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Evaluation of antibacterial activity of the endophytic fungi isolated from spice plants

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Abstract
Endophytic fungi are defined as fungi that grow in healthy plants tissues without causing any signs of disease or damage to the host plants. In the present study we isolated endophytic fungi from three different spice plants such as Clove (*Syzygium aromaticum*), Cinnamon (*Cinnamomum verum*) and Pepper (*Piper nigrum*) and cultivated in potato dextrose broth. The extracellular metabolites were extracted using ethyl acetate solvent. Further we evaluated the antimicrobial activity of the ethyl acetate extract using Kirby-Bauer disc diffusion technique. In total 18 endophytic fungi were isolated from the spice plants and identified morphologically as *Fusarium* spp. and *Colletotrichum* spp. The thin layer chromatography of the ethyl acetate extract showed 3 prominent bands having a Rf value of 0.31, 0.43 and 0.52, respectively. In conclusion, the *Fusarium* sp. isolated from clove plant showed significant antibacterial activity against *Klebsiella* sp. and *Citrobacter* sp.

Keywords: endophytic fungi, spice plant, crude extract, antimicrobial assay, MIC, TLC

Introduction

Endophytes are defined as the "microbes that colonize living, internal tissues of plants without causing any immediate, overt negative effects". The term endophyte was originally defined by De Bary in 1866 as "any organism occurring within plant tissues". The word endophyte means in the plant (derived from the Greek- endo = within, phyton = plant) this term can be used for a wide spectrum of potential hosts inhabitants, e.g. bacteria, fungi, etc, (Stone *et al*., 2000) [11].

Endophytic fungi are defined as fungi that grow in healthy plants tissues without causing any signs of disease or damage to the host plants (Bacon and White *et al*., 2000: Porras-Alfora and Bayman *et al*., 2011) [2]. Endophytes are ubiquitous and have been found in all the species of plants and their tissues such as stem, leaves, roots and petioles etc. (Jasim *et al*., 2013) [6]. Endophytes may benefit host plants by preventing pathogenic organisms from colonizing them. The relationship between host plant and endophytes are usually symbiotic, in which the endophytic get nutrients from plants (Verma *et al*., 2011) [12].

A spice is a seed, fruit, root, bark or plant substance primarily used for flavouring, colouring or preserving food. For example, cloves are flower buds, cinnamon is bark, ginger is a root, pepper crones are berries, nigella is a seed, cumin is a fruit, saffron is a stigma, and cardamom is a pods and seeds.

The leaves, flowers or stems of spice plants have the antimicrobial properties and antioxidant activity. The bioactive compounds of spice plants are products of the plant itself or the endophytes living inside the plants. The isolation of endophytic fungi from spice plants to produce bioactive compounds is an efficient method in search for new active compounds. In the last two decades, novel bioactive compounds from endophytic fungi have been found and have potential as antimicrobial, insecticidal, cytotoxic and anticancer (Aly *et al*., 2010) [1].

Materials and Methods

Collection of Plant Samples

The leaf, stem and root samples of *Syzgium aromaticum* (Clove), *Piper nigrum* (Pepper) and *Cinnamomum verum* (Cinnamon) were collected from the Lalbagh botanical garden Bangalore, Karnataka, India.

Collection of Bacterial Isolates

The bacterial isolates (*Bacillus sp, Klebsiella sp, Pseudomonas sp and Citrobacter sp*) were collected from the Garden city University Bangalore, Karnataka, India.

Isolation of Endophytic Fungi

Initially the plant samples were washed with the running tap water for 10 minutes to remove the dirt, and then dried at room temperature using sterile filter paper. Each piece of the plant organ was cut into 2cm long segments for roots, stems and 2cm square pieces for leaves. Surface sterilization of each sample was performed by soaking the samples in 4% of sodium hypochlorite for 3-4 min, followed by 70% ethanol wash for 1 min and then rinsed three times using sterile distillated water. All the samples were dried on sterile filter paper in a safety cabinet. The rinsed water was streaked on the PDA medium and sterilized segments were imprinted on PDA medium to ensure the surface sterilization. The surface sterilized segments were cut into 1 cm and placed in the petri dishes containing PDA medium supplemented with chloramphenicol 100 mg/ml. Then the petri dishes were kept for incubation at room temperature and monitored every day to check the growth of fungal colonies from the sample segments. The fungi that grew from the tissue fragments were sub cultured onto fresh PDA plates, isolates that failed to produce reproductive structures were sub cultured onto Malt extract agar or Yeast extract agar. Cultures that failed to sporulate even under these conditions were considered sterile mycelia (Petrini 1991; Guo *et al*., 2000; Photita *et al*., 2001) [9].

Identification of Endophytic Fungal Isolates

Identification was conducted by observing the morphological traits and characters of fungal colonies grown on PDA at room temperature, both macroscopically and microscopically. Macroscopic characters observed were colour and surface colonies (granular, mounting, slippery), texture, zonation, growth area, the lines of radial and concentric, reverse colour and exudate drops. Microscopic identification was done by the microscopic slides of each fungal endophytes were prepared; the slides were prepared by tease mount method using lactophenol cotton blue attaining and observed under microscope (Barnett and Hunter *et al*.,1998).

Fermentation and Extraction of Fungal Metabolites

The endophytic fungi were inoculated into 250ml of PDB medium in 500ml Erlenmeyer flasks. The inoculated flasks were incubated at 28-30˚C under both the shaking condition at 150 rpm and under static condition for 21 Days. The fungal biomass was removed from the conical flasks by filtration. The filtration was done by using the sterile muslin cloth using Whatman filter paper number 1. Then the filtrate was extracted and the equal volume of the ethyl acetate was added (1:1) in the separating funnel, shaken well for 15-20 min then allowed to settle down and collect the upper layer which contains the ethyl acetate dissolved metabolites and dried using rotary vacuum evaporator then the crude extract was obtained (Verma *et al*.,2011) [12].

Antibacterial Activity

Kirby Bauer Method

The agar well diffusion and disc diffusion method were employed for preliminary screening for endophytic fungal extracts against selected Bacteria. *Bacillus. sp, Klebsiella. sp, Pseudomonas aeruginosa and Citrobacter. Sp.* were grown in 10ml of Nutrient broth (NB) medium incubated for 24h at 37℃. The bacterium swabbed on the Muller Hinton Agar plate and the disc is made by using sterile Whatman filter paper number 1, and the disc is dipped into 10ul of extract and placed on the agar medium, similarly corkborer is used to make the well and the well is sealed by 10ul of MHA and 40ul of extract is added to the well and incubate the plates at 37℃ for 24h and the zone of inhibition is a circular area around the well and the disc in which bacteria colonies were absent. Place the metric ruler across the zone of inhibition to measure. (Deepthi *et al*., 2018) [3].

Determination of Minimm Inhibitory Concentration (MIC)

The crude ethyl acetate extract was determined by a broth microdilution method against the bacteria. Prepare Muller Hinton broth and dispense 140ul to each well and add 50ul of fungi extract and two-fold serial dilution was performed. Create the inoculum by taking a few colonies from an agar plate with sterile swab, preparing a 0.5 McFarland turbidity standard [0.05ml of 1.175% of Barium chloride (BaCl2.2H2O) in 9.95ml of 1%Sulphuric acid (H2SO4)] the standard can be compared visually to a suspension of bacteria in sterile saline/nutrient broth. Then dispense the 10ul of inoculum into microdilution plate. Then incubated the microdilution plate at 37℃ for 24h. ELISA READER is used to determine the MIC value.

Analysis of the Chemical Compounds of Endophytic Fungal Extract

The chemical compounds of endophytic fungi (*Fusarium*. sp) extracts were analysed by Thin Layer Chromatography (TLC) and eluted with a mobile phase Ethyl acetate: Chloroform: Ethanol (7:2:1). Chromatogram was observed under UV Light at wavelength of 254nm and 366nm. Place the chromatogram in the Iodine chamber and calculate the RF value.

$$RF = \frac{\text{Distance travelled by sample.}}{\text{Distance travelled by solvent}}$$ (Praptiwi et al., 2015).

Results and Discussion

Total 18 endophytic fungi were isolated from various parts (leaf, stem, root) of spice plants *viz.* Clove (*Syzygium aromaticum L.*), Pepper (*Piper nigrum L.*), Cinnamon (*Cinnamomum verum L.*) (Figure 1). Based on morphological characters of endophytic fungi, they were identified as *Colletotrichum.sp* and *Fusarium.sp*, isolated from Clove plant, *Colletotrichum.sp* and *Sterile mycelia* isolated from Pepper plant and *Fusarium Sp*. was isolated from Cinnamon plant (Figure 2). Fungal identification was done based on the morphological characteristics but most of the endophytic fungi did not sporulate on the PDA hence it is classified under *sterile mycelia* (Table 1).

Further the cultivation (figure 3) and extraction of metabolites from the endophytic fungi was carried out (figure 4). The result showed 1.36g of crude extract was obtained from *Colletotrichum* sp., 0.87g of crude extract was obtained from *sterile mycelia* and 0.67g of crude sample was obtained from *Fusarium.sp*. (Figure 5) (Table 2).

Preliminary antimicrobial assay (disc diffusion method and agar well diffusion method) was performed using the crude extract against the bacteria. The crude extract 1is *sterile mycelia*, 2 is *Colletotrichum sp* and 3 is *Fusarium sp* and C is control. Only *Fusarium sp* extract showed the zone of inhibition against the bacteria and the result was interpreted *Bacillus sp (3.cm and 3cm), Pseudomonas sp (2.5cm and 3cm), Klebsiella sp (3cm and 3.5cm) and Citrobacter sp (1cm and 1.2cm)*. (Figure 6) (Table 3).

MIC was determined by microbroth dilution method using the *Fusarium sp* metabolite extract and result was recorded using ELISA READER. The MIC of Bacillus sp (3.47mg/ml), Klebsiella sp (6.95mg/ml), Pseudomonas sp (13.9mg/ml) and Citrobacter sp (1.73mg/ml). (Figure 7) (Table 4).

Thin layer chromatography (TLC) is a method to identify the compounds and purity of a compound from the *Fusarium sp* extract. And the Rf value obtained 0.39, 0.59, 0.70 and 0.79. (Figure 8)

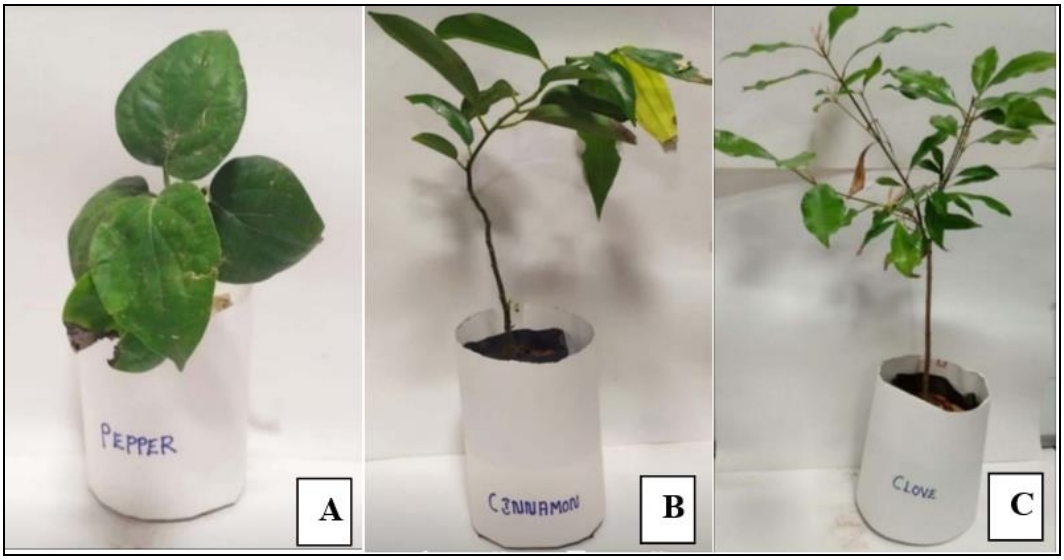

Fig 1: Spice plants: A) *Piper nigrum, B) Cinnamomum verum and C) Syzygium aromaticum*

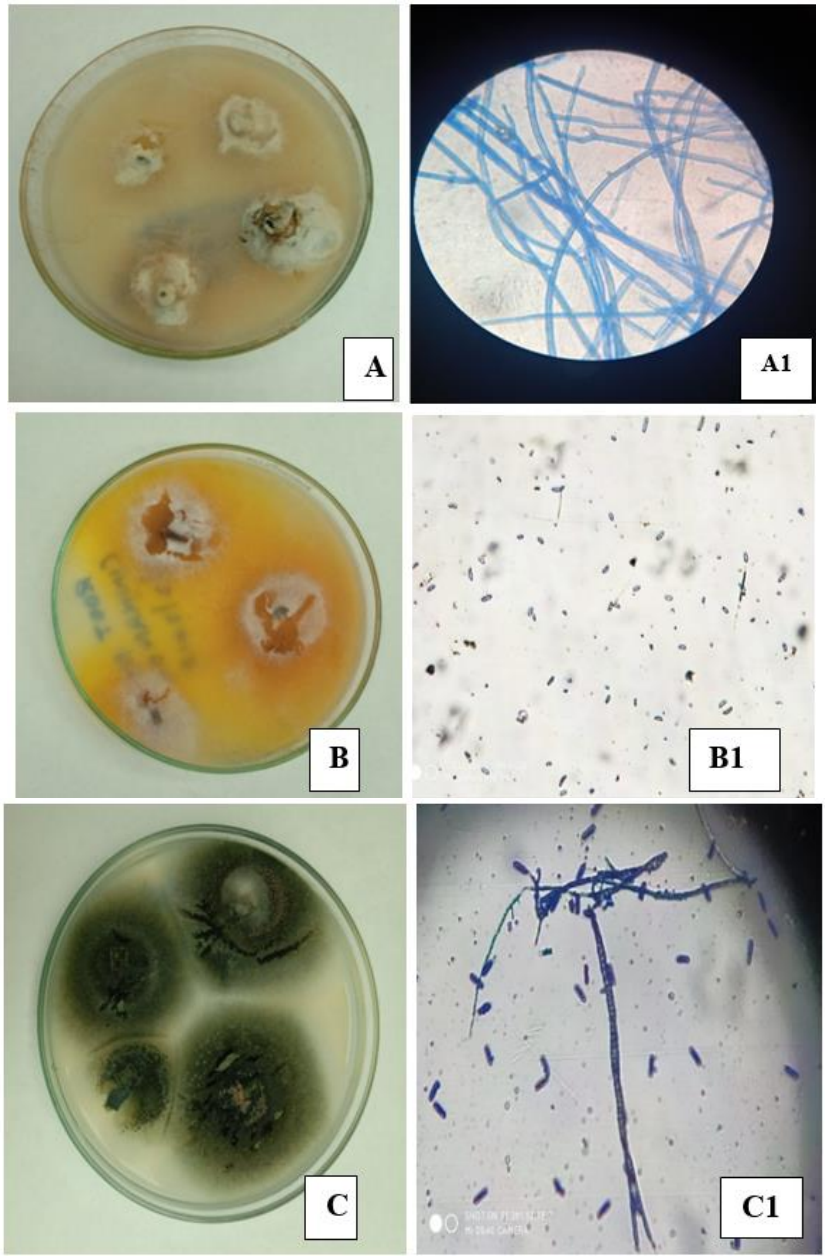

Fig 2: Macroscopic and Microscopic view of endophytic fungal, (A) and (A1) Sterile mycelia, (B) and (B1) *Fusarium* sp. and (C) and (C1) *Colletotrichum*sp.

Table 1: Macroscopic and microscopic identification

Sl. No	Name of the plant	Botanical name	Parts used	No of isolates	Texture	Obverse Colour	Reverse colour	Name of the species
1	Clove Plant	*Syzygium aromaticum*	Leaf	1	Cottony	White	Orange	*Sterile mycelia*
				2	Hairy cottony	Grey-green	Black	*Colletotrichum* sp.
				3	Cottony	White	Orange	*Sterile mycelia*
			Stem	1	Cottony	White	Orange	*Fusarium* sp.
			Root	1	Cottony	White	Orange	*Fusarium* sp.
2	Pepper Plant	*Piper nigrum*	*Leaf*	*1*	*Hairy cottony*	*Grey- green*	*Black*	*Colletotrichum sp.*
			Stem	*1*	*Cottony*	*Cream*	*Black Orange*	*Sterile mycelia*
			Root	*2*	*Cottony*	*Grey*	*Brown*	*Sterile mycelia*
				1	*Powdery*	*Cream*		*Sterile mycelia*
3	Cinnamon plant	*Cinnamomum verum*	Leaf	1	Hairy cottony	Grey	Cream	*Sterile mycelia*
			Stem	2	Cottony	White	White	*Sterile mycelia*
			Root	1	Cottony	White	White	*Sterile mycelia*
				1	Cottony	White	Yellow-Orange	*Fusarium* sp.

Fig 3: Cultivation of endophytic fungi: (a) Static condition and (b) Shaking condition

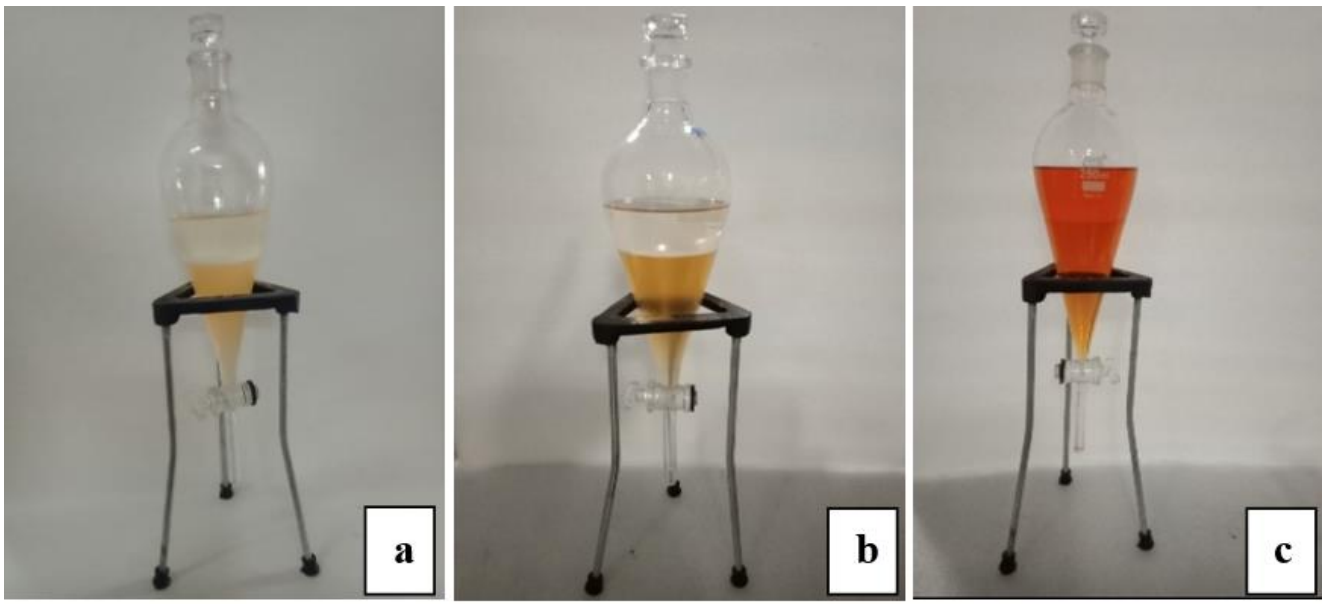

Fig 4: Extraction of metabolites from endophytic fungi: (a) *Sterile mycelia* from stem of pepper, (b) *Colletotrichum* sp. from leaf of Pepper, (c) *Fusarium* sp. from stem of clove.

Fig 5: Crude extract of sterile mycelia, *Colletotrichum* sp. and *Fusarium* sp.

Table 2: crude extract of fungal metabolites.

Serial Number	Name of plants	Botanical name	Plant parts	Name of endophytic fungi	Weight of metabolite
1	Pepper	*Piper nigrum*	Leaf	*Colletotrichum sp*	1.36 g
2	Clove	*Syzygium aromaticum*	Stem	*Sterile mycelia*	0.87g
			Stem	*Fusarium sp*	0.67g

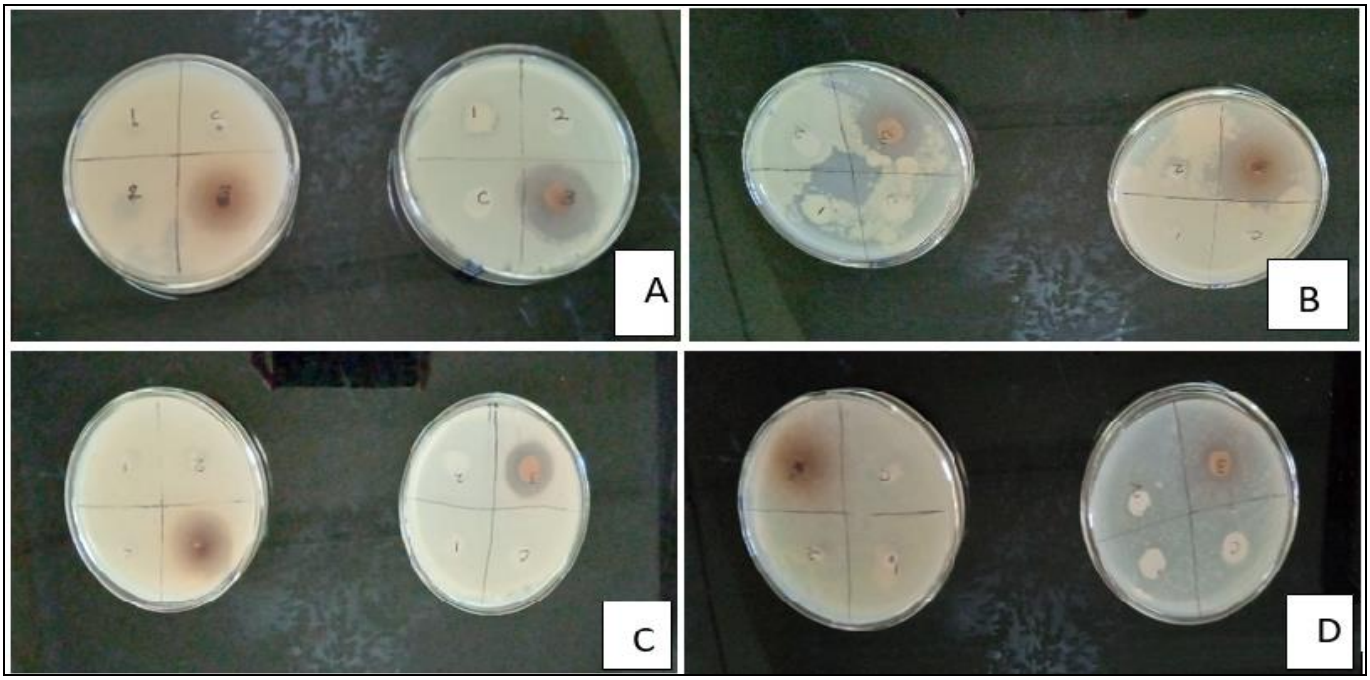

Fig 6: Preliminary antimicrobial test againts the bacteria. A (Bacilus sp), B (Klebsiella sp), C(Pseudomonas sp) and D(Citrobacter sp).

Table 3: resut for priliminary antimicrobial test

Sl. No	Organisms	Disc diffusion method	Agar well diffusion
0.1	*Bacillus sp*	3.1cm	3cm
0.2	*Klebsiella sp*	3cm	3.5cm
0.3	*Pseudomonas sp*	2.5cm	3cm
0.4	*Citrobacter sp*	1.8cm	2.2cm

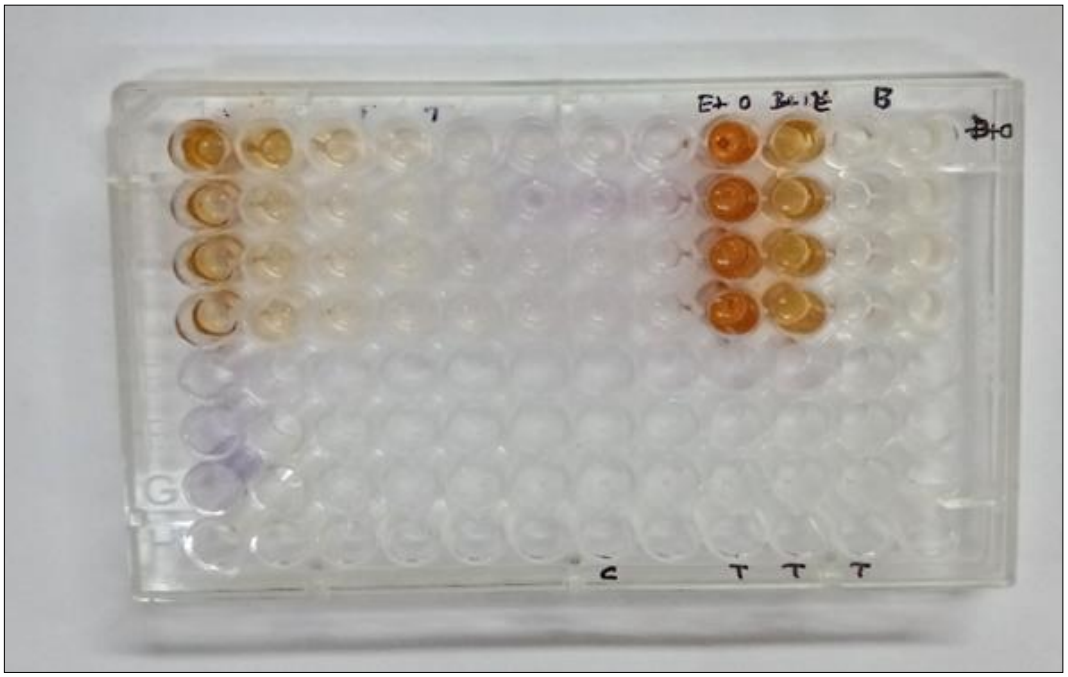

Fig 7: MIC by Microbroth Dilution Method

Table 4: Determination of Minimum inhibitory concentration (MIC)

Bacillus. Sp	0.233	0.236	0.127	0.136	0.655	0.704	0.246	0421	0.113	0.726
Klebsiella. Sp	0.218	0.238	0.118	0.672	1.285	0.128	0.145	0.149	0.133	0.894
Pseudomonas. Sp	0.218	0.182	0.46	0.711	0.64	0.74	0.424	0.258	0.099	0.46
Citrobacter sp	0.15	0.172	0.288	0.509	0.537	0.573	0.572	0.475	0.123	0.592

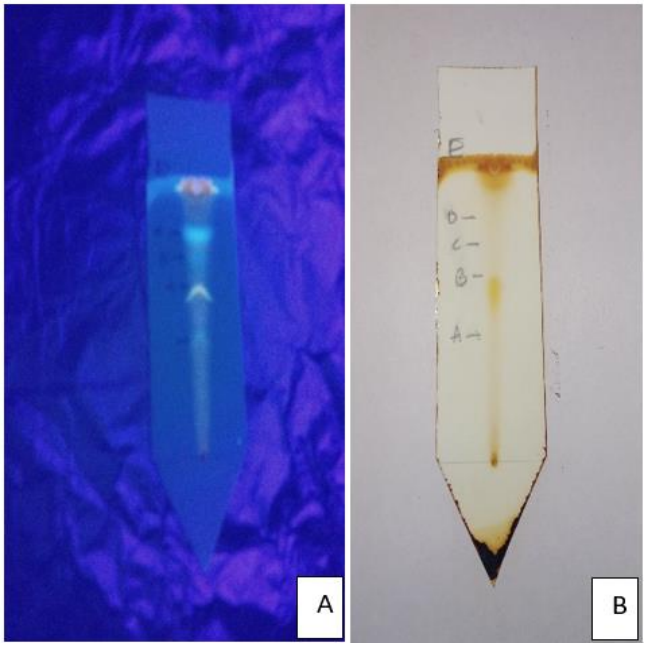

Fig 8: A (Chromatogram viewed under UV light at 365nm). B (Chromatogram treated with iodine chamber). Rf values 0.39, 0.53, 0.70 and 0.79.

Conclusion

Isolation and identification of endophytic fungi by conventional agar plate method. The results of this study showed that three endophytic fungi were isolated from *Syzygium aromaticum*, *Piper nigrum* and *Cinnamomum verum* spice plants. Among them pepper (*Piper nigrum*) and clove (*Syzygium aromaticum*) produced bioactive metabolites.

The weight of metabolite was more in *Colletotrichum sp* isolated from leaf of pepper (*Piper nigrum*) (1.36 gram) compared to *sterile mycelia* isolated from stem (0.87gram). Similarly, the amount of crude metabolite in *Fusarium* species isolated from stem of *Syzygium aromaticum* was found to be 0.67grams. Preliminary antimicrobial assay (disc diffusion method and agar well diffusion method) was performed using the crude extract against the bacteria. The result was interpreted *Bacillus sp (3.cm and 3cm), Pseudomonas sp (2.5cm and 3cm), Klebsiella sp (3cm and 3.5cm) and Citrobacter sp (1cm and 1.2cm)*. MIC was determined by microbroth dilution method and result was recorded using ELISA READER. The MIC of *Bacillus sp* (3.47mg/ml), *Klebsiella sp* (6.95mg/ml), *Pseudomonas sp* (13.9mg/ml) and *Citrobacter sp* (1.73mg/ml). Thin layer chromatography (TLC) is a method to identify the compounds and purity of a compound from the extract. And the Rf value obtained 0.39, 0.59, 0.70 and 0.79. The present study concludes that the endophytic fungi obtained from Clove plant i.e., *Fusarium sp* can be used to treat the bacterial diseases caused by *Bacillus sp, Klebsiella sp, Pseudomonas sp and Citrobacter sp.*

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