2(1), 26–38, Doi 10.5943/sif/2/1/4

**Abstract**

Enormous fungal species live within the healthy plant tissues, some of which presumably occur in a symbiotic association with host. Some fungal endophytes are widespread and can be found in many different plant species, whereas others are highly specific to single hosts. In this study, we isolated three endophytic fungi from the medicinal plant *Vitex negundo*. They were identified based on morphological characteristics such as size, shape, and colour of the spore and it was reinforced by 18s rRNA gene sequence analysis. The phylogenetic tree showed that C. *gloeosporioides* VN1 and *Pestalotiopsis virgatula* VN2 were closely relationship between. But they were not closely relationship between the other endophytic fungal species that were obtained from geographically different part of the world. This aspect can be further explored to understand the relationships between plant hosts and their fungal endophyte.

**Key words** – Endophytic fungi – MEGA 6.0 – phylogenetic relationship – rRNA

## Introduction

Endophytes are to be found in virtually every plant on earth. They reside in the living tissues of the host plant and do so in a variety of relationships ranging from symbiotic to pathogenic (Strobel et al. 2004). Endophytes receive nutrition and protection from the host plant, while the host plant may benefit from enhanced competitive abilities and increased resistance to herbivores, pathogens, and various abiotic stresses by attaining the metabolic substances of endophytes (Saikkonen et al. 1998, Tan & Zou 2001, Zhang et al. 2006). Endophytic fungi have been found in all plant families so far investigated, which represent many species in different climatic regions of the world (Spurr & Welty 1975, Petrini & carroll 1981, Petrini et al. 1992). Endophytes have been reported from all major groups of plants including algae (Zuccaro et al. 2008, Suryanarayanan et al. 2010), lichens (Suryanarayanan et al. 2005), mosses (Schulz et al. 1993), ferns (Petrini et al. 1992), conifers (Giordano et al. 2009) and angiosperms (Saikkonen 2007), and may persist even in aseptically cultured plants (Lucero et al. 2008). Endophytic fungi are reported from plants that grow in various environments including tropic (Mohali et al. 2005), temperate (Ganley et

Submitted 16 August 2016, Accepted 20 February 2017, Published online 27 March 2017
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al. 2004), xerophytic (Suryanarayanan et al. 2005) coastal mangroves (Kumaresan & Suryanarayanan 2001, Okane et al. 1998) and aquatic environment (Sati & Belwal 2005). Environment plays an important role on endophyte biodiversity, while the species diversity is dependent upon the nature of the host plant and their ecological location.

Medicinal plants have been recognized as a repository of fungal endophytes with novel metabolites of pharmaceutical importance (Strobel et al. 2004, Wiyakrutta et al. 2004, Kumar et al. 2005, Tejesvi et al. 2007). Plant with pharmaceutical importance is being exploited because of their healing properties. However, large scale harvesting of medicinal plants has already become a major threat to biodiversity. As an alternative, microbe which lives inside the plants (endophytes) may often become a tremendous potential source of therapeutic compounds.

Traditional classification and identification of endophytic fungi depends upon microscopic features, colony characteristics on artificial media and biochemical reactions (Sutton & Cundell 2004). This kind of methods have served in the past but they have major drawbacks as they cannot be applied to non cultivatable organisms and occasionally biochemical characteristic of some organisms do not fit into the patterns of any known genus and species. Amplification and sequencing of target regions within the ribosomal DNA gene complex has emerged as a useful adjunctive tool for the identification of endophytic fungi and does not depend on fungus sporulation for identification (Buzina et al. 2001, Iwen et al. 2002, Rakeman et al. 2005, Schwarz et al. 2006).

The key elements for the evolution of the endophytes are quite complex, involving various types of interactions between the host plant, numerous levels of happenstance, and multidirectional flows, they are also influenced by random events, such as living and non living factors, which guide the process of co-evolution between endophytic fungi and their hosts (Saikkonen et al. 2004).

Eventhough knowledge regarding the ecology, life cycle and phylogeny of endophytic fungi has quickly increased and accumulated over the last three decades, questions concerning their evolutionary origin, species and ecological role are not yet completely understood (Saikkonen et al. 2004). There is good reason to believe that partnership co-evolution was essential for the survival of both, and in this case, the symbiosis was mutualistic (Read et al. 2000).

The ribosomal DNA (rDNA) is present in all organisms and its evolution is rapid, so it is used to discriminate related species or even varieties of the same species. The ITS regions are flanked by preserved segments (18S, 5.8S and 28S genes). These preserved regions provide the information about the phylogeny and the taxonomic level, since their evolution is slow and they are highly similar within different taxa

Considering the importance of the *Vitex negundo* L. as a medicinal plant, the aim of the present work was to determine the phylogenetic relationship of three endophytic fungi comparison with other endophytes from different geographic region that were deposited in NCBI database.

## Materials & Methods

### Plant material and study area

Healthy leaves of medicinal plant *Vitex negundo* L. were collected from various seasons in Botanical garden, Department of Botany, VHNSN College, Virudhunagar, Tamil Nadu, India (Fig.1) Leaves were cut from the plants and placed in plastic bags after removal of excess moisture. The leaf samples were stored at 4°C.

**Isolation of endophytic fungi**

The leaf samples were washed thoroughly under running tap water and air dried before they were processed. An endophytic fungus was isolated according to the reported protocol (Petrini 1986), which was modified slightly based on preliminary testing. All the leaf samples were washed twice in distilled water and then surface sterilized by immersion for 1 min in 70% v/v ethanol, 4 min in sodium hypochlorite (3% v/v available chlorine) and 30 s in 70% v/v ethanol, and further washed three times in sterilized distilled water for 1 min each time. After surface sterilization, the samples were cut into 5-7 mm pieces and aseptically transferred to Petri plates containing potato dextrose agar (PDA) with 50 µg/mL of streptomycin to suppress bacterial growth. The Petri plates were incubated at 30°C with normal daily light and dark periods. The plates were examined daily for up to 1 month for the development of fungal colonies growing on the leaf segments. The fungi growing on the leaf tissue were subsequently transferred onto fresh PDA plates without antibiotics.

**Microscopic analysis**

The endophytic fungi were grown on PDA at 30 $^{o}$C for 7 - 9 d, and the formation of conidia was examined under a microscope. Moreover, slide culture technique was also used to observe the morphology of the fungi. For spore dimensions determinations we were used 50 spores. Lacto phenol cotton blue and distilled water were used as mounting media for microscopic analysis. Photography was carried out with the assistance of light microscope and binocular microscope (COSLAP) with computer attached. The isolated endophytic fungi were identified at Centre for Advanced Studies in Botany, University of Madras, Tamil Nadu, India.

**DNA extraction, amplification and sequencing**

Fungal isolates were incubated a week at 30 ˚C on PDA. The mycelia were harvested and transferred into 2 ml plastic tubes using a sterile spatula and lyophilized for DNA isolation. Genomic DNA was isolated by using the method of Doyle & Doyle **(**1987**).** Further, the ribosomal DNA amplification, ITS1-5.8S-ITS2 region, was carried out and primers ITS1 and ITS4 were used as described by White et al. (1990). Isolates of 18s rRNA fungal sequences obtained were submitted to GenBank (NCBI, USA) (accession numbers: HQ191217, JF795287 and JF795288). All the studies of DNA isolation and sequencing were done by Synergy Scientific Services, Chennai.

**Phylogenetic analysis**

Phylogenetic analysis was conducted in MEGA 6 software (Tamura et al. 2007). Sequenced ITS1-5.8S-ITS2 regions were aligned initially using the alignment algorithm Clustal W (Thompson et al. 1997) with the gap open penalty 7.0 and gap extension penalty 4.0. Due to some variation in areas of ITS1 and ITS2 regions, an alignment was then improved manually. The evolutionary history was inferred using the neighbor joining method (Saitou & Nei 1987). All positions containing gaps with missing data were eliminated from the dataset. Strengths of internal branches of resulting trees were statistically tested by the bootstrap analysis of 1000 replications **(**Felsenstein 1985). Additional sequences were retrieved from GenBank (Table 1).

## Results

**Taxonomy**

**Fungal isolate VN1**

The morphological characteristics of the endophytic fungal isolate VN1 was observed

on PDA after 7 days of growth at 30 $^{o}$C. Colonies on PDA was circular, raised, at first orange-white, sometimes grey and becoming pale orange with age, aerial mycelia white dense, cottony without visible conidial masses, reverse bright orange but sometimes yellowish-brown to olive-brown and very slow-growing. Acervuli and Setae were absent in culture. Conidia were hyaline, unicellular and cylindrical with obtuse apices and tapering bases. Average conidial size was 14.7 × 3.8 µm. (Fig. 2).

Traditionally, identification of *Colletotrichum* sp. have been based on size and shape of conidia and culture characteristics such as colony colour, growth rate and texture (Smith et al. 1990). Morphological characteristics allowed the identification of the endophytic fungal isolate VN1 as *Colletotrichum gloeosporioides*, which was reinforced by the sequence of its 18S rRNA that gave a 91% sequence similarity to those accessible at the BLAST of *Colletotrichum gloeosporioides* (Fig. 3). The endophytic fungal sequence was deposited at GenBank with Accession No. HQ191217.

**Fungal isolate VN2**

The fungus growing on PCA was pale buff with sparse aerial mycelium and acervuli containing black, slimy spore masses (Fig.4). All isolates had 5-celled conidia, apical and basal cells were hyaline, while the three median cells were olivaceous; the upper two were slightly darker than the lower one. Conidia were 20.3 × 6.8 µm. They were typically three apical appendages averaging 16.8 µm long. The average basal appendage was 3.8 µm long (Fig. 4). The fungal isolate was initially identified by comparing morphological and cultural characteristics (Size of the conidia, color and length of median cells, length and number of apical appendages and length of basal appendage) to those described in Guba's monograph of *Monochaetia* and *Pestalotia* (Guba 1961).

Morphological characteristics allowed the identification of the endophytic fungal isolate VN2 as *Pestalotiopsis virgatula* which was reinforced by the sequence of its 18S rRNA gene that gave a 98% sequence similarity to those accessible at the BLAST of *Pestalotiopsis virgatula* (Fig. 5). The endophytic fungal sequence was deposited at GenBank Accession No. JF795287.

Based on the above morphological and molecular characteristics the endophytic fungus was identified and designated as *Pestalotiopsis virgatula* VN2. The endophytic fungus *P. virgatula* VN2 belongs to the class Ascomycota.

**Fungal Isolate VN3**

The fungal isolate VN3 grows rapidly on PDA medium and matures within 5 d. The colony is flat, downy to cottony and may eventually be covered by greyish, short, aerial hyphae. The reverse side is typically brown to black due to pigment production (Fig. 6). They have septate, dark hyphae. They bear simple or branched large conidia (8-16 × 23-50 µm) which have both transverse and longitudinal septations. These conidia may be observed singly or in acropetal chains and may produce germ tube. They are ovoid to obclavate, darkly pigmented muriform, smooth or roughened. The end of the conidium nearest the conidiophere is round while it tapers towards the apex (Fig. 6).

Morphological characteristics of fungus allowed the identification of the endophytic fungal isolate VN3 as *Alternaria alternata* which was reinforced by the sequence of its 18S rRNA gene that gave a 95% sequence similarity to those accessible at the BLAST of *Alternaria alternata*. The endophytic fungal sequence was deposited at GenBank with Accession No. JF795288 (Fig. 7). This endophytic fungus *A. alternaria* VN3 belongs to the class Ascomycota.

**Phylogenetic analysis**

Phylogenetic relationships inferred from ITS1-5.8S-ITS2 region sequences of three species are shown in Figure 8. The tree is divided into three main clusters (A, B and C) and further each one divided into two sub-clusters like A1, A2, B1, B2, C1 & C2. Based on the evolution, among the present three fungal endophytes *Colletotrichum gloesporoides* VN1 and *Pestalotiopsis virgatula* VN2 were grouped into the single sub cluster B2. Another present endophytic fungal species of *Alternaria alternata* VN3 was located in the subclaade A1. In sub-cluster A1 *Colletotrichum gloeosporoides, Pestalotiopsis funerea* strain SYJM13, *Pestalotiopsis* sp. Strain F4875 and *Alternaria alterna* VN3 were grouped together. *Pestalotiopsis* sp. MA165, *Pestalotiopsis* sp. MA129 and *A. compacta* strain IR13 were grouped together in sub-cluster A2. In sub-cluster B1 *C. gloeosporiodes* strain FL1-ML2, *Alternaria* sp. Abs and *Pestalotiopsis* sp. Z4-08 were grouped together. The phylogenetic tree results showed *C. gloeosporioides* VN1 and *Pestalotiopsis virgatula* VN2 were closely relationship between. But they were not closely relationship between the other endophytic fungal species that were obtained from geographically different parts of the world.

**Discussion**

*Vitex negundo* L. (*Verbenaceae*) is a woody, aromatic shrub growing to a small tree. It commonly bears tri or penta foliate leaves on quadrangular branches, which give rise to bluish purple colored flowers in branched tomentose cymes. It thrives in humid places or along water courses in wastelands and mixed open forests and has been reported in many countries. It is grown commercially as a crop in parts of Asia, Europe, North America and the West Indies (de Padua et al. 1999). It is an important medicinal plant used in the traditional medicine and has a variety of pharmacological activities. Hence, in the present study we were used this plant as a host for endophytic fungal isolation. Three endophytic fungal species were isolated from leaves of *V. negundo*.

**Table 1** Species and GenBank accession number used in the study

| Name of the Species | Geographic origin | GenBank No |
|---|---|---|
| *Colletotrichum gloeosporioides* VN1* | India | HQ191217 |
| *Colletotrichum gloeosporioides* Strain JS1-SAS12 | China | KP900300 |
| *Colletotrichum gloeosporioides* Strain FL1-ML2 | China | KP900236 |
| *Colletotrichum gloeosporioides* Strain W-2 | China | HQ845101 |
| *Colletotrichum gloeosporioides* Strain JL5 | China | KM513573 |
| *Colletotrichum gloeosporioides* Strain FL1-CJL1 | China | KP900235 |
| *Pestalotiopsis virgatula* VN2* | India | JF795287 |
| *Pestalotiopsis funerea* strain SYJM13 | India | JF923833 |
| *Pestalotiopsis* sp. Z4-08 | China | HQ262524 |
| *Pestalotiopsis* sp. 1 AE-2013 Strain F4875 | Panama | KF746126 |
| *Pestalotiopsis* sp. MA129 | Thailand | GQ254681 |
| *Pestalotiopsis* sp. MA165 | Thailand | GU592005 |
| *Alternaria alternata* VN3* | India | JF795288 |
| *Alternaria compacta* Strain IR13 | Iran | KU323573 |
| *Alternaria* sp. HT-M18-LS | China | KJ527010 |
| *Alternaria* sp. HT-M18-L | China | KJ527009 |
| *Alternaria* sp. Abs | Serbia | JF742668 |
| *Alternaria* sp. B5A | USA | EF432299 |

Asterisks indicate the sequences obtained from the present study

Endophytic fungal species are complex anamorphic genus. For example *Pestalotiopsis* was established by Steyaert (1949). It can be lived as saprobes, plant pathogens or endophytes (Suto & Kobayashi 1993, Rivera & Wright 2000, Karakaya 2001, Gonthier et al. 2006, Sousa et al. 2004). The identification of endophytic fungal species based on morphology is however, complicated because there are few morphological characters available to distinguish taxa at the species level. Hence, nuclear small subunit ribosomal RNA gene regions are usually used as a molecular tool to analyze fungal taxa at a family or order level and ITS regions are commonly used to examine phylogenetic positions or relationship at a species or intra species level. Morphological characters are important in identifying *Pestalotiopsis* species (Steyaert 1949, Guba 1961, Sutton 1980, Nag Raj 1993). Characters used however are few and often overlap. This results in identification problems and difficulties in differentiating species. In the case of *Colletotrichum* sp. molecular phylogeny has been helpful in establishing species concepts (Photita et al. 2005).

Our fungal strains formed a segregated claade with *A. alternata*, *C. gloeosporioides* and *P. virgatula* supported by low bootstrap values of 38, 18 and 46 %, respectively. Similar results were obtained in the phylogenetic analysis of *Xylaria* species from Western Gahts of Curtallum Hills (Ramesh et al. 2012). Phylogenetic analysis, based on rDNA sequencing, enabled us to show that there is genetic variability among the isolates of the three endophytic fungi. Moreover, they were not closely related between the other endophytic fungal species that were obtained from geographically different parts of the world.

**Fig. 1 -** Medicinal plant *Vitex negundo* L.

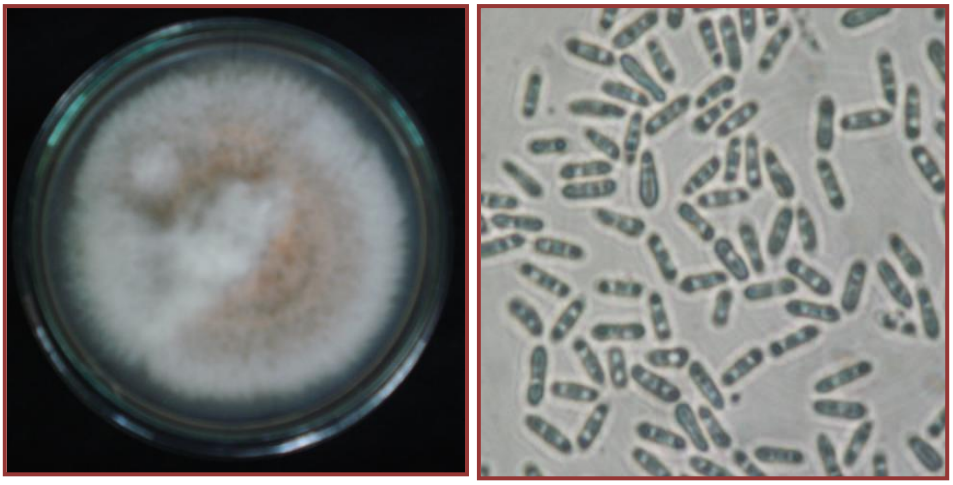
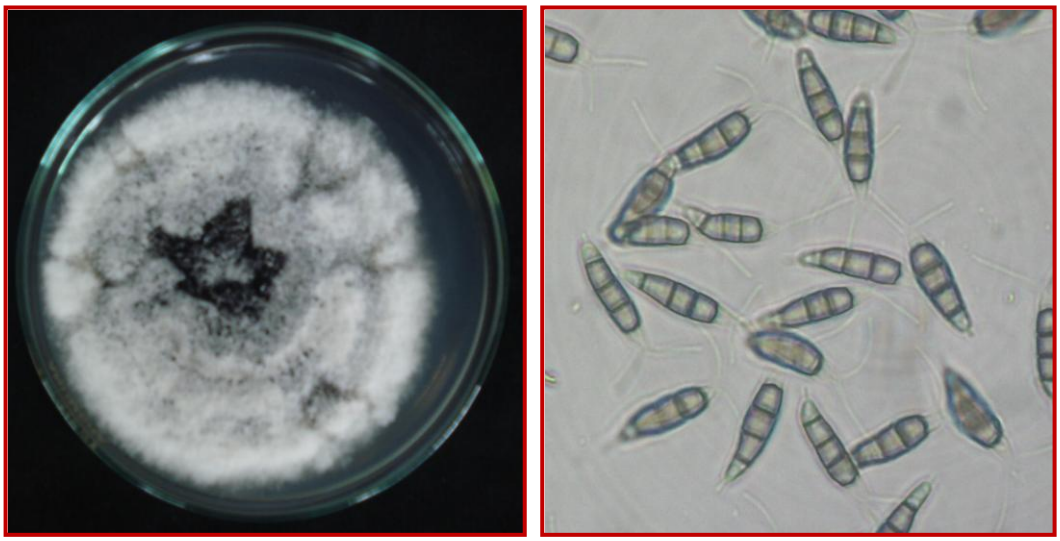


**Fig. 2 -** Morphology characteristics of *Colletotrichum gloeosporioides*

TCTACACCCTTTGTGACATACCTATAACTGTTGCTTCCGCGGGTAAGGTCCCCGT
GACCCTCCCGGGCTCCCGCCCCCCGGGCGGGTCGGCGCCCGCCCGAAGAAAACC
CAACTCTGATTTAACGACCTTTCTTCTGAATGGTACAAGCAAATAATCCAAACTT
TTAACAACGGATCTCTTGGTTCTGGCATCCATGAAAAACGCAGCGAAATGCGAT
AAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGC
GCCCGCCCACATTCTGGCGGGCATGGCTGTTCCAACGTCCTTTTCAACCCTCAAG
CTCTGCTTGGTGGTGGGGGCCCTACACTGATGTTAGGCCCTCAAGGTAATGGCGG
AACCCTCCCCGAACCCCCTTTGCGTTATAACTTTTACGTCTCGCACTGGGGATCC
GGAAGGGACTCCTTGCCCGAAAACCCCCAATTTTTCCAAAGGTTGACCTCGGATC
AGGTAAGAAATACCCCGCTGAACTTTAACATATCAATAACCGGAAGA

**Fig. 3 -** 18S rRNA sequence of *Colletotrichum gloeosporioides*



Colony morphology Conidial spore (200 X)

**Fig. 4 -** Morphology characteristics of *Pestalotiopsis virgatula*

TGTGAACTTACCTTTTGTTGCCTCGGCAGAAGTTATAGGTCTTCTTATAACTGCTG
CCGGTGGACCATTAAACTCTTGTTATTTTATGTAATCTGAACGTCTTATTTTAATA
AGTCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGC
GAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAA
CGCACATTGCGCCCATTAATATTCTAGTGGGCATGCCTGTTCGAGCGTCATTTCA
ACCCTTAAGCCTAACTTAGTGTTGGGGAATCTACTTCTTTATAGTTGTAGTTCCTG
AAATACAACGGCGGATTTGTAGTATCCTCTGAGCGTAGTAATTTTTTTCTCGCTTT
TGTTAAGTGCTATAACTCCCAGCCGCTAAACCCCCAATTTTTTGTGGTTGACCTCG
GATCACGTAGGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAAATAA
CCTTTTTTAGTTTTCTAATCTCCATCCATGTGACTTACCTTTAGTTGACTCGCAAA
GTTATATGTCTTCTT

**Fig. 5 -** 18S rRNA sequence of *Pestalotiopsis virgatula*

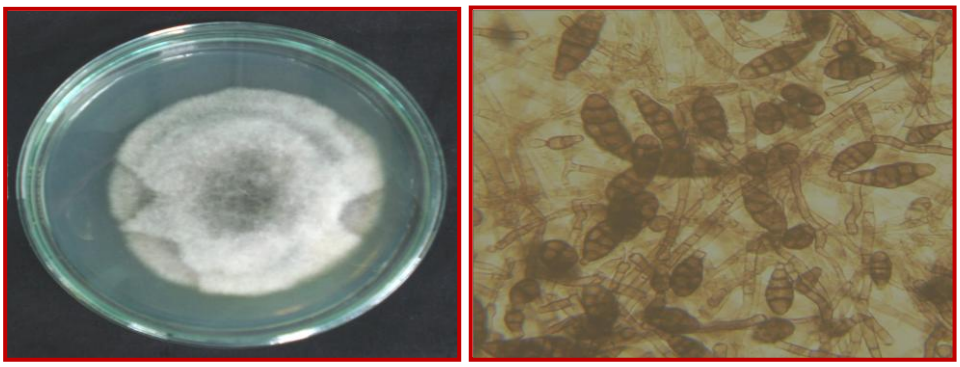


**Fig. 6 -** Morphology characteristics *Alternaria alternata*

TCTCGGGGTTACAGCCTTGCTGAATTATTCACCCTTGTCTTTTGCGTACTTCTTGTT
TCCTTGGTGGGTTCCCCCCCCACTAAGACAAACATAAACCTTTTGTAATTGCAAT
CCGCGTCAGTAACAAATTAATAATTACAACTTTCAACAACGGATCTCTTGGTTCT
GGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAATTGCAGAATTC
AGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATTCCAAAGGGCA
TGCCTGTTCGAGCGTCATTTGTACCCTCAAGCTTTGCTTGGTGTTGGGCGTCTTGT
CTCTAGCTTTGCTGGAGACTCCCTTAAAGTAATTGGGCAGCCGGCCTACTGGTTT
TCGGAGCGCAGCACAAGTCCCCACTCTCTATCAGCAAAGGTCTAACCATCCCATT
AAGCCTTTTTTTTTCAACTTTTTGACCCTCGGGATCCAGGTAGGGAATACCCCGCT
GAAACTTAAACCATAATCAATAAGCGGAAGAAAAAAATCATTACACAAATAATG
AAAGGGCGGGCTGGAATC

**Fig. 7 -** 18S rRNA sequence of *Alternaria alternata*

Endophytic fungi are everywhere and occur within all plant parts in various ecosystems, but the geographic differences in endophyte diversity, community composition and host preference have not been well documented. To understand the ecology of fungal endophytes, data regarding fundamental parameters of endophyte symbiosis are require from regional to continental scales and encompassing entire ecosystems (Peay et al. 2010). It is hoped that powerful, high throughput molecular techniques like sequencing technology will make the global assessment of endophyte diversity a reality and open up the ‘black box’ of fungal ecology.

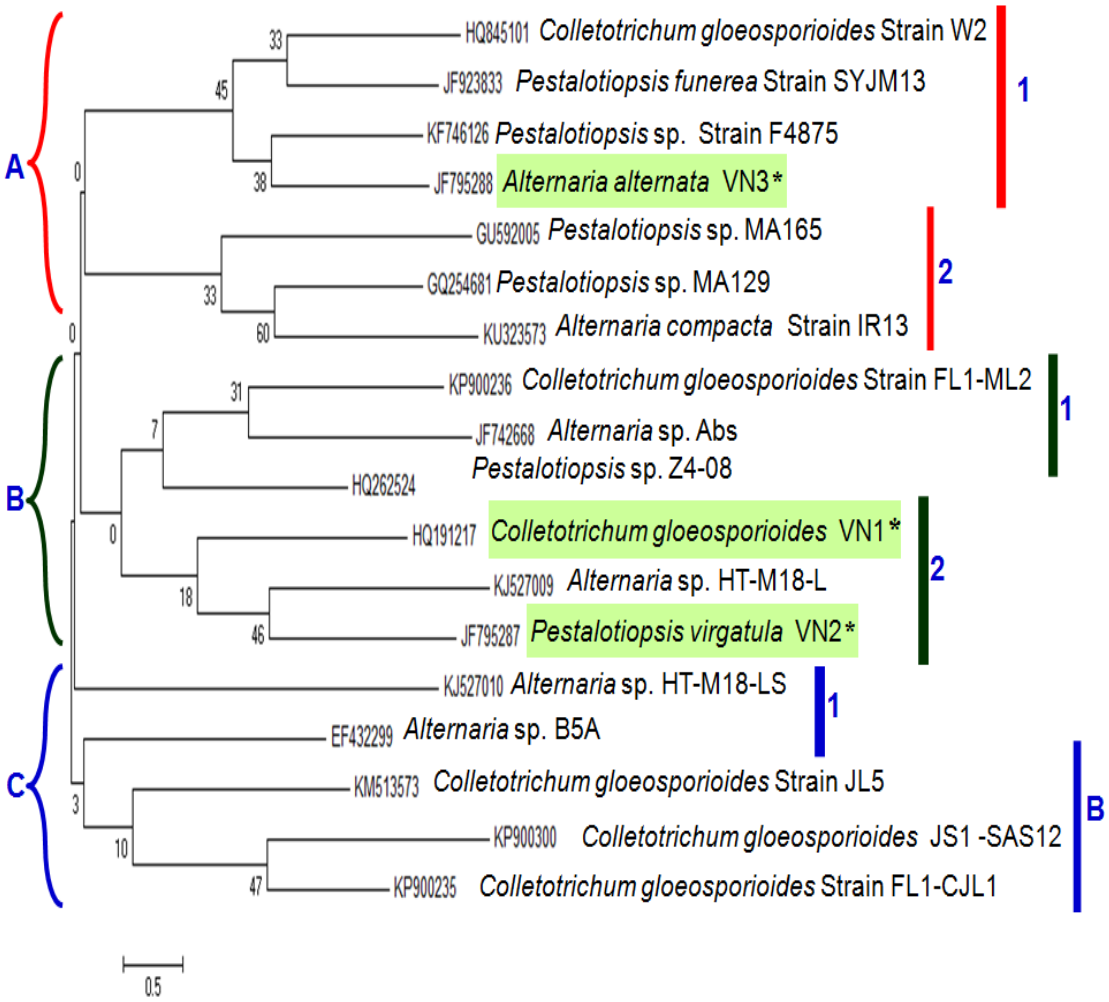



**Fig. 8 -** Phylogenetic relationship between three endophytic fungal species, inferred from ITS nucleotide sequence data. Bootstrap values are shown for those branches that had >10% support in a bootstrap analysis of 1000 replicates. The numbers of nucleotide changes among taxa are represented by branch length and scale bar equals the number of nucleotide substitutions per site. Asterisks indicate the sequence obtained from the present study. A, B & C indicates major clusters and 1 & 2 indicates sub-clusters referred to in the text.

**Acknowledgments**

Author thanks the Managing Board of Virudhunagar Hindu Nadar's Senthikumara Nadar College, Virudhunagar-626 001, Tamil Nadu, India for providing research facilities.

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