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Insecticidal Potential of an Endophytic Fungi *Colletotrichum Falcatum* against *Spodoptera Litura*

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Abstract: Pest insects significantly impair and devastate the world's agriculture, yet the major method used to manage them by using chemical insecticides which are extremely damaging to both human health and the environment. End ophytes are abundant sources of secondary metabolites with biologically active properties that have the potential to be effective biocontrol agents. To isolate endophytic fungi *Colletotrichum falcatum* (OQ553930)from medicinal plant *Mukia maderaspatana*(L.) Roem. The *Colletotrichum falcatum* (OQ553930) ethyl acetate extract was extracted and analysed for TLC and Phytochemical analysis. The extracts were applied to *Spodoptera litura* using residual film and dipping methods. The results demonstrated that the larval mortality in the dipping method increased by a percentage as 85%, 65%, and 25% when the extract concentration was increased (1mg/ml, 0.5mg/ml, 0.25mg/ml), whereas the larval mortality in the residual film method showed the mortality percentage as 20%, 12%, 7% when the same extract concentration was maintained. Therefore, *Colletotrichum falcatum* (OQ553930) ethyl acetate extract can be used as an alternative to chemical insecticides to control *Spodoptera littoralis*and possibly other lepidopterans. This could be a significant contribution to integrated pest management strategies.

Keywords: Endophyte, Spoduptera litura, Ethylacetate , Insectcidies

Introduction:

One of the most dangerous pests of cotton, maize, rice, alfalfa, potato, tomato, and ornamental plants is the African cotton leafworm, *Spodoptera littoralis* (1). Significant interest has been shown in the use of microbial pesticides for the biological control of insect pests. because they do not generate resistance in their insect hosts or leave behind toxic chemical residues in the environment (2). Biological plant protection utilizing entomopathogenic fungi is an essential part of a long-term pest management approach. Entomopathogens have a variety of advantages over conventional insecticides as biocontrol agents. Affordable, extremely efficient, safe for beneficial creatures, decreasing environmental

residues, and enhancing biodiversity in human-managed settings are a few of these attributes (3).

Microorganisms known as endophytes develop inside plants without displaying any outward signs of illness or disease. Some endophytes help their host plants develop faster and absorb more nutrients (4).By creating secondary metabolites, several endophytes are thought to defend their host from attack by fungi, insects, and animals. interact closely with their host plants, offering the possibility of using them as biological control agents in the production of sustainable crops (5).In this study, endophytic fungi from the medicinal plant *Mukia maderaspatana* (L.,) Roem were isolated, their secondary metabolites were extracted and investigated for their constituent chemicals using TLC, GC-MS, and Phytochemical analysis, their capacity to kill *Spodoptera litura* larvae was evaluated.

Materials and Methods:

Isolation of Endophytic Fungi:

The fresh leaves of *Mukia maderaspatanus* (L.,) Roem. were collected from Anna Herbal garden. The larvae of *Spodoptera litura* used for the bioassays were collected from Department of Entomology, Agricultural Research Organization ,Tamilnadu . The larvae were reared on castor bean leaves under laboratory conditions.

To get rid of the surface impurities, the segments were successively submerged for 2 minutes in 70% ethanol and 4% sodium hypochlorite solution, both of which are surface sterilization agents, and finally it was once again rinsed three times in sterilized distilled water. and dried using sterile blotting paper(6). With sterile blades, the sterilized leaf pieces were divided into tiny pieces (about 1.0 x 1.0 cm). To avoid bacterial contamination, potato dextrose agar (PDA) media were given a 100 µg/mL dose of streptomycin. Five tissue segments were included in each medium plate, which was parafilm-sealed before being incubated for a week (7). After incubation, the fungal endophytes that emerged from the tissue were purified on PDA plates (8).

Identification of Fungal isolates:

Microscopic Observation:

Based on their morphological and cultural traits, the fungi were identified. A drop of distilled water was added to the fungal samples before they were placed on the slides and examined using a phase contrast microscope.

DNA extraction and PCR amplification:

Add 500 mL of the lysis buffer to the fungus broth, and centrifuge at 13,000 rpm for 10 minutes. 2ml of RNase A was added to the supernatant and incubated for 15 minutes. Then, phenol, chloroform, and isoamyl alcohol (25:24:1) were mixed in an equal volume and centrifuged at 13000 rpm for 10 minutes. Separately, the upper aqueous layer was added to an equivalent volume of 100% ethanol. After 30 minutes at -20°C, the DNA was then pelleted by centrifuging for 10 minutes at 12000 rpm. After being dried, the pellet was dissolved in TE buffer (9).

For a chosen strain, Polymerase Chain Reaction (PCR) was carried out in a T-100 Thermal Cycler, TTC100 in a total volume of 25µl. The PCR mixture included 4µl of 2X Taq buffer (0.4 mM), 6 ng of template DNA, each primer at a concentration of 0.8 M, and 0.75 units of Taq DNA polymerase. With 19 and 20 bases, respectively, two primers, ITS1 (5'TCCGTAGGTGAACCTGCGG3') and ITS2 (5'GCTGCGTTCTTCATCGATGC 3') were employed.DNA amplification was carried out using 30 cycles of initial denaturation at 94 °C for 30 sec, 59 °C for 55 sec, 72 °C for 90 sec, and a final extension at 72 °C for 1 min. The PCR for ITS regions was run at 95 °C for two minutes as a hot start, then 30 cycles of 94 °C for one minute, 56 °C for thirty seconds, 72 °C for two minutes, and finally 72 °C for a final extension of ten minutes. The amplified product was purified, put through a 1.2% (w/v) agarose gel analysis, and then put through DNA sequencing (10).

Phylogenetic analysis:

The ITS nrDNA sequences of the isolates were compared with those reported in GenBank databases using the BLAST search program to find probable homologous sequences of the recently sequenced species for fungi. The sequences of closely related fungi acquired from GenBank were aligned to lessen the amount of inferred gaps. The sequences underwent several alignments using the ClustalW software. The evolutionary trees were reconstructed using the MEGA5.1 program. The bootstrap determined the trustworthy level of the tree's nodes using 1,000 replications (11).

Crude Extraction Of Bioactive Compounds:

Colletotrichum falcatum (OQ553930) isolated endophytic fungi, was grown in potato dextrose broth and cultured at 28 °C for two weeks at 120 rpm (12). To get culture filtrate devoid of mycelia, the culture broth was next filtered through four layers of cheesecloth and Whatman filter paper No. 1. The fungal filtrate was extracted using a separating funnel and ethylacetate at a 1:2 ratio. To create the crude broth extract, the organic phase was dried under reduced pressure using a rotary evaporator at 40 °C (10).

Thin Layer Chromatography:

Over 1 cm from one end of the TLC plate, a line was drawn in the TLC plate. After applying the *Colletotrichum falcatum* (OQ553930) ethyl acetate extract to the plate's line with a capillary tube, the plates were allowed to dry for a little while. The TLC tank was closed for a short period to allow the reagent to fully permeate the internal environment. Here, three different TLC reagents, including 100% ethylacetate, a 2:1 ratio of toluene to ethyl acetate, and a 1.5:1.5 ratio of toluene to ethyl acetate, were utilized. To prevent the reagent from crossing the sample line and the TLC plate from touching the tank's wall, it was carefully positioned inside the tank. Once it hits the top, the reagent is taken out and allowed to dry. After the plate had dried, it was looked at under UV light to determine the Rf value and to identify the bands.(13)

Rf value = Distance traveled by the compound / Distance traveled by the solvent

Phytochemical analysis:

Alkaloids

The small portions of solvent-free extract were stirred separately with a few drops of dilute HCl, filtered, and treated with Dragendroff's reagent (Potassium Bismuth iodide) to produce an orange-brown precipitate indicating the presence.(14)

Carbohydrates:

The extract was dissolved in 5 ml of distilled water, filtered, and then a small amount of the filtrate was treated with Fehling's reagent (Fehling's reagent A - Copper sulfate in water and Fehling's reagent B - Sodium Potassium Tartrate), heated, and the mixture precipitated brick red, indicating the presence of reducing sugars.(14)

Glycosides:

The extract was filtered after being hydrolyzed with con HCL for two hours in a water bath. 3ml of chloroform was added to 2ml of filtrate hydrolysate and thoroughly mixed. After separating the chloroform layer, ammonia solution was added to it. Glycosides are present when the color is pink. (15)

Saponins:

Diluted 1ml of the extract with distilled water to 20ml and shake for 15min. A 1cm layer of foam indicates the presence of saponins.

Tannins:

Extract treated with ferric chloride solution, the blue color indicates the presence of tannins.

Terpenoids:

The presence of terpenoids was demonstrated by the production of a yellow to crimson precipitate after 2ml of trichloroacetic acid was added to 1ml of the extract.(15)

Flavonoids:

A few drops of sodium hydroxide solution were added to the test solution; this caused the creation of a strong yellow color, which changed to colorless when a few drops of diluted acid were added, demonstrating the presence of flavonoids.(14)

Pathogenicity Of Endophyte Secondary Metabolites:

The dipping and residual film techniques evaluated the effectiveness of ethyl acetate extracts against *S. littoralis* third-instar larvae. In sterile distilled water, concentrations of ethyl acetate crude extracts (250, 500, 1000, and 2000 mg/l) were produced. In thedipping method, five *S. littoralis* larvae were immersed for five seconds in each extract concentration, dried on filter paper for ten seconds, and then put into bottles containing castor leaves.For the residual film bioassay, the same ethyl acetate extract concentrations were employed. Five *S. littoralis* larvae that had been fasted for 4-5 hours received castor leaves that had been dipped in each extract concentration for 20 seconds, dried, and then placed in bottles. Castor leaves treatedwithsterilized distilled water were used in the control treatments. Over the course of a week, the mortality rate was tracked everyday, and the mortality percentage was computed (12).

Results and Discussion:

Identification of Fungal endophytes:

From *Mukia maderaspatanus* (L.,) Roem., endophytic fungi from several genera were isolated (fig 1,2). fungi that displayed a promising outcome were chosen for investigation and were distinguished by their morphological traits that resembled the structure of *Colletotrichum* sp (fig 3).

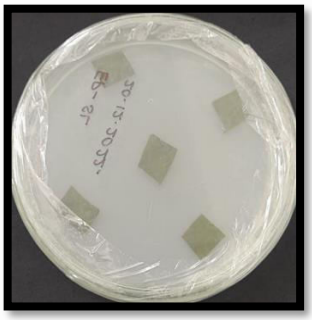


Fig 1: PDA plates with leaf pieces

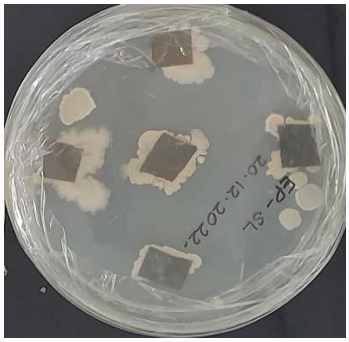


Fig 2: plates after incubation

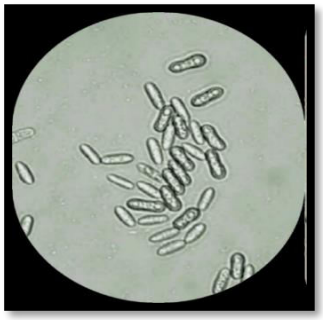
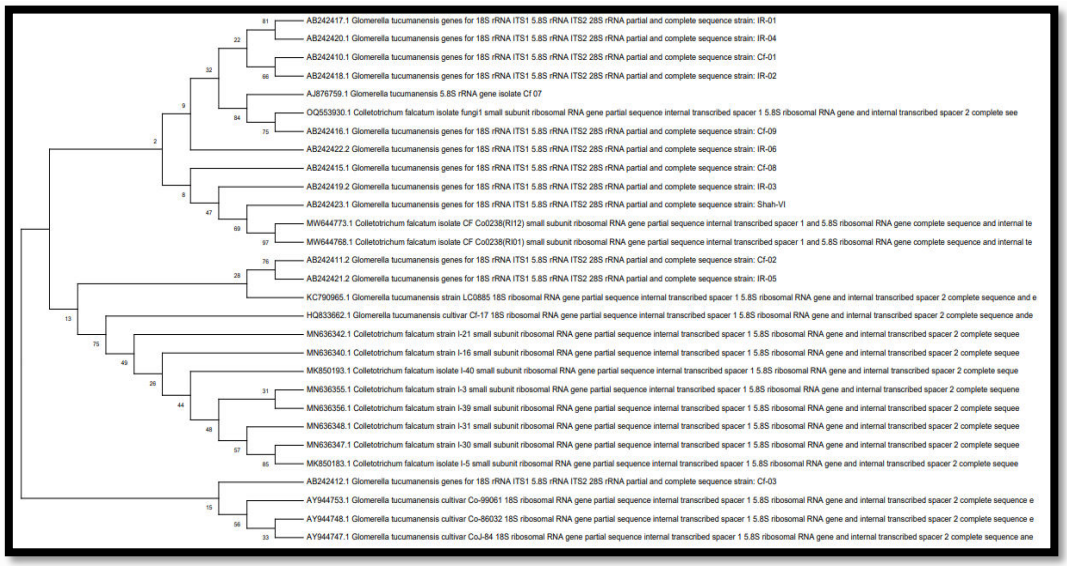


Fig 3 :Phase contrast microscopic observation of *Colletotrichum falcatum* OQ553930

Phylogenetic Analysis:



About 541 base pairs were obtained in the sequence through forward and reverse primer sets. The sequenced organism was found to be *Colletotrichum falcatum.*(Fig 4) illustrates the phylogenetic tree of the isolated endophytic fungi, respectively.

Thin Layer Chromatography:

The active principle on the thin layer plates appeared as dark bands. TLC thus provided the initial confirmation for the presence of components in the organic extracts of fungal isolates. Table 1 illustrate the TLC report of Ethyl acetate crude extract of *Colletotrichum falcatum* OQ553930 and their Rf values. In comparison with the analysis of H. Sheeba, *et al*., 2019 Three types of solvent systems were chosen to obtain a good result. From the Chloroform: Methanol (8:2), the crude extract of sterile mycelia revealed 1 compound with Rf values of 0.880. In the Benzene: Ethyl acetate (1:1), the crude extract of *Trichoderma viride* revealed the presence of 1 compound with Rf values of 0.790 and 2 compounds from white sterile mycelia Rf values of 0.76, 0.397; 3 spots were detected from Sterile mycelia with Rf values of 0.166, 0.93, 0.89; the crude extract of *Nigrospora sphaerica* revealed with Rf values of 0.54, 0.906, 0.52 respectively. Finally in Butanol: Ethyl acetate (1:1) the crude extract of *Trichoderma viride* develops 2 bands from UV with Rf values of 0.1612, 0.790, from the extract of white sterile mycelia-3 spots with Rf values of 0.54, 0.76, 0.1505, from Sterile mycelia -2 spots withRf values 0.755 and 0.76 were identified, and in the extract of *Nigrospora sphaerica* 2 spots were obtained Rf values 0.767 and 0.72 were detected.

Solvent used	**Band number**	**Rf value**
100% Ethylacetate	1	0.767
	2	0.833
	3	0.933
Toluene: Ethylacetate (2:1)	1	0.138
	2	0.4
	3	0.707
	4	0.876

	5	0.969
Toluene: Ethylacetate (1.5:1.5)	1	0.171
	2	0.671
	3	0.757
	4	0.914

Table 1: TLC report Ethyl acetate crude extract of *Colletotrichum falcatum* OQ553930 and their Rf values

Phytochemical Analysis :

Colletotrichum falcatum (OQ553930) fungal extract underwent qualitative analysis using ethyl acetate, the color change of the fungi extracts reveals the presence or absence of phytochemical components (Table 2). The amount of secondary metabolites in the *Colletotrichum falcatum* (OQ553930) ethyl acetate extract is greater. The endophytic fungi *Penicillium* sp. isolated from *Centella asiatica* showed positive findings for alkaloids, phenols, flavonoids, tannin, and glycosides in ethyl acetate extract when compared to the study of Nameirakoam Nirjanta devi (2012). The findings of the phytochemical experiments demonstrate that the same solvents have the same ability to extract active chemicals.

Table 2: A phytochemical analysis result indicates the presence of various compounds

S NO	**Phytochemical Test**	**Observation**
1	Alkaloids	Presence
2	Carbohydrates	Presence
3	Glycosides	Absence
4	Saponins	Presence
5	Tannins	Absence
6	Terpinoids	Absence
7	Flavonoids	Presence

Biological activity of *Colletotrichum falcatum* crude extract against *S. Littoralis*

Following feeding on the three concentrations of ethyl acetate extracts of *Colletotrichum falcatum* (OQ553930) (1, 0.5, and 0.25 mg/ml, respectively), *S.*

littoralis show a considerable increase in mortality (20%, 60%, and 80%) when compared to the control.

Fig 4: Dead larva of *Spodoptera litura*

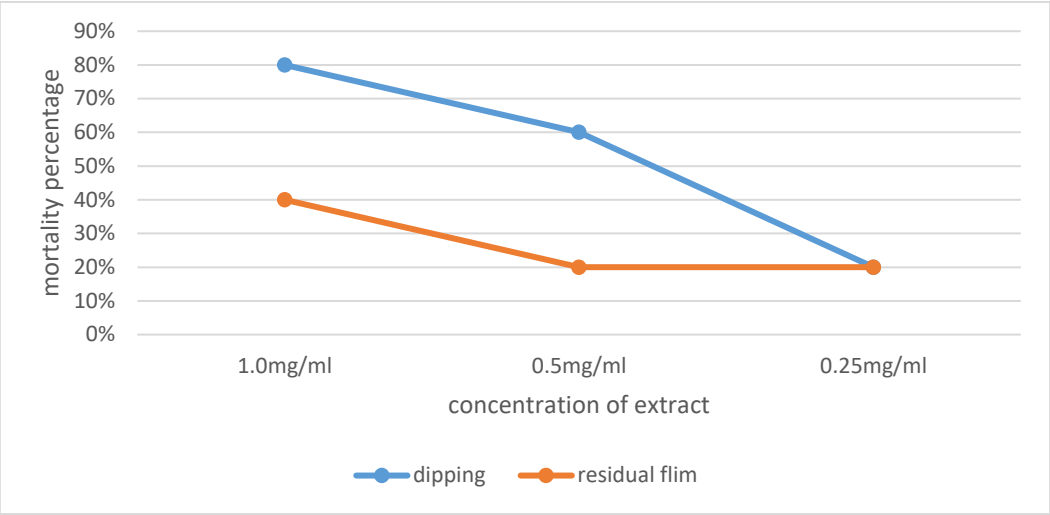
All of the endophytes examined for concentration in the residual film method displayed very low pathogenicity. The partial pupae that were produced after 15 days in larvae given (1 & 0.5mg/ml) of *Colletotrichum falcatum* (OQ553930) crude ethyl acetate extracts for 8 days show that the larvae were unable to finish their development. The larva, which serves as a controlled adult, has reached the moth stage of development.

Fig 5: Incomplete pupae of *Spodoptera litura*

Fig 6: Adult moth stage of *Spodoptera litura*

Senthilkumar et al., 2014 reported that in methanol extracts of *Aspergillus flavus* and *Nigrospora sphaerica* isolated from *Tectona grandis*, 65% and 62%, respectively, of *Hyblea purea*, 59% and 56% of *Eligma narcissus*, and Han et al., Han et al., 2013, respectively, reported that ethyl acetate extracts of 16 mycelial strains and 9 fermentation broth strains showed larvicidal activity against third-instar larvae of *Culex pipiens*. These active strains were concentrated in the genera Aspergillus, Penicillium, Foma and Pesarotyopsis. However, the results

obtained showed that *Colletotrichum falcatum* OQ553930 could infect and kill the S. littoralis larvae by direct contact rather than ingestion of metabolites.



Graph 2: Pathogenicity of *Colletotrichum falcatum* crude extract against S. littoralis

The activity of *Colletotrichum falcatum* extracts against *Spodoptera litura*

Conclusion:

The results demonstrate that *Colletotrichum falcatum* (OQ553930) extract has the potential to function as an entomopathogenic fungal against *Spodoptera litura*. When compared to the residual film method, the dipping method has a higher possibility for *Spodoptera litura* deaths (85%, 65%, and 25%, respectively). It is possible to use the recently discovered *Colletotrichum falcatum* (OQ553930) strains as biological control agents to manage S. littoralis larvae. As a result, endophytic fungi constitute a class of organisms that could be used as a biocontrol agent for insect pests.

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