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Isolation of Endophytic Fungi from Green Pepper (Piper Nigrum) and Analysis of Phytochemicals and their Antioxidant and Antibacterial Activity

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Abstract: ***Endophytic fungi have been a great interest as they produce biologically active secondary metabolites like flavonoids, terpenoids, sterols etc. and they can synthesize compounds associated with their host plants. The present study aimed to isolate Endophytic fungi from green pepper corn (Piper nigrum). The isolated endophytic fungi were processed for fungal extracts and they were screened for the presence of phytochemicals and their Antioxidant and Antibacterial activity were tested***

Keywords: Green Pepper, Endophytic fungi, Phtochemicals, Antioxidant activity and Antibacterial activity

1. Introduction

Endophytic fungi are ubiquitous in plants and they belong to a group of fungi in specific ecosystem mainly inside the plant tissues and produces varieties of secondary metabolites which plays an important role for biological activities such as antioxidant, antivirus, antituberculosis, antimycotics, anti - parasite, insecticides, immunodulatory i.e., modulation of immune system and anticancer. Therefore, Endophytic fungi can be used as a source for producing active metabolites leading for the drug development and the secondary metabolites produced from endophytic fungi are like the host plants. (**Prabukumar S *et al., 2015*)**

Piper nigrum is a flowering vine in the family *Piperaceae* and its fruit is known as Pipercorn. It is usually dried and used as spice. The fruit is a drupe (stonefruit) which is 5mm (0.20 in) diameter (fresh and fully mature). Black pepper is native to the Malabar coast of India. Black pepper is the worlds most traded spice and is one of the most common spices added to cuisines around the world. Its spiciness is due to the chemical compound *piperine*. It is ubiquitous in the western world as a seasoning. The endophytic fungi found in *Piper nigrum* are *Aspergillus, Penicillium and Curvularia species.*

Aspergillus is one of the most well - known and extensively studied groups of fungi because of their abundance and the ease with which they can be cultivated in laboratory conditions. Many species of *Aspergillus* have economic importance ranging from harmful to beneficial effects involve their use in industrial production of enzymes, therapeutic molecules like lovastatin and their harmful effects include them being the causative agents of disease (*Aspergillosis*, allergic reactions).

Penicillium is among the most abundant and ubiquitous groups of soil fungi that exist in nature. Several species of Penicillium are being widely used in industry, such as *Penicillium rubens is used for penicillin production, Penicillium camemberti, and Penicillium roqueforti, are used as cheese starters, and Penicillium nalgiovense is* used in fermenting sausages (**Bhardwaji et al., 2015).**

In the present study, the endophytic fungi are isolated from green pepper corn (Piper nigrum) and the antibacterial and antioxidant activity of endophytic fungal extracts are determined.

2. Methodology

2.1 Collection of Sample

Tender pepper, *Piper nigrum* was collected from Idukki district, Kerala.

2.2 Isolation of Endophytic Fungi

2.2.1 Surface Sterilization

The general procedures adopted for isolation of the fungal endophytes were followed as described in Suryanarayanan *et al*., 2010. The tender pepper was cleaned in sterile distilled water. Aseptically, the sample was fragmented with scalpel and surface sterilized by 2 methods: 15 s in 70% ethanol and 5 s in 1% sodium hypochlorite (NaOCl).

2.2.2 Isolation of fungal strains

After each surface sterilization, fragments of samples were rinsed in sterile distilled water and inoculated in potato - dextrose - agar (PDA) medium, supplemented with chloramphenicol (200 mgL−1). The plates were incubated at 28 °C, and periodically observed.

2.2.3 Maintenance of isolated fungal cultures

The isolated endophytic fungal strains were sub cultured in PDA medium to achieve purity and pure mother cultures were flooded with 20% glycerol and stored in - 20° C.

2.3 Extraction of crude endophytic fungal extracts

The endophytic fungi isolated from tender pepper were inoculated into Potato Dextrose broth separately and were incubated for 15 days at 28°C (Zhang *et al*., 2009). The cultures were centrifuged at 5000 rpm at 4°C to separate the mycelia and the culture filtrate. The culture filtrate was extracted with ethyl acetate (1: 1 v/v), evaporated to remove the organic solvent and 1 mg of each crude endophytic fungal extract was dissolved in DMSO and stored in 4 °C for further study.

2.4 Qualitative Analysis of Phytochemicals in the Endophytic Fungal Crude Extract:

A series of qualitative tests was carried out using the method described by Ashwani *et al (*2015)

2.4.1 Ninhydrin test for amino acids:
2ml of the plant extract was taken in a test tube and few drops of 0.2% Ninhydrin reagent was added and heated for 5 minutes. Formation of blue colour indicates the presence of amino acid.

2.4.2 Test for flavonoids:
1ml of the plant extract was taken in a test tube, 1ml of 10% lead acetate was added and shaken for 30 secs and allowed to stand. Formation of yellow precipitate represents a positive result for flavonoids.

2.4.3 Test for alkaloids:
2ml of plant extract was added with 2ml of concentrated hydrochloric acid. Then few drops of Mayer reagent were added. The Presence of green colour or white precipitate indicates the presences of alkaloids.

2.4.4 Test for coumarins:
1ml of 10% Sodium hydroxide was added to 1ml of the plant extract and observed for the formation of yellow colour.

2.4.5 Test for quinones:
1ml of the extract was added with 1ml of concentrated sulphuric acid and observed for the formation of red colour.

2.4.6 Test for saponins:
2ml of plant extract was added with 2ml of distilled water and shaken in a graduated cylinder for 15 mins and observed for the formation of foam.

2.4.7 Test for terpenoids:
0.5ml of the plant extract was treated with 2ml of Chloroform and conc. Sulphuric acid and observed for the formation of red brown colour at the interface.

2.4.8 Test for cardiac glycosides:
To 2ml of plant extract, 2ml of acetic acid, 1ml 1% ferric chloride and 1ml sulphuric acid were added and observed for the formation of brown ring.

2.4.9 Test for anthrocyanin:
To 1ml of extract, 1ml of 2N NaOH was added and heated at 100°C for 15 mins and observed for the formation of bluish green colour.

2.4.10 Test for Phenol:
2 ml of the extract, 2 ml of distilled water and 10% of FeCl3 were added in the test tube and mixed well and observed for the formation of black colour.

2.5 Antioxidant Test by DPPH Assay

The effect of endophytic fungi crude extract and fraction were determined by the method of Yen and Chen (1995). Briefly, 2ml of 6×10^{-5}M methanolic solution of DPPH (2, 2 Diphenyl, 1 - Picryl hydrazyl hydrate) were added to 50µl of crude fungal extract (F1, F2 and F3) sample. The mixture was vortexed for 1 minute and kept at room temperature for 30 minutes in the dark. The absorbance of all sample solution was measured at 517nm. The scavenging effect (%) was calculated by using the formulae given by Duan *et al., (*2009).

% Antioxidant activity = (Absorbance at blank) - (Absorbance at test) x100 (Absorbance at blank)

2.6 Antibacterial activity Assay:

2.6.1 Test Bacteria: *Escherichia coli ESBL, Staphylococcus aureus ATTC, Pseudomonas aeruginosa, Methicillin resistant Staphylococcus aureus (MRSA)* were obtained from Ramachandran medical college, (Chennai). Bacterial strain was grown and maintained on Muller Hinton Agar medium.

2.6.2 Preparation of Inoculum
The inoculum was prepared for each bacterium by using the direct colony suspension procedure (CLSI). The inoculum density was standardized using a barium sulphate turbidity standard, equivalent to 0.5 Mcfarland standards. The absorbance was measured using a spectrophotometer at 625 nm should be 0.08 - 0.13 for the 0.5 Mcfarland standard.

2.6.3 Antibacterial activity
The endophytic fungal extract was screened for their antibacterial activity by Agar well diffusion method. Mueller - hinton agar was prepared, autoclaved and the cooled medium was added to sterile petri dishes to give a uniform depth of 4mm and allowed to set. Optimally within 15 min after adjusting the turbidity of the inoculum suspension, a sterile cotton swab was dipped into the adjusted suspension and pressed firmly on the inside wall of tube to remove excess inoculum form the swab. The swab was streaked over the entire sterile agar surface.1 mg/ml of endophytic fungal extract was prepared for each solvent as the stock solution. Then a hole with a diameter of 6 to 8 mm was punched aseptically with a sterile cork borer. Five wells were made and a volume of 100 µl of different fungal extracts were added into the well. Gentamycin (1mg/ml) was used as positive control. The extracts diffuse in the agar medium and inhibits the growth of the microbial strain tested. The plates were incubated at 37˚C for 16 - 18 hrs and the diameter of the zone of inhibition was measured around the well.

2.7 Identification of Fungi

The endophytic fungal isolates were identified based on microscopic appearance by Lactophenol Cotton blue staining and colony appearance on Potatao Dextrose agar medium.

3. Results

Figure 1: *Piper nigrum (*Green pepper seeds)

Figure 2: Front and back view of endophytic fungal isolates from green pepper.

Figure 3: Endophytic fungal isolates - F1 (Front view)

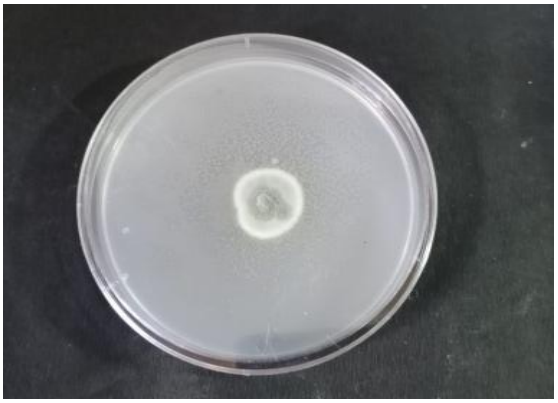

Figure 4: Endophytic fungal isolate - F2 (Front view)

Figure 5: Endophytic fungal isolate - F3 (Front view)

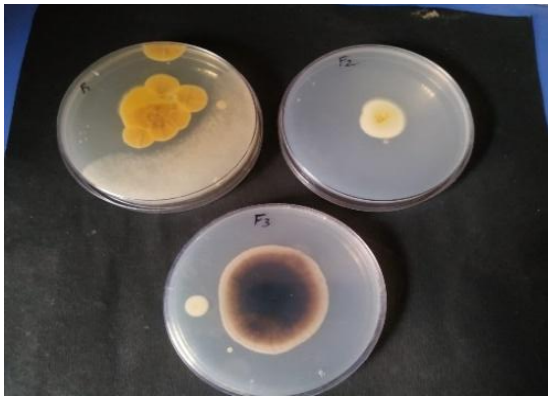

Figure 6: Endophytic fungal isolates – F1, F2 and F3 (back view)

Figure 7: Cultivation of endophytic fungal isolates - F1, F2 and F3 in Potato Dextrose Broth (PDB).

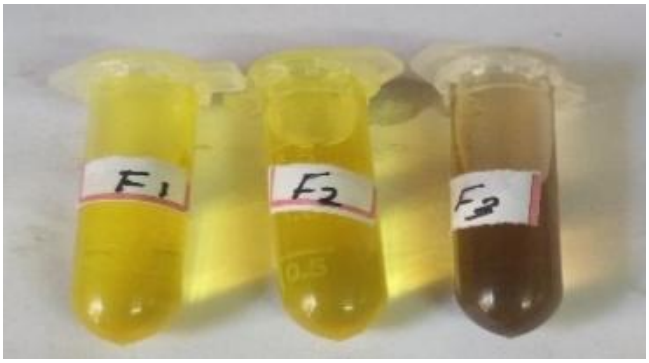

Figure 8: Endophytic fungal extracts of F1, F2 and F3 dissolved in DMSO

Table 1: Screening of phytochemicals of endophytic fungal extracts of F1, F2 and F3

S. No	Phytochemicals	F1	F2	F3
1.	Alkaloids	_	_	_
2.	Flavonoid	+	+	+
3.	Caumanis	+	+	+
4.	Terpenoids	_	+	_
5.	Quinones	+	+	+
6.	Amino acid	_	_	_
7.	Anthra Cynin	_	_	_
8.	Saponin	_	_	_
9.	Cardiac glyceroids	+	+	_
10.	Phenol	+	+	+

Table 2: Antioxidant activity of endophytic fungal extracts – F1, F2 and F3

S. No	Endophytic fungal extracts	OD Value	DPPH Activity (%)
1	F1	0.126	86.66%
2	F2	0.553	41.48%
3	F3	0.650	31.21%

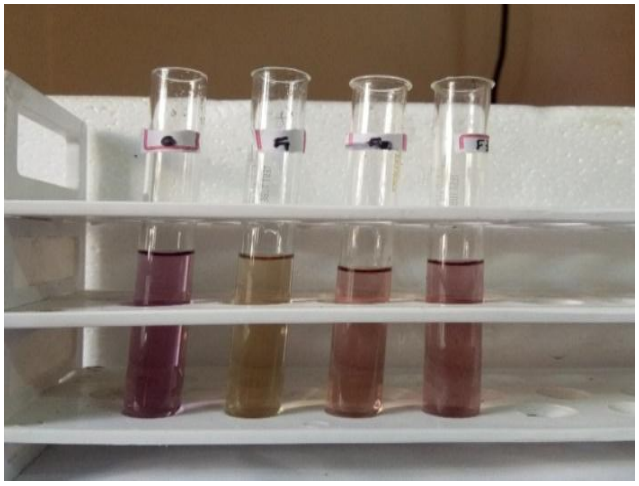

Figure 9: Antioxidant activity of endophytic fungal extracts - F1, F2 and F3 by DPPH assay

Figure 10: Antibacterial activity of Endophytic Fungal Extracts of F1, F2 and F3 against *Staphylococcus aureus ATCC 25923*

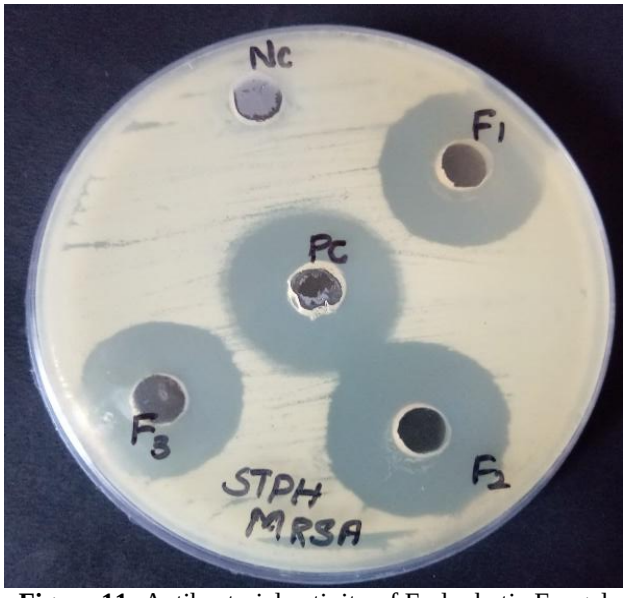

Figure 11: Antibacterial activity of Endophytic Fungal Extracts of F1, F2 and F3 against *Methicillin resistant Staphylococcus aureus* (MRSA)

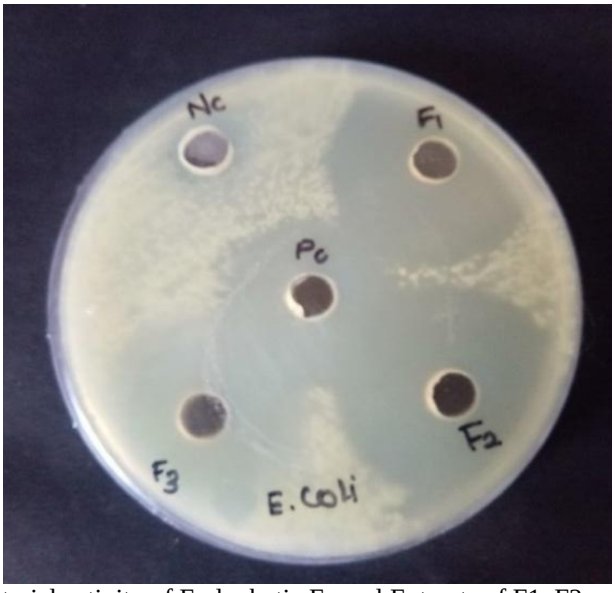

Figure 12: Antibacterial activity of Endophytic Fungal Extracts of F1, F2 and F3 against *E. coli*

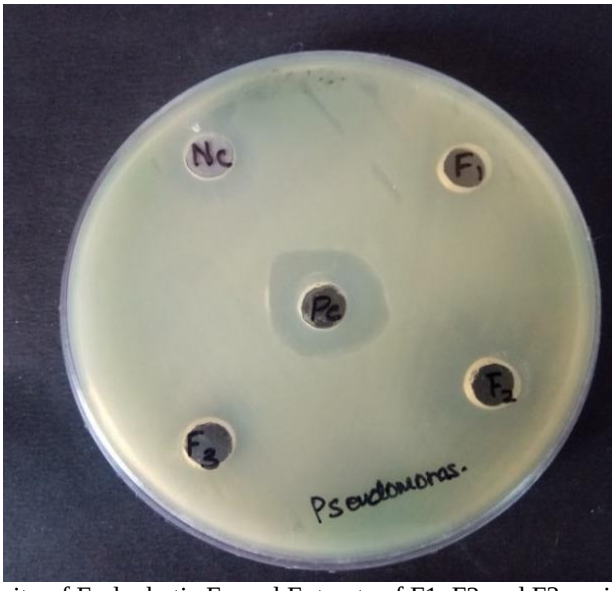

Figure 13: Antibacterial activity of Endophytic Fungal Extracts of F1, F2 and F3 against *Pseudomonas aeruginosa*

Table 3: Antibacterial activity of endophytic fungal crude extracts of F1, F2 and F3

S. No.	Test Organism	Zone of inhibition (mm) for 100 µl from 1mg/ml stock			Zone of inhibition (mm) for Positive Control (Gentamicin)
		F1	F2	F3	
1.	*MRSA*	26	27	25	26
2.	*Staphylococcus aureus ATCC 25923*	29	32	29	20
3.	*Escherichia coli*	37	36	35	25
4.	*Pseudomonas aeruginosa*	-	15	-	20

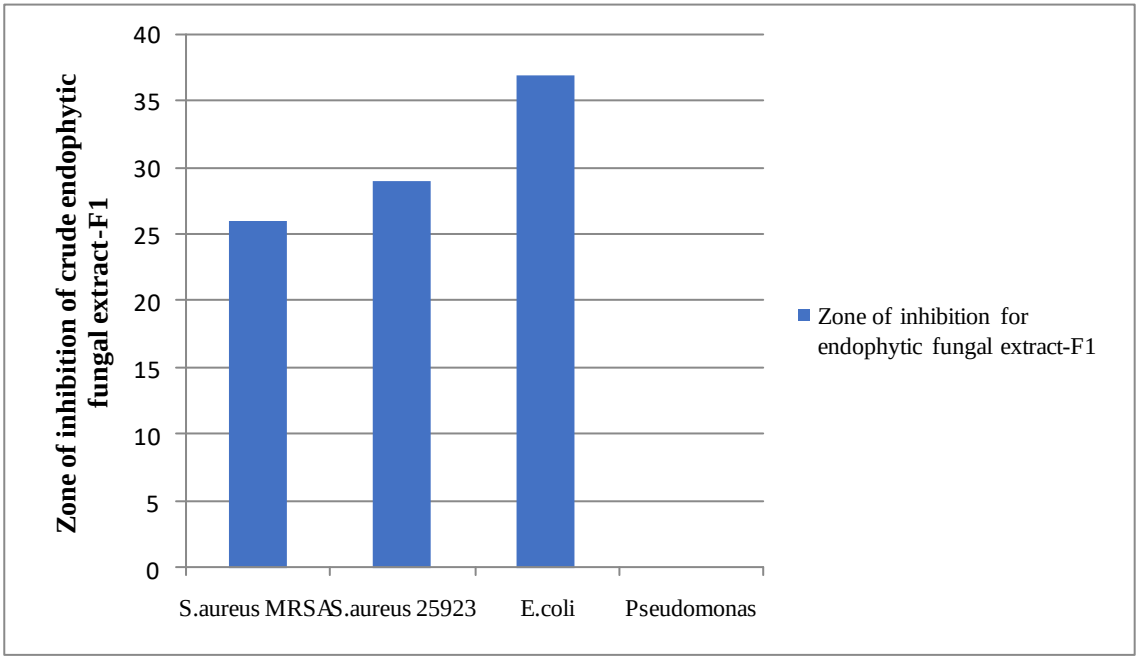

Chart 1: Antibacterial activity for endophytic fungal extract - F1

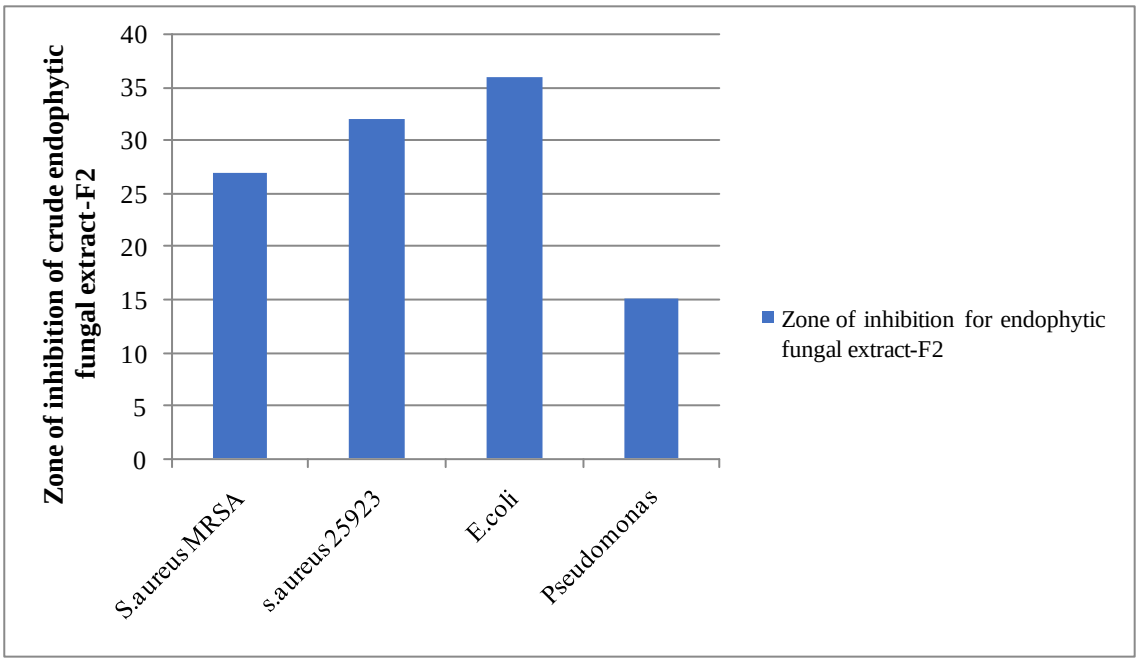

Chart 2: Antibacterial activity for endophytic fungal extraxt - F2

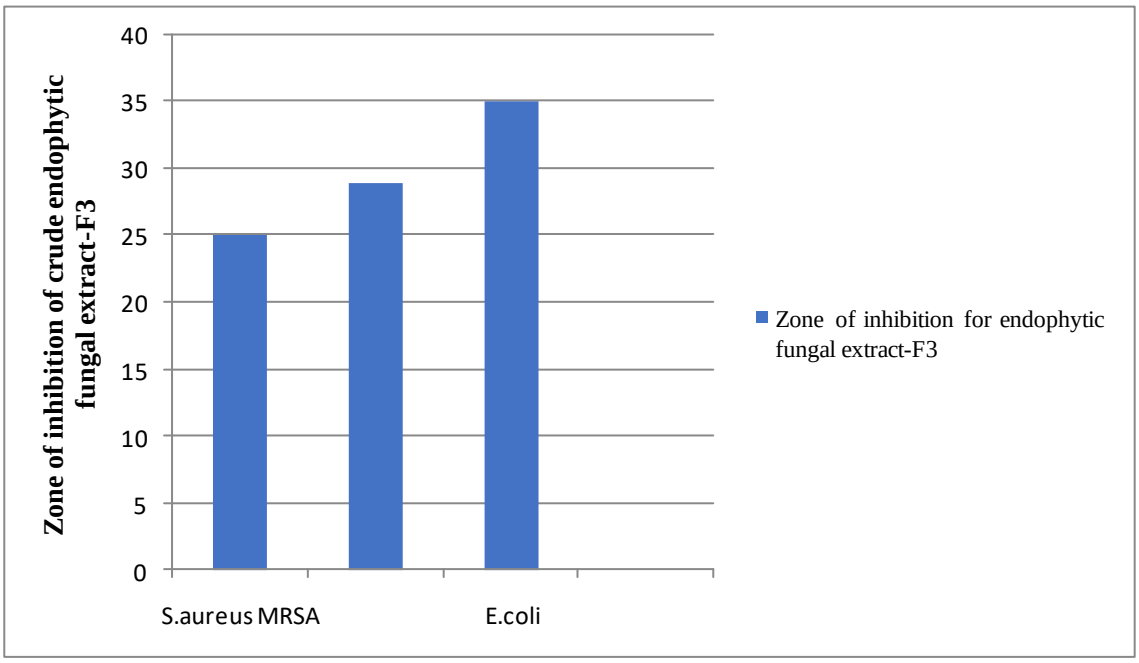

Chart 3: Antibacterial activity for endophytic fungal extract - F3

Identification of Endophytic Fungi:

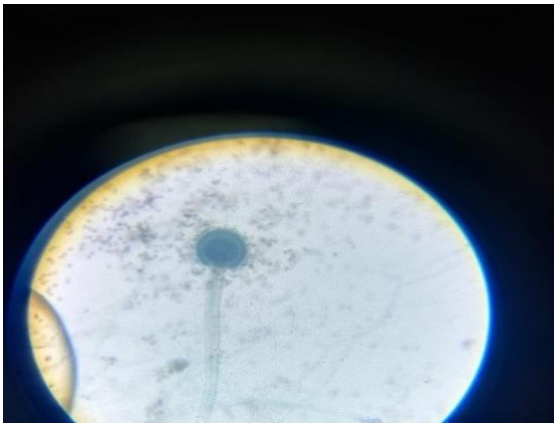

Figure 14: *Aspergillus species*

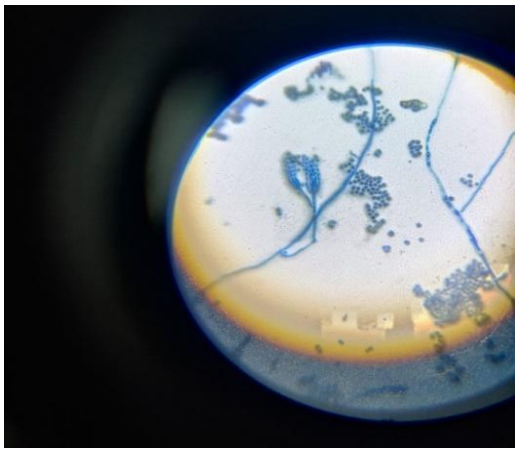

Figure 15: *Penicillium species*

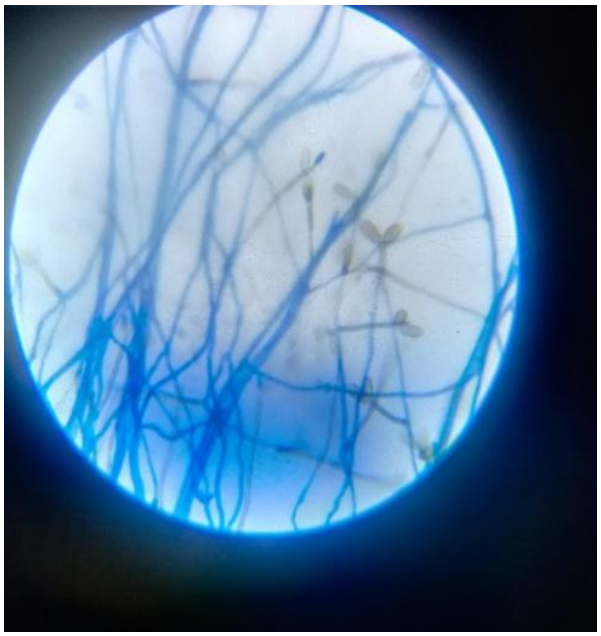

Figure 16: *Curvularia species*

4. Discussion

The three fungal isolates F1, F2 and F3 were identified based on Lactophenol Cotton blue staining and colony morphology on PDA.
F1 - *Aspergillus*
F2 - Penicillium
F3 - Curvularia species.

The endophytic fungi were isolated from green pepper seeds and totally three fungi were isolated and the three fungal isolates were named as F1, F2 and F3 (Fig 2, 3, 4, 5 &6). The three endophytic fungi were grown in Potato Dextrose broth medium separately (Fig 7). The ethylacetate extracts of the endophytic fungi isolated from green pepper seeds were used for analysing the presence of phytochemicals and determination of Antioxidant activity by DPPH Assay.

The Phytochemical screening revealed the presence of phenols and flavonoids in the three endophytic fungal extracts (Table 1). Flavonoids are the most known for their antioxidant activity and act as transformers which modify the body's reactions to carcinogens, viruses, and allergens (Balch and Balch, 2000). The presence of phytochemicals in endophytes is an indicator that they can be potential source of precursors in the development of synthetic drugs (Castillo et al., 2007).

The stable compound DPPH gets reduced by gaining a hydrogen or electron and the violet color of the DPPH radical was reduced to yellow color. The percentage scavenging of DPPH radical for the three endophytic fungal extracts of F1, F2 and F3 were determined to be 86.6%, 41.48% and 31.21% (Fig 9 & Table 2). The change in color indicates that the endophytic fungal extracts were found to contain an antioxidant.

In the present study, Ethyl acetate extracts of Endophytic fungi – F1, F2 and F3 were tested for Anti - bacterial activity by Agar well diffusion method against *MRSA, Staphylococcus aureus ATCC 25923, E. coli and Pseudomonas aeruginosa*.1 mg/ml concentration acts as stock. Gentamicin was used as a positive control. The endophytic fungal extracts of F1 inhibited *MRSA, Staphylococcus aureus ATCC 25923, E. coli* with a zone of inhibition 26 mm, 29 mm, and 37 mm for 100 µl of fungal extracts**,** but does not inhibited *Pseudomonas aeruginosa (*Chart 1*)*
The endophytic fungal extracts of F2 inhibited all the four tested bacteria with a zone of inhibition 27 mm, 32 mm, 36 mm, and 15 mm (Table 3 & Chart 2) for *MRSA, Staphylococcus aureus ATCC 25923, E. coli and Pseudomonas aeruginosa*.

The endophytic fungal extracts of F3 inhibited *MRSA, Staphylococcus aureus ATCC 25923, E. coli* with a zone of inhibition 25 mm, 29 mm, and 35 mm (Table 3 & Chart 3) for 100 µl of fungal extracts**,** but does not inhibited *Pseudomonas aeruginosa. The endophytic fungal extracts of F1 and F2 showed good antibacterial activity than the fungal extracts of F3. The F2 fungal extract has inhibited all the tested bacteria.*

The endophytic fungal isolates – F1, F2 and F3 were identified as *Aspergillus species, Penicillium species and Curvularia species (*Fig 14, Fig 15 & Fig 16) based on Lactophenol cotton blue staining and colony morphology on Potato Dextrose Agar medium. John *et al.,* 2012 reported the presence of endophytic fungi, Penicillium species from the pepper fruit and it has shown good antibacterial activity. Prabukumar et al., 2015 reported that the endophytic fungi such as *Aspergillus species, Fusarium species, Trichoderma species* possessed excellent antibacterial activity against Gram positive and Gram - negative bacteria. From these two studies, the endophytic fungi - *Aspergillus species* and *Penicillium* species exhibited good antibacterial activity which correlate with my study.

Endophytic fungal species are considered as exciting novel sources for obtaining new bioactive compounds and have been reported from several hosts (Castillo et al., 2007). Alfi et al 2021 stated that the secondary metabolites from endophytic fungi can play a role in plant defense and many have pharmaceutical potential.

5. Conclusion

In this study, Endophytic fungi have been successfully isolated from the green pepper corn. The endophytic fungi were identified as Aspergillus species, Penicillium species and Curvularia species. The ethyl acetate extracts of Aspergillus species showed prominent antioxidant activity than Penicillium species and Curvularia species. The extracts of Penicillium inhibited all the tested bacteria and had shown good antibacterial activity followed by Aspergillus species and Curvularia species. The Endophytic fungi, Aspergillus species and Penicillium species isolated from green pepper corn act as a potential source of antibacterial compound and further deep investigation should be needed to exploit their potentiality to develop as a chemotherapeutic agent.

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