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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 4^{o}C 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 30^0C. 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 35^0C. 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 30^0C 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 30°C 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.

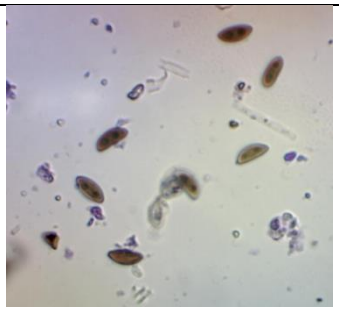
3. Results:

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.

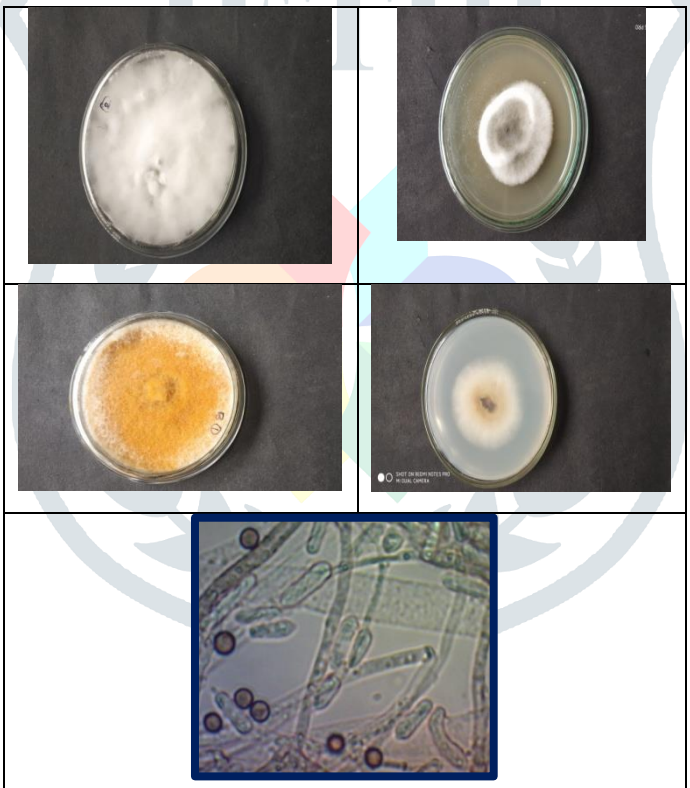
Mycelial biomass can be harvested in significant amounts and used for biological activity. The ideal conditions were established after considering how environmental variables like pH, temperature, and culture media affected the situation. The morphological characteristics of the *Xylaria* sp. strain 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.

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