CHAPTER- IV

RESULTS

RESULTS

4.1 Collection and identification of plant samples

Altogether three medicinal plant species were selected for the endophytic study. The selected plant species with their medicinal properties is presented in **Table 4.1**. Samples of the plants were collected from different sites of Assam (**Fig. 4.1**) and identified as *Houttuynia cordata* Thunb., *Eryngium foetidum* L. and *Zanthoxylum oxyphyllum* Edgew. A voucher specimen of each plant selected plant species was deposited in Gauhati University Botanical Herbarium (GUBH) with accession numbers (**Fig. 4.2**). The sampling sites and their geographical coordinates are presented in **Table 4.2**.

4.2 Isolation and identification of endophytic fungi

In the present study, a total of 214 endophytic fungal isolates belonging to different genera were recovered from healthy surface sterilized leaf tissues of the three plant species on different mycological media namely, Potato Dextrose Agar (PDA), Malt Extract Agar (MEA) and Water Agar (WA) medium. However, for the plant species, *Z. oxyphyllum*, a modified medium amended with the plant extract and agar (PEA) was used for the isolation of endophytic fungi in addition to the above mentioned media for better recovery of endophytic fungi. The mycological culture media were supplemented with Streptomycin Sulphate (50µg/ml) to suppress bacterial contamination. It was observed that the surface sterilization protocol varied for different plant species. Further, different genera of endophytic fungi were isolated from the sampling sites and the endophytes also varied along with the plant species. The isolated endophytic fungi were identified based

Table 4.1 Plant species selected for endophytic study with their medicinal properties

Plant Species	Vernacular Name	Family	Medicinal Properties
Houttuynia cordata Thunb.	Musundari, Musandari	Saururaceae	Anti-cancer, anti-viral, anti-bacterial, anti-inflammatory, anti-microbial, anti-allergic, anti-leukemic, chronic sinusitis, against dysentery (Yang and Jiang, 2009; Kumar *et al*., 2014).
Eryngium foetidum L.	Ban dhania, Maan dhania	Apiaceae	Against constipation, asthma, stomachache, antihelmenthic, infertility complications, snakebites, diarrhoea and malaria (Garcia *et al*., 1999; Paul *et al*., 2010).
Zanthoxylum oxyphyllum Edgew.	Mezenga/ Mejenga, Onger	Rutaceae	Stimulant, stringent, anti-diarrhoeaic, anti-dysenteric, blood purifier, carminative, rheumatism, arthritis, against dyspepsia and leucoderma (Medhi *et al*., 2009; Buragohain *et al*., 2011).

Table 4.2 Sampling sites of the selected plant species

Plant species	Sampling sites	Geographical coordinates
H. cordata	Howly	26°25'16.5"N 90°58'50.2"E
	Jalukbari	26°09'20.9"N 91°40'18.0"E
	Sonapur	26°06'57.5"N 91°58'44.8"E
	Tezpur	26°37'35.5"N 92°47'19.0"E
E. foetidum	Amingaon	26°11'05.0"N 91°40'09.0"E
	Sonapur	26°06'57.5"N 91°58'44.8"E
	Baihata Chariali	26°20'41.0"N 91°43'35.0"E
Z. oxyphyllum	Jonai	27°49'56.2"N 95°13'17.2"E
	Boko	25°58'38.4"N 91°14'0.95"E
	Goalpara	26°25'59.9"N 90°21'59.9"E

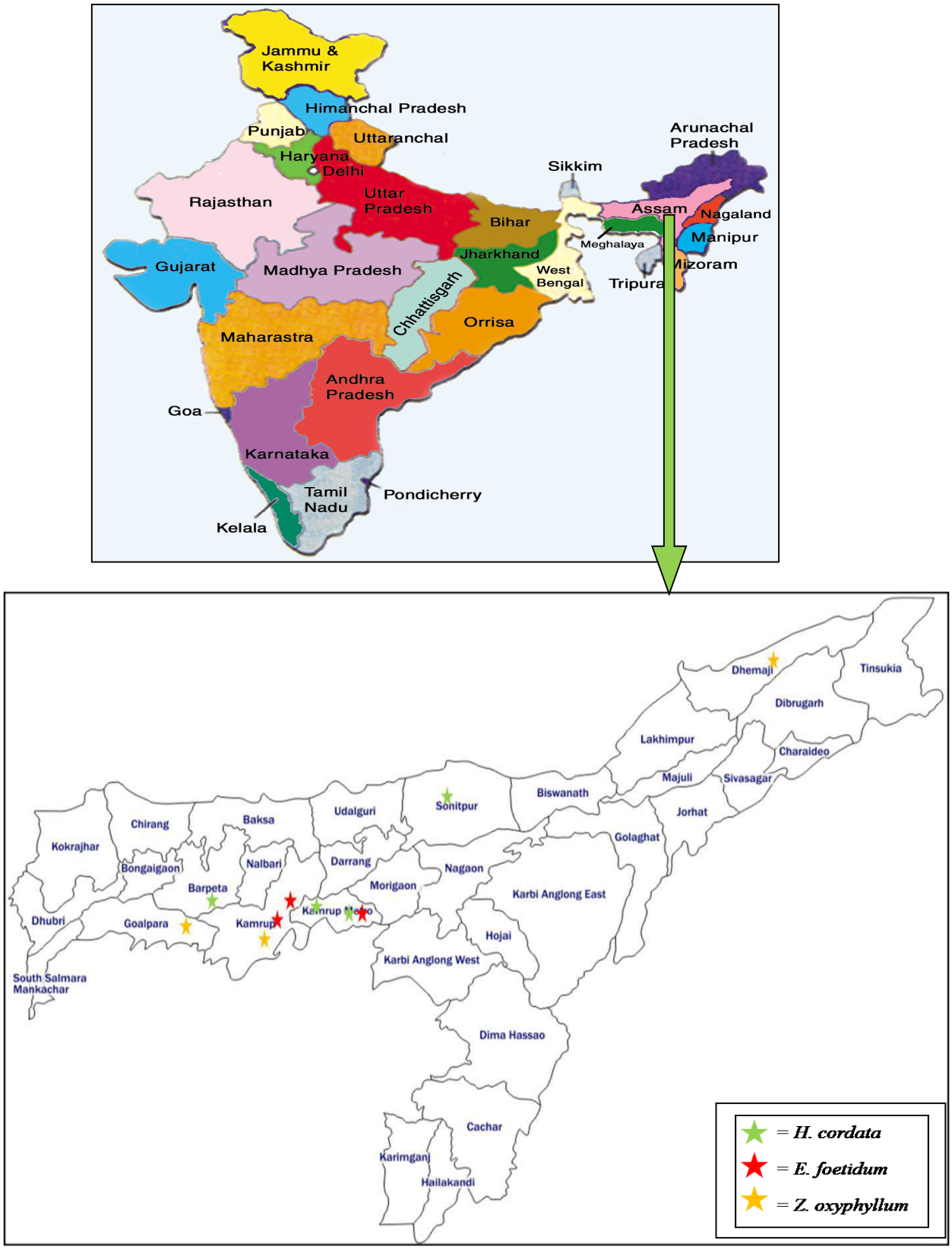
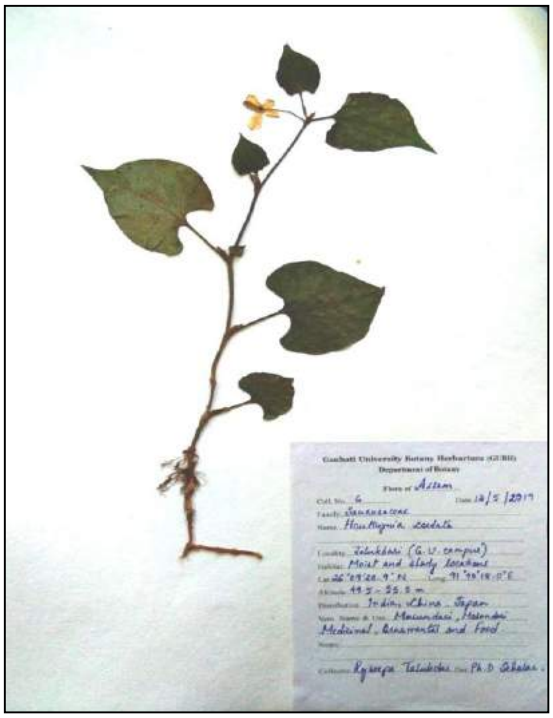
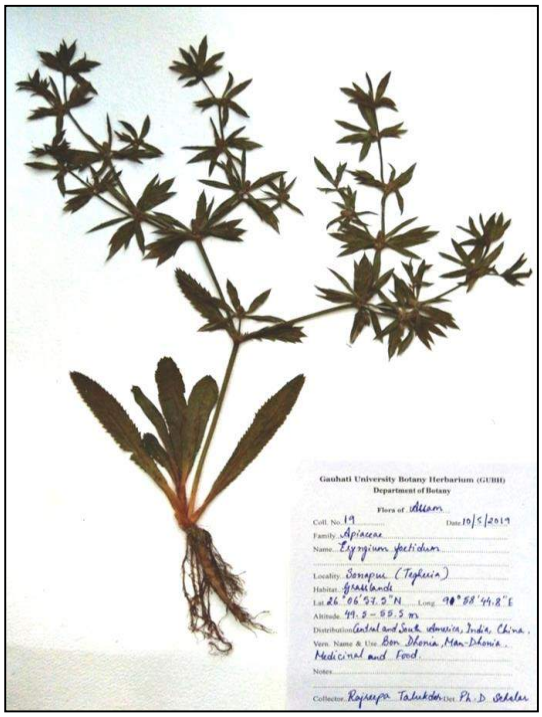


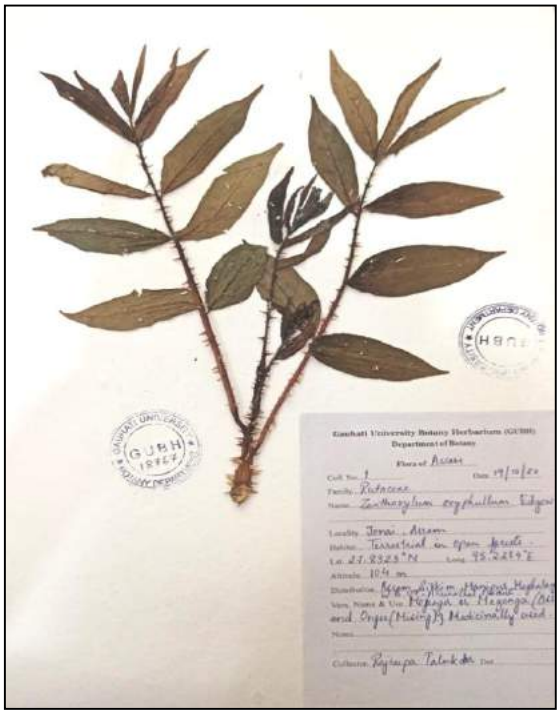
Figure 4.1 Map of Assam indicating sampling sites.



A) *H. cordata* Accession no.-18538



B) *E. foetidum* Accession no.-18539



C) *Z. oxyphyllum* Accession no.-18767

Figure 4.2 Voucher specimen of the plant species deposited at GUBH with accession numbers

Table 4.3 Recovery of endophytes from *H. cordata* leaf on different media from various sites

Sample	Media	No. of colonies recovered per location				Total no. of colonies recovered/150 segments	% of Recovery
		HW	JL	SN	TZ		
Leaf	PDA	4	8	3	7	22	14.67
	MEA	5	6	3	6	20	13.34
	WA	3	5	3	3	14	09.34
	Total isolates	12	19	9	16	56	37.34

PDA=Potato Dextrose Agar; MEA=Malt Extract Agar; WA=Water Agar; HW=Howly, JL=Jalukbari, SN=Sonapur, TZ=Tezpur

Table 4.4 Recovery of endophytes from *E. foetidum* leaf on different media from various sites

Sample	Media	No. of colonies recovered from sites			Total no. of fungal colonies recovered/150 segments	% of Recovery
		AN	BH	SN		
Leaf	PDA	7	5	17	43	28.67
	MEA	5	9	14	25	16.67
	WA	4	9	12	16	10.67
	Total isolates	16	25	43	84	56.01

PDA=Potato Dextrose Agar; MEA=Malt Extract Agar; WA=Water Agar; AN=Amingaon, BH=Baihata, SN=Sonapur

Table 4.5 Recovery of endophytes from *Z. oxyphyllum* leaf on different media from various sites

Sample	Media	No. of colonies recovered from sites			Total no. of fungal colonies recovered/150 segments	% of Recovery
		JO	BO	GO		
Leaf	PEA	4	13	15	32	21.33
	MEA	2	9	3	14	09.33
	WA	1	7	5	13	08.67
	PDA	6	7	2	15	10.00
	Total isolates	13	36	25	74	49.33

PDA= Potato Dextrose Agar; MEA=Malt Extract Agar; WA=Water Agar; PEA= Plant Extract Agar; JO= Jonai, BO=Boko, GO=Goalpara

on their colonial morphology and reproductive structures referring standard fungal identification manuals. The fungal isolates which did not sporulate were categorized as Mycelia Sterilia and each sterile isolate with distinct morphological features was designated as morphotype.

4.2.1 Endophytic fungi from *H. cordata*

Healthy leaf tissues of *H. cordata* were found to harbour different genera of endophytic fungi. From the surface-sterilized leaf fragments, a total of 56 endophytic fungal isolates were recovered (**Fig. 4.3**). The highest recovery of endophytes was observed in PDA medium, followed by MEA and least was observed in WA medium (**Table 4.3**). The endophytic fungi from *H. cordata* belonged to 6different fungal genera and the overall Colonization Frequency (CF%) was found to be 38.09%. The highest colonization frequency was found to be that of Mycelia sterilia (19.71%) followed by the genus *Colletotrichum* (14.03%) and *Corynespora* (1.34%). The genus *Colletotrichum* is represented by 5 species namely, *Colletotrichum siamense* (0.67%), *C. fructicola* (0.67%), *C. capsici* (2%), *C. acutatum* (1.34%) and *C. dematium* (4%). Other endophytic fungi isolates include like *Curvularia lunata* (1.67%), *Bipolaris* sp. (0.84%), *Corynespora* sp. (0.67%) and a dimorphic yeast species *Pseudozyma* sp. (0.84%). The dominant fungal genera obtained from all of the sampling sites was *Colletotrichum* and non-sporulating fungi categorised as Mycelia sterilia (**Table 4.6**) (**Fig. 4.4**).

Figure 4.3 Photo-plates showing growth of endophytic fungi from *H. cordata* on various media

Table 4.6 Composition of endophytic fungi in healthy leaf tissues of *H. cordata* isolated from four different sites of Assam

Endophytic fungi	Locations				Total isolates/ 150 fragments	CF (%)	Frequency of dominant endophytes
	HW	JL	SN	TZ			
Colletotrichum siamense	1	-	-	-	1	0.67	1.77
Colletotrichum fructicola	1	-	-	-	1	0.67	1.77
Colletotrichum capsici	3	-	-	-	3	2.00	5.36
Colletotrichum acutatum	-	-	2	-	2	1.34	3.58
Colletotrichum dematium	-	-	-	6	6	4.00	10.71
Colletotrichum sp.1	-	-	1	-	1	0.67	1.77
Colletotrichum sp.2	-	-	4	-	4	2.67	7.15
Colletotrichum sp.3	-	2	-	-	2	1.34	3.58
Colletotrichum sp.4	-	1	-	-	1	0.67	1.77
Curvularia lunata	-	-	-	2	2	1.67	3.58
Bipolaris sp.	-	-	-	1	1	0.67	1.77
Corynespora casiicola	-	-	-	1	1	0.67	1.77
Corynespora sp.	-	-	-	1	1	0.67	1.77
Pseudozyma sp.	-	-	1	-	1	0.67	1.77
Morphotype sp.1	2	-	-	-	2	1.34	3.58
Morphotype sp.2	2	-	-	-	2	1.34	3.58
Morphotype sp.3	3	-	-	-	3	2.00	5.36
Morphotype sp.4	-	3	-	-	3	2.00	5.36
Morphotype sp.5	-	2	-	-	2	1.34	3.58
Morphotype sp.6	-	1	-	-	1	0.67	1.77
Morphotype sp.7	-	4	-	-	4	2.67	7.15
Morphotype sp.8	-	2	-	-	2	1.67	3.58
Morphotype sp.9	-	4	-	-	4	2.67	7.15
Morphotype sp.10	-	-	1	-	1	0.67	1.77
Morphotype sp.11	-	-	-	2	2	1.34	3.58
Morphotype sp.12	-	-	-	3	3	2.00	5.36
Total no. of isolates	12	19	9	16	56	38.09	100

CF= Colonizing frequency, HW= Howly, JL= Jalukbari, SN= Sonapur, TZ= Tezpur

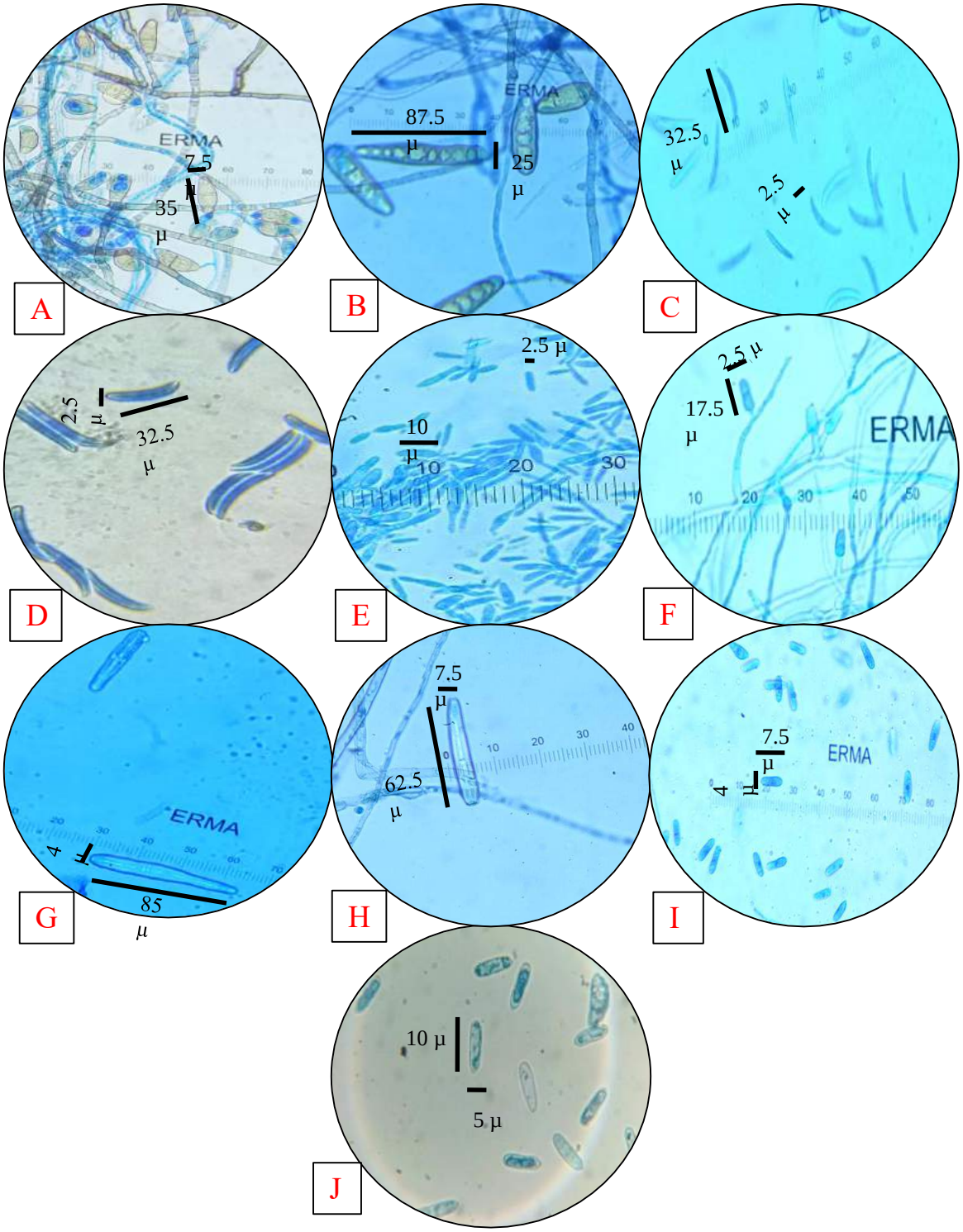


Figure 4.4 Microscopic photographs of some endophytic fungi isolated from *H. cordata*- a) *Curvularia lunata*, b) *Bipolaris* sp., c) *Colletotrichum dematium*, d) *C. capsici* e) *Pseudozyma* sp., f) *Colletotrichum acutatum*, g) *Corynespora casiicola*, h) *Corynespora* sp., i) *Colletotrichum gloeosporoides*, j) *Colletotrichum* sp.1 (under 40x and 100x)

4.2.2 Endophytic fungi from *E. foetidum*

From the surface-sterilized leaf fragments of *E. foetidum*, a total of 84 endophytic fungal isolates were recovered (**Fig. 4.5)**. The highest recovery of endophytes was observed in PDA medium, followed by MEA and the least was in WA medium (**Table 4.4**). The endophytic fungi belonged to 8 different genera with an overall CF% of 56.01%.

The highest colonization frequency was found to be of the genus *Colletotrichum* (32%), followed by Mycelia Sterilia (4.67%) and *Stemphylium* (4%). The genus *Colletotrrichum* was further represented by 12 different species with varied colonization frequencies. Some of the identified species were *Colletotrichum tropicale* (0.67%), *C. acutatum* (0.67%), *C. gloeospoiroides* (0.67%), *C. boninense* (0.67%), *C. siamense* (0.67%) and *C. karstii* (2.67%). Other identified fungal species were *Cladosporium macrocarpum* (2.67%), *C. fulvum* (0.67%), *Scopulariopsis brevicaulis* (0.67%), *Penicillium chrysogenum* (0.67%), *Alternaria alternata* (0.67%), *Alternaria* sp. (0.67%) and *Purpureocillium lilacinum* (0.67%). (**Table 4.7**) (**Fig. 4.6**).

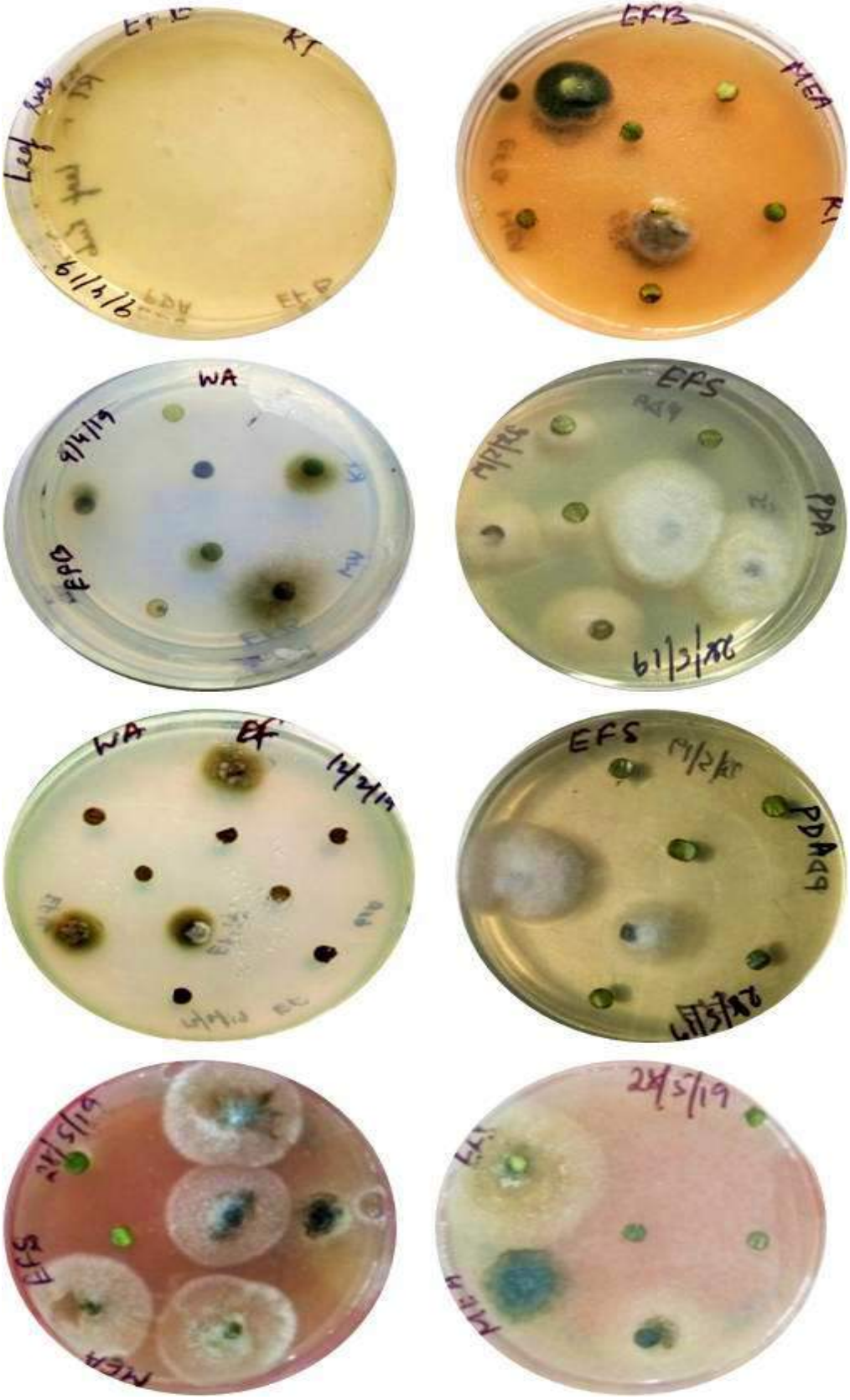


Figure 4.5 Photo-plates showing growth of endophytic fungi from *E. foetidum* on various media

Table 4.7 Composition of endophytic fungi in healthy leaf tissues of *E. foetidum* isolated from three different sites of Assam

Endophytic fungi	Locations			Total isolates/150 fragments	CF (%)	Frequency of dominant endophytes (%)
	AN	BH	SN			
Scopulariopsis brevicaulis	1	-	-	1	0.67	1.19
Cladosporium macrocarpum	4	-	-	4	2.67	4.76
Cladosporium fulvum	1	-	-	1	0.67	1.19
Stemphylium botryosum	-	4	1	5	3.33	5.94
Stemphylium sp.	-	1	-	1	0.67	1.19
Penicillium chrysogenum	-	1	-	1	0.67	1.19
Alternaria alternata	-	1	-	1	0.67	1.19
Alternaria sp.	-	1	-	1	0.67	1.19
Purpureocillium lilacinum	10	4	-	14	9.33	16.66
Colletotrichum tropicale	-	1	-	1	0.67	1.19
Colletotrichum acutatum	-	1	-	1	0.67	1.19
Colletotrichum goleospoiroides	-	1	-	1	0.67	1.19
Colletotrichum boninense	-	1	-	1	0.67	1.19
Colletotrichum siamense	-	1	-	1	0.67	1.19
Colletotrichum karstii	-	4	-	4	2.67	4.76
Colletotrichum sp.1	-	2	-	2	1.33	2.37
Colletotrichum sp.2	-	-	2	2	1.33	2.37
Colletotrichum sp.3	-	-	8	8	5.33	9.52
Colletotrichum sp.4	-	-	17	17	11.33	20.23
Colletotrichum sp.5	-	-	5	5	3.33	5.94
Colletotrichum sp.6	-	-	5	5	3.33	5.94
Morphotype sp.1	-	2	-	2	1.33	2.37
Morphotype sp.2	-	-	2	2	1.33	2.37
Morphotype sp.3	-	-	1	1	0.67	1.19
Morphotype sp.4	-	-	1	1	0.67	1.19
Morphotype sp.5	-	-	1	1	0.67	1.19
Total no. of isolates	16	25	43	84	56.01	100

CF= Colonization Frequency; AN= Amingaon, BH= Baihata, SN=Sonapur

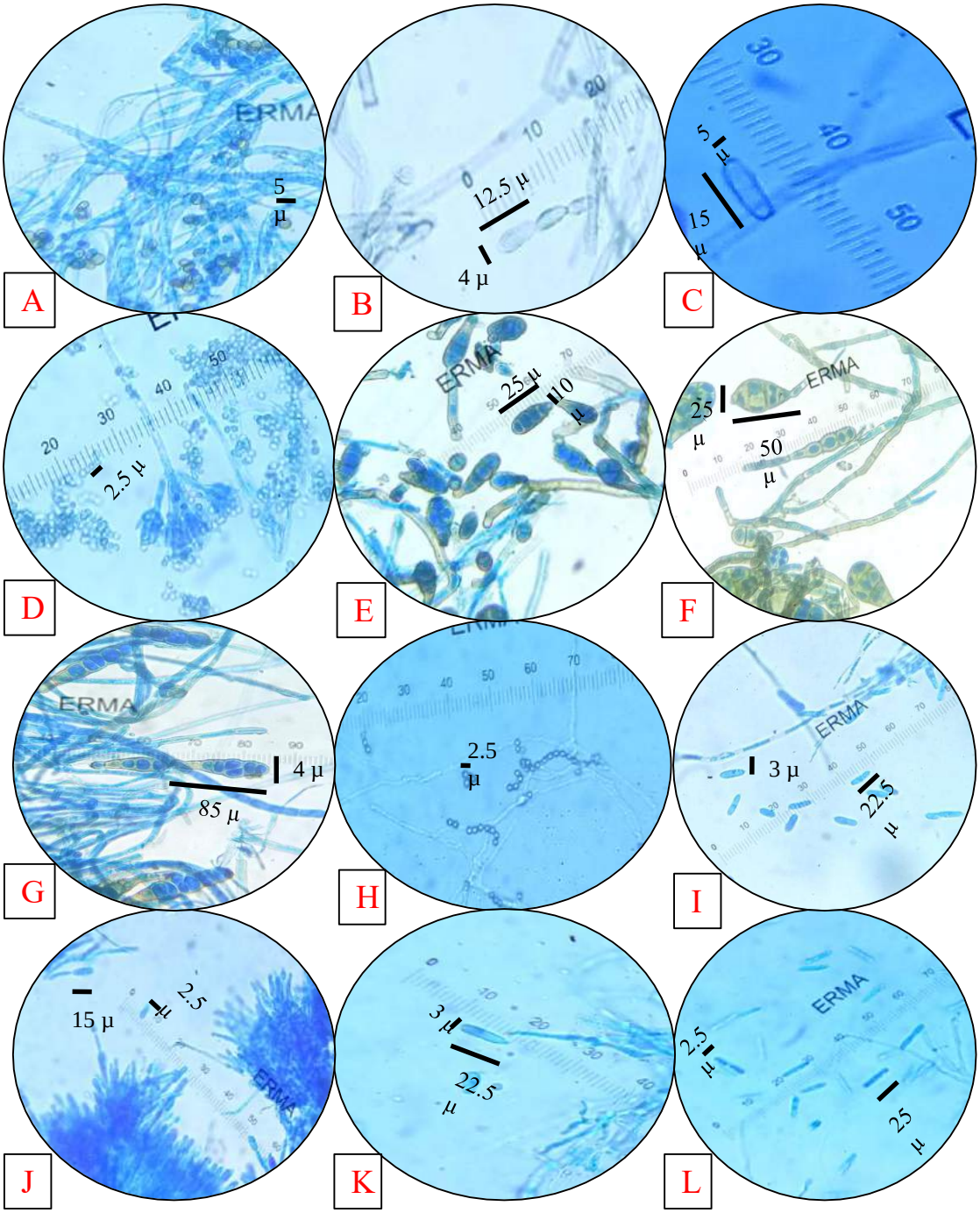


Figure 4.6 Microscopic photographs of some endophytic fungi isolated from *E. foetidum*- a) *Scopulariopsis brevicaulis*, b) *Cladosporium macrocarpum*, c) *C. fulvum*, d) *Penicillium chrysogenum*, e) *Stemphylium botryosum*, f) *Stemphylium sp*., g) *Alternaria alternata*, h) *Purpureocillium lilacinum*, i) *Colletotrichum tropicale, j) C. boninense*, k) *C. orbiculare*, l) *C. hippeastri* (under 40x and 100x)

4.2.3 Endophytic fungi from *Z. oxyphyllum*

From the surface-sterilized leaf tissues of *Z. oxyphyllum*, out of the 150 leaf segments plated, a total number of 74 endophytic fungal isolates were recovered (**Fig. 4.7**). The highest recovery of endophytes was obtained in PEA medium, followed by PDA, MEA and the least was observed in WA medium (**Table 4.5**). From healthy leaf tissues of *Z. oxyphyllum* endophytic fungi belonging to 6 different genera were isolated with a total colonization frequency of 49.33%. Here also, the highest colonization frequency was found to be that of the genus *Colletotrichum* (22.01%), followed by Mycelia Sterilia (12.66%) and *Curvularia* (6.67%). The genus included *Colletotrichum* 7 different species, namely, *Colletotrichum dematium* (3.33%), *C. siamense* (4%), *C. gloeosporioides* (4.67%), *C. acutatum* (2.67%), *C. boninense* (4.67%), *C. asianum* (2.67%) and *C. nymphae* (3.33%). Further, the genus *Curvularia* included three different species namely, *Curvularia pallescens* (2%), *C. protuberata* (2.67%) and *C. geniculata* (2%). Other identified fungal isolates were *Fusarium oxysporum* (2%), *Aspergillus flavus* (1.33%) and *Corynespora cassicola* (1.33%). (**Table 4.8**) (**Fig. 4.8**).

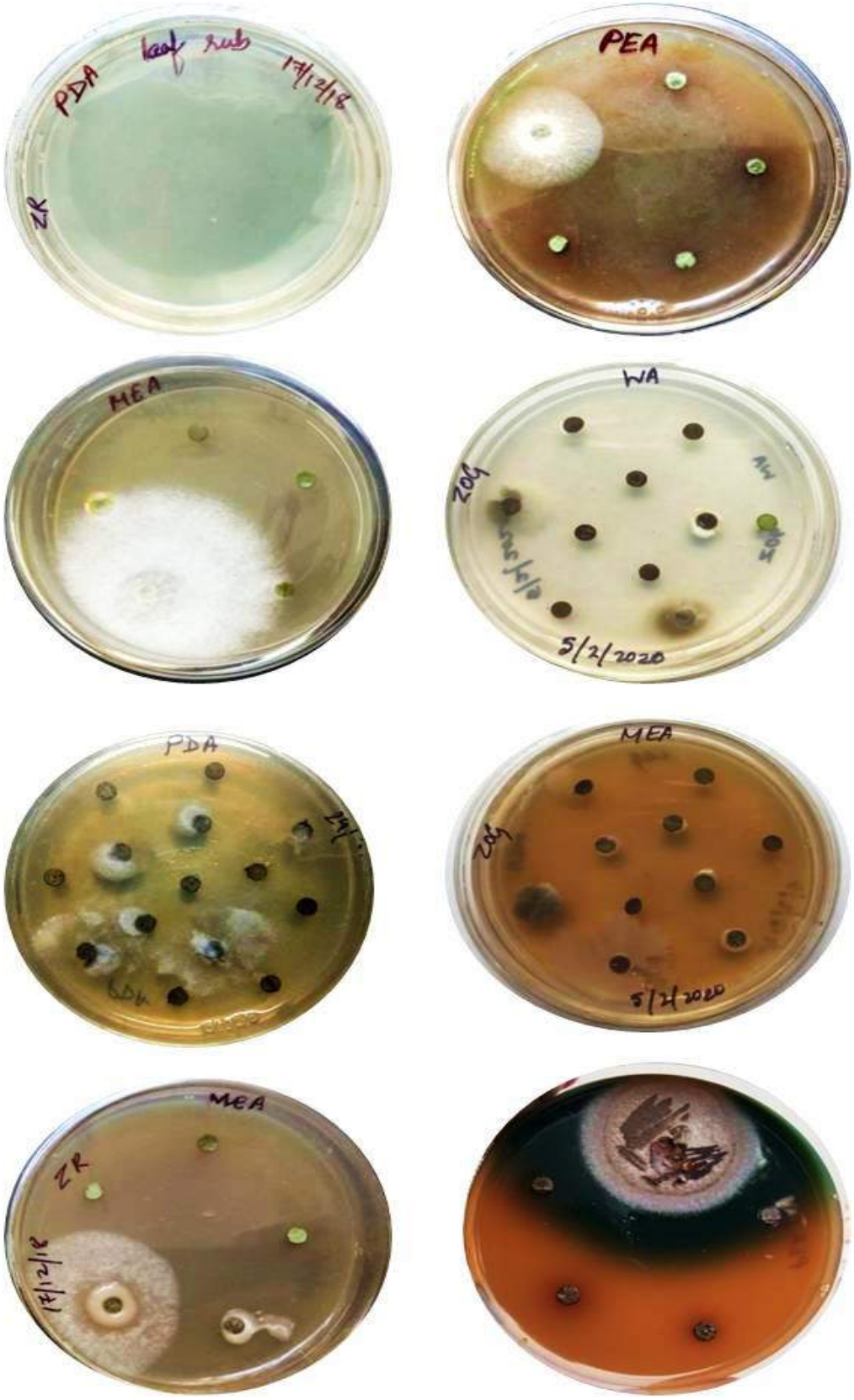


Figure 4.7 Photo-plates showing growth of endophytic fungi from *Z. oxyphyllum* on various media

Table 4.8 Composition of endophytic fungi in healthy leaf tissues of *Z. oxyphyllum* isolated from three different sites of Assam

Endophytic fungi	**Locations**			**Total isolates/150 fragments**	**CF (%)**	**Frequency of dominant endophytes (%)**
	JO	**BO**	**GO**			
Fusarium oxysporum	-	3	-	3	2.00	4.05
Curvularia pallescens	-	3	-	3	2.00	4.05
Curvularia protuberata	-	-	4	4	2.67	5.41
Curvularia geniculata	-	-	3	3	2.00	4.05
Aspergillus flavus	-	2	-	2	1.33	2.70
Corynespora cassicola	2	-	-	2	1.33	2.70
Colletotrichum dematium	5	-	-	5	3.33	6.75
Colletotrichum siamense	-	6	-	6	4.00	8.10
Colletotrichum gloeosporioides	-	7	-	7	4.67	9.47
Colletotrichum acutatum	-	4	-	4	2.67	5.41
Colletotrichum boninense	-	-	7	7	4.67	9.47
Colletotrichum asianum	-	-	4	4	2.67	5.41
Colletotrichum nymphae	-	-	5	5	3.33	6.75
Morphotype sp. 1	3	-	-	3	2.00	4.05
Morphotype sp. 2	3	-	-	3	2.00	4.05
Morphotype sp. 3	-	5	-	5	3.33	6.75
Morphotype sp. 4	-	6	-	6	4.00	8.10
Morphotype sp. 5	-	-	2	2	1.33	2.70
Total no. of isolates	13	36	25	74	49.33	100

CF= Colonization Frequency; JO= Jonai, BO= Boko, GO= Goalpara

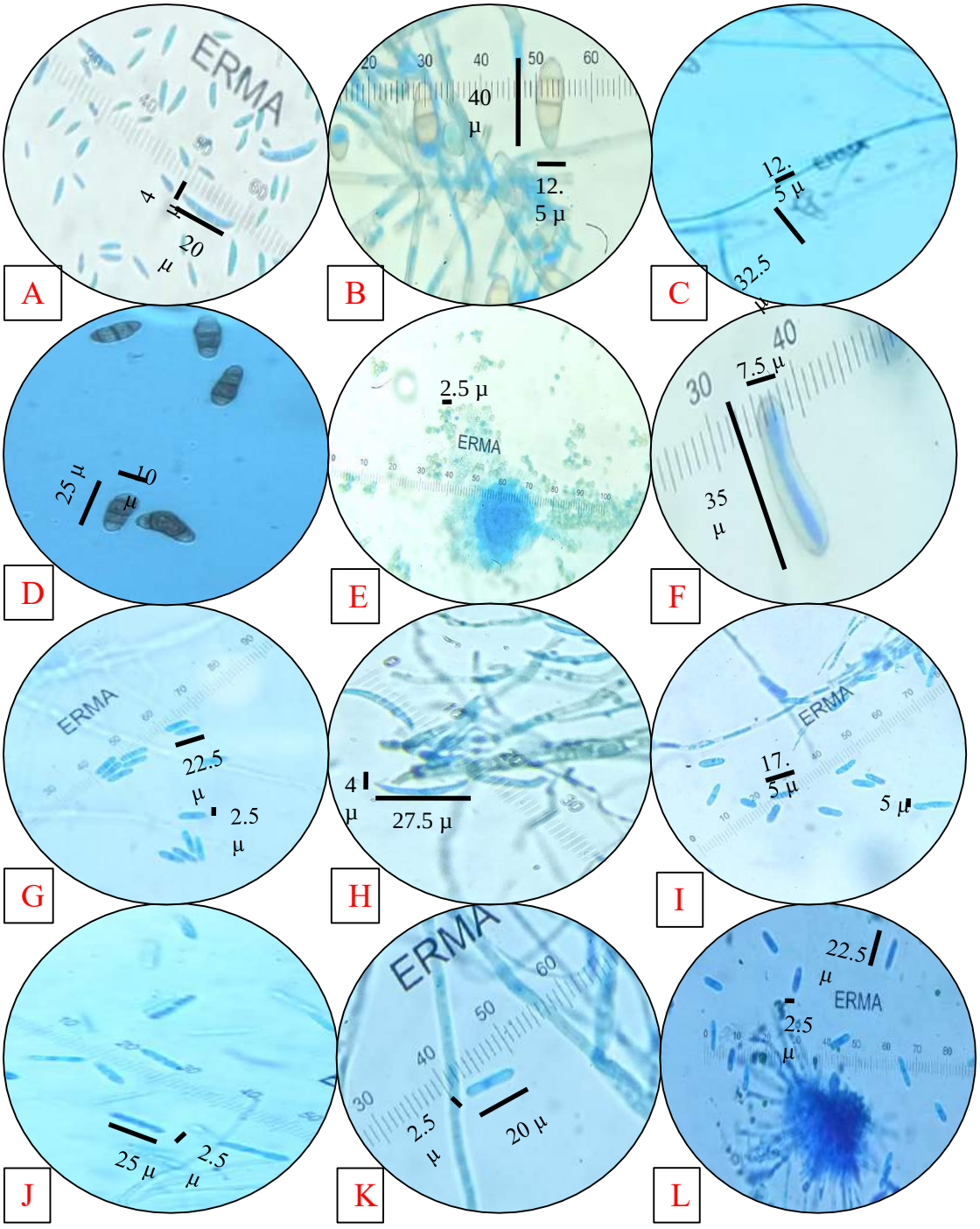


Figure 4.8 Microscopic photographs of some endophytic fungi isolated from *Z. oxyphyllum*- a) *Fusarium oxysporum*, b) *Curvularia pallescens*, c) *C. protuberata*, d) *C. geniculata*, e) *Aspergillus flavus*, f) *Corynespora casiicola*, g) *Colletotrichum boninense*, h) *C. dematium*, i) *C. siamense*, j) *C. nymphae*, k) *C. asianum*, l) *C. gloeosporioides* (under 40x and 100x)

It was observed that the highest colonizing fungal genera in all the three plant species was *Colletotrichum* (50%), followed by Mycelia Sterilia (25.7%) which were also commonly isolated from all the three investigated plant species. Other endophytic genera were *Purpureocillium* (6.54%), *Curvularia* (5.61%) and *Corynespora* (1.87%). The percentage occurrence of endophytic fungal genera in the three studied plant species is presented in **Figure 4.9**. It can be concluded that PDA medium was found to be the most suitable nutrient for obtaining maximum endophytic fungal isolates from all the plant species except that of *Z. oxyphyllum*.

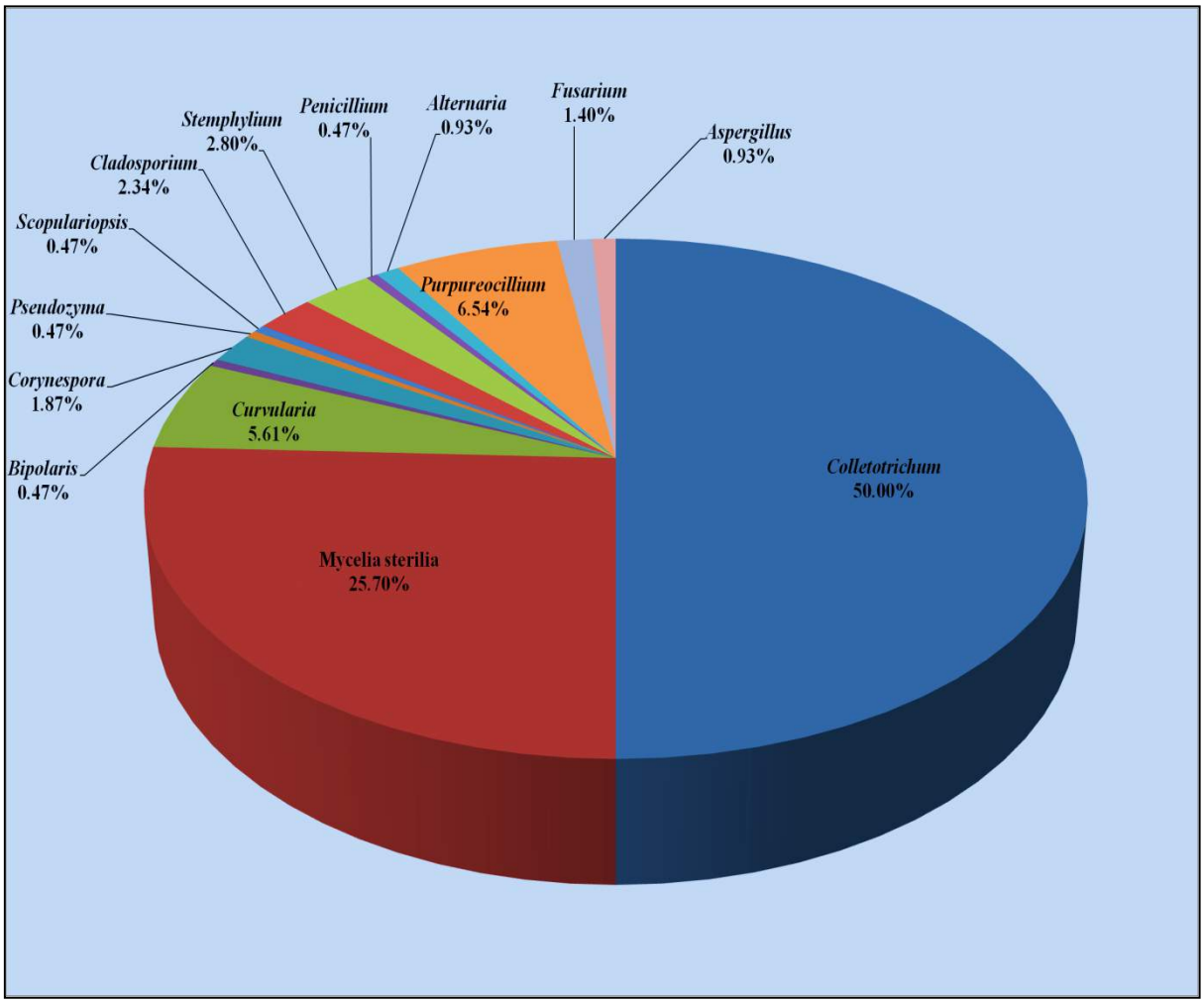


Figure 4.9 Percentage colonization of endophytic fungal genera occurring in the three selected ethno-medicinal plants

4.3 Antimicrobial activity of the isolated endophytic fungi

Crude secondary metabolites obtained from endophytic fungi were determined for antimicrobial activity against a panel of clinically significant human test pathogenic microorganisms, namely, *Staphylococcus aureus* (MTCC 737), *Pseudomonas aeruginosa* (MTCC424), *Escherichia coli* (MTCC 443) and *Candida albicans* (MTCC 227). The test pathogens were from the Institute of Microbial Technology (IMTECH), Chandigarh, India and revived on respective media to obtain the pure cultures (**Fig. 4.10**). The result of antimicrobial activity indicated that 78.57% of the isolates showed antimicrobial activity by inhibiting atleast one of the four test pathogens whereas 58.57% of the isolates showed antimicrobial activity by inhibiting all the test pathogens. It was observed that 57.14% of the isolates showed antifungal activity while 41.42% of the isolates showed antibacterial activity.

Endophytic fungi isolated from *H. cordata* showed noticeable antifungal and antibacterial activities against all the test pathogens. All of the isolates showed antifungal activity against *C. albicans* and antibacterial activity against *S. aureus*. Further 76.19% of isolates showed antibacterial activity against all the test bacterial pathogens. Amongst the endophytes, the genus *Colletotrichum* was isolated from all the sampling sites. Out of these, an isolate designated as *Colletotrichum* sp. HCS3 showed maximum activity against all the test pathogens. Another non-sporulating endophytic fungal isolate designated as Morphotype HCS6 showed considerable antifungal activity against *C. albicans*. The isolate also showed promising antibacterial activity against both Gram-positive and Gram-negative tested bacterial pathogens (**Table 4.9**).

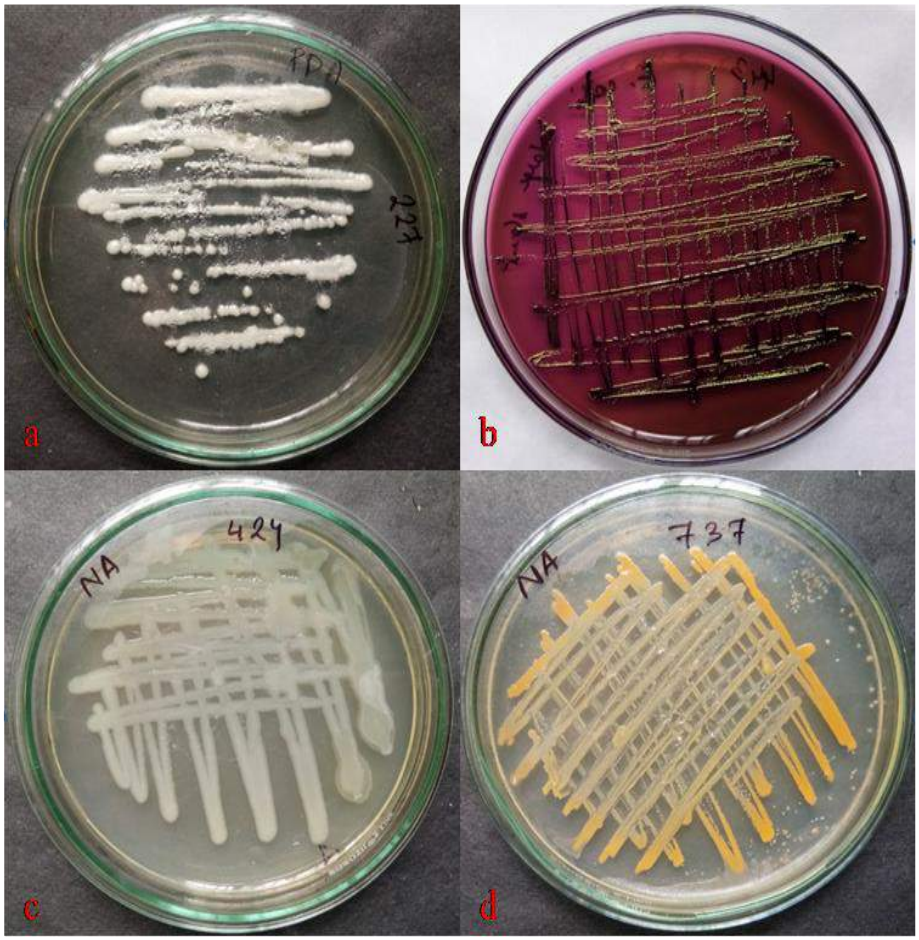


Figure 4.10 Test pathogens procured from IMTECH, Chandigarh: a) *Candida albicans*, b) *Escherichia coli*, c) *Pseudomonas aeruginosa*, d) *Staphylococcus aureus*.

Amongst the endophytic fungi isolated from *E. foetidum*, almost all the isolates showed significant antifungal and antibacterial activities. However, it was observed that the isolates higher antifungal activity against *C. albicans* as compared to the bacterial test pathogens. The result also indicated that 94.11% of the isolates showed antibacterial activity against *E. coli* and 70.58% of isolates showed inhibitory effect against all the test bacterial pathogens. Out of all the isolates, *Scopulariopsis* sp. (EF1), *Purpureocillium lilacinum* (EF6) and *Penicillium chrysogenum* (EFB9) showed highest antifungal activity against *C. albicans* and good antibacterial activity against both Gram-positive and Gram-negative tested bacterial pathogens (**Table 4.10**).

Similarly, the crude metabolites obtained from endophytic fungal isolates of *Z. oxyphyllum* also showed significant inhibitory effects against all the test pathogens. All of the isolates showed antifungal activity against *C. albicans*, 94.11% of the isolates showed antibacterial activity against *E. coli* while 76.47% of isolates showed antibacterial activity against all the pathogens. Amongst the isolates, maximum antimicrobial activity was observed in *Colletotrichum gloeosporoides* (ZOB3) against both fungal and bacterial pathogens (**Table 4.11**).

Thus, the result of antimicrobial assay revealed that from the three selected medicinal plants six endophytic fungal isolates, namely, *Colletotrichum* sp. (HCS3), Mycelia sterilia (HCS6), *Scopulariopsis brevicaulis* (EF1), *Purpureocillium lilacinum* (EF6), *Penicillium chrysogenum* (EFB9) and *Colletotrichum gloeosporioides* (ZOB3) showed promising antimicrobial activity against all the clinically significant test pathogens (**Fig. 4.11**).

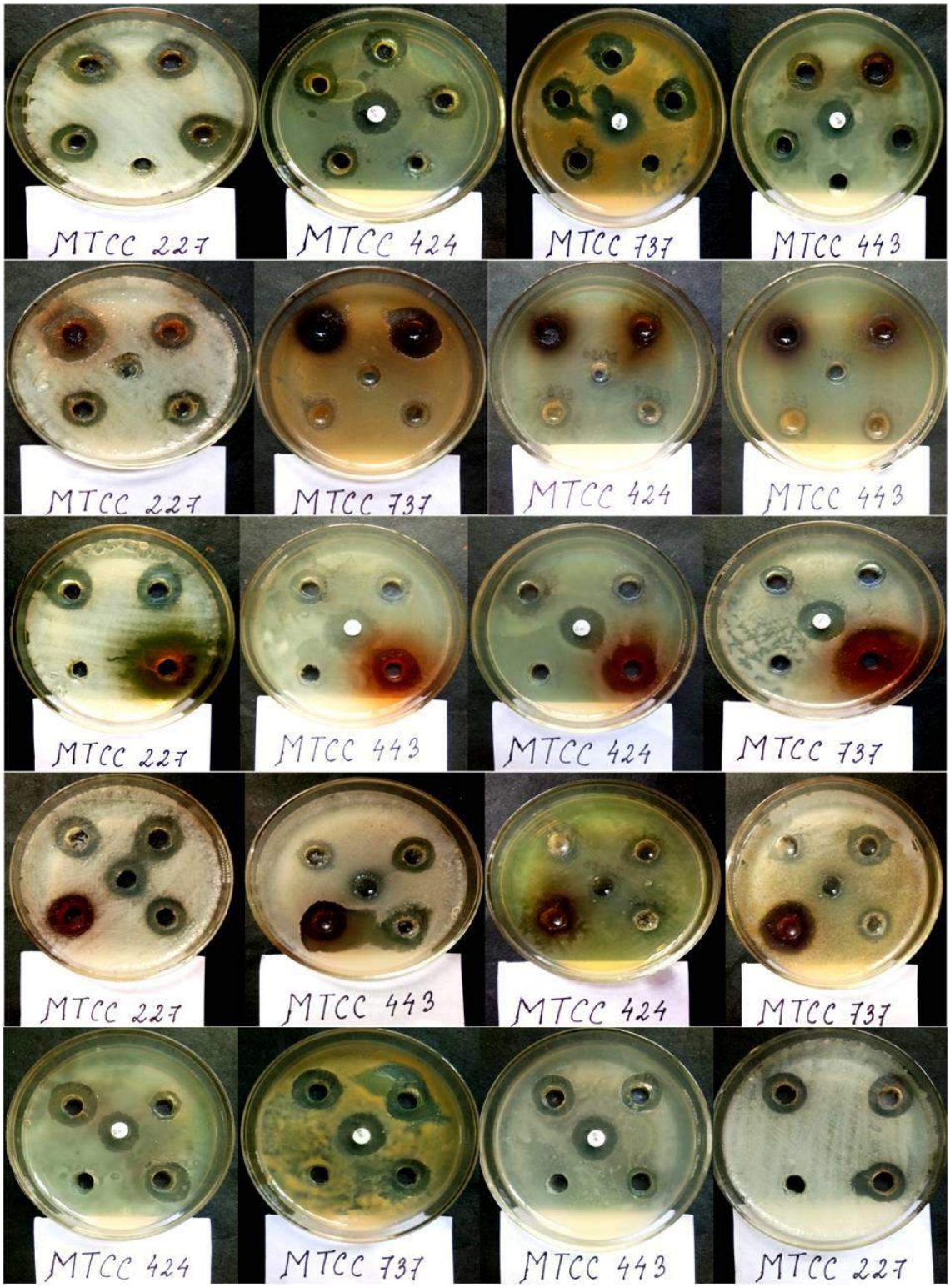


Figure 4.11 Antimicrobial activities of some potent endophytic fungi against test organisms.

Table 4.9 Antimicrobial activity of endophytic fungi of *H. cordata* against test organisms

Isolate code	Endophytic fungi	Zone of Inhibition (ZoI)			
		CA	**SA**	**EC**	**PA**
HC3	*Colletotrichum siamense*	+	+	-	+
HC6	*Colletotrichum fructicola*	+	+	+	-
HC7	*Colletotrichum capsici*	+	+	+	+
HCS4	*Colletotrichum acutatum*	+	+	+	+
HCT13	*Colletotrichum dermatium*	+	++	+	+
HCS3	***Colletotrichum* sp. 1**	**+++**	**+++**	**+++**	**+++**
HCS8	*Colletotrichum* sp.2	+	+	+	+
HCJ7	*Colletotrichum* sp.3	+	+	+	+
HCJ19	*Colletotrichum* sp.4	+	++	-	+
HCT1	*Curvularia lunata*	++	+	+	+
HCT10	*Bipolaris* sp.	+	+	++	+
HCT8	*Corynespora casiicola*	+	+	+	+
HCT9	*Corynespora* sp.	+	+	+	+
HCS2	*Pseudozyma* sp.	+	+	+	+
HC1	Morphotype sp.1	-	+	+	+
HCJ2	Morphotype sp.4	+	+	+	+
HCJ14	Morphotype sp.8	+	+	+	-
HCJ18	Morphotype sp.9	+	+	+	+
HCS6	**Morphotype sp.10**	**+++**	**+++**	**+++**	**+++**
HCT5	Morphotype sp.11	+	+	+	+
HCT11	Morphotype sp.12	++	+	+	+

"-" indicates no inhibition; "+" indicates ZoI > 10 mm; "++" indicates ZoI >15 mm; "+++" indicates ZoI >20 mm. CA= *C. albicans*, SA= *S. aureus*, EC= *E. coli*, PA= *P. aeruginosa*.

Table 4.10 Antimicrobial activity of endophytic fungi of *E. foetidum* against test organisms

Isolate code	Endophytic fungi	Zone of Inhibition (ZoI)			
		CA	**SA**	**EC**	**PA**
EF1	***Scopulariopsis brevicaulis***	**+++**	**+++**	**+++**	**+++**
EF2	*Cladosporium macrocarpum*	++	+	+	+
EF6	***Purpureocillium lilacinum***	**+++**	**+++**	**+++**	**+++**
EF12	*Cladosporium fulvum*	++	+	++	+
EFB4	*Stemphylium botryosum*	+++	+	+	+
EFB9	***Penicillium chrysogenum***	**+++**	**+++**	**++**	**+++**
EFB16	*Alternaria alternata*	++	+	+	+
EFB2	*Colletotrichum acutatum*	++	+	+	+
EFB3	*Colletotrichum goleospoiroides*	++	+	+	+
EFB15	*Colletotrichum siamense*	++	+	++	+
EFB18	*Colletotrichum karstii*	++	+	++	+
EFS1	*Colletotrichum* sp.2	++	+	+	++
EFS14	*Colletotrichum* sp.5	+	-	+	+
EFS28	*Colletotrichum* sp.6	++	-	+	+
EFB6	Morphotype sp.2	++	-	+	+
EFS35	Morphotype sp.5	+	++	-	+
EFS41	Morphotype sp.6	+	-	+	-

"-" indicates no inhibition; "+" indicates ZoI > 10 mm; "++" indicates ZoI >15 mm; "+++" indicates ZoI >20 mm. CA= *C. albicans*, SA= *S. aureus*, EC= *E. coli*, PA= *P. aeruginosa*.

Table 4.11 Antimicrobial activity of endophytic fungi of *Z. oxyphyllum* against test organisms

Isolate code	**Endophytic fungi**	**Zone of Inhibition(ZoI)**			
		CA	**SA**	**EC**	**PA**
ZOB2	*Fusarium oxysporum*	++	+	++	+
ZOB4	*Curvularia pallescens*	+	+	+	+
ZOG3	*Curvularia protuberata*	+	+	++	+
ZOG4	*Curvularia geniculata*	+	-	+	-
ZOB14	*Aspergillus flavus*	+	+	+	+
ZOJ2	*Corynespora cassicola*	+	-	-	+
ZOJ1	*Colletotrichum dematium*	+	+	++	+
ZOB3	***Colletotrichum gloeosporioides***	+++	+++	+++	+++
ZOB9	*Colletotrichum siamense*	+	++	+	+
ZOB21	*Colletotrichum acutatum*	+	+	+	+
ZOG1	*Colletotrichum boninense*	++	+	+	+
ZOG8	*Colletotrichum asianum*	++	+	++	++
ZOG11	*Colletotrichum nymphae*	+	+	+	+
ZOJ3	Morphotype sp.1	+	+	+	-
ZOJ4	Morphotype sp.2	+	-	+	-
ZOB7	Morphotype sp.4	+	++	+	+
ZOG2	Morphotype sp.5	+	+	+	+

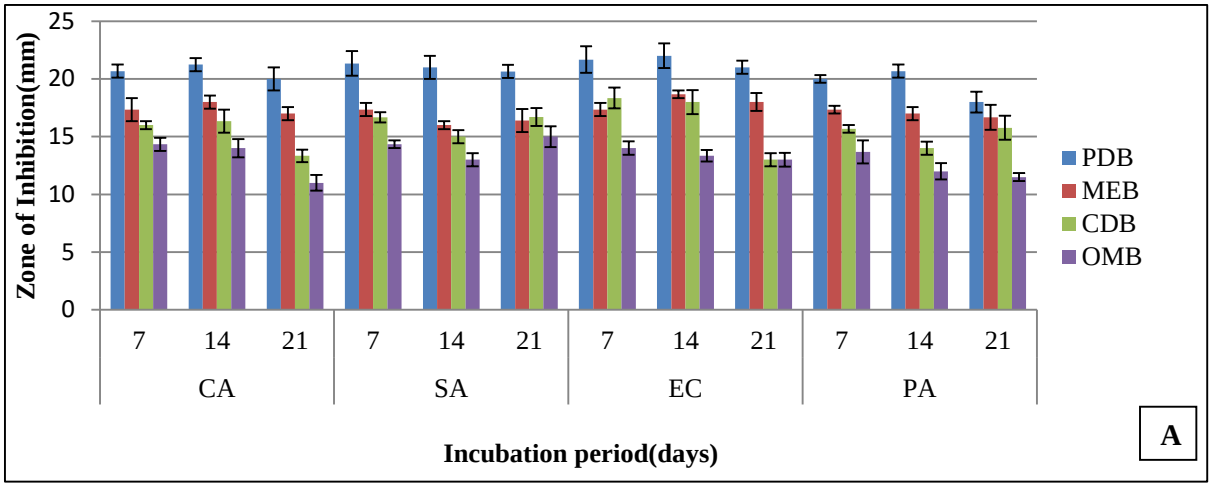
"-" indicates no inhibition; "+" indicates ZoI > 10 mm; "++" indicates ZoI >15 mm; "+++" indicates ZoI >20 mm. CA= *C. albicans*, SA= *S. aureus*, EC= *E. coli*, PA= *P. aeruginosa*.

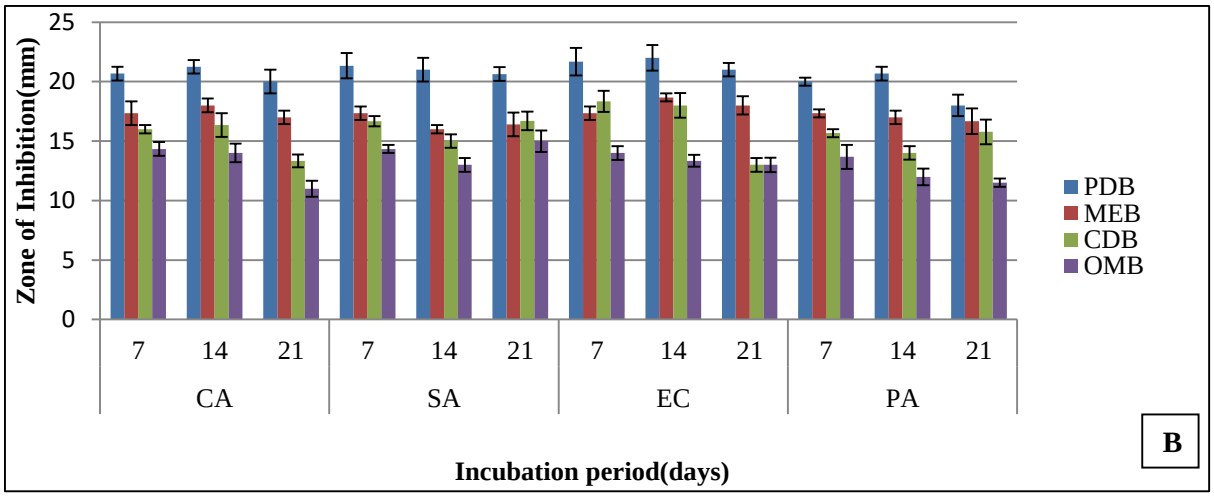
4.4 Process optimization of potent endophytic isolates

Based on preliminary antimicrobial activity, six potent endophytic fungal isolates namely *Colletotrichum* sp. (HCS3), Mycelia sterilia (HCS6), *Scopulariopsis brevicaulis* (EF1), *Purpureocillium lilacinum* (EF6), *Penicillium chrysogenum* (EFB9) and *Colletotrichum gloeosporioides* (ZOB3) were selected for further study. These endophytes were studied for enhanced production of antimicrobial metabolites under different cultural and environmental conditions.

To study the effect of growth medium on enhanced metabolites production, the strains were cultivated in four different media viz. PDB, MEB, CDB and OMB for 3 weeks and the metabolites were extracted after an interval of every 7 days following solvent extraction with ethyl acetate. The extracts were tested for antimicrobial activity against the test pathogens and assays were carried out in triplicates. The results showed that maximum antimicrobial activity was observed in PDB medium for the isolates *Colletotrichum* sp. (HCS3), Morphotype (HCS6), *Scopulariopsis brevicaulis* (EF1) and *Colletotrichum gloeosporioides* (ZOB3) on incubation for 14 days **(Fig. 4.12 A, B, C, F)**. While for *Penicillium chrysogenum* (EFB9) enhanced antimicrobial activity was observed in 21 days (**Fig. 4.12 E**). Again for the isolate *Purpureocillium lilacinum* (EF6) maximum antimicrobial activity was observed in CDB medium when incubated for 14 days (**Fig. 4.12 D**). It was observed that the isolates showed variable antimicrobial activity against all the test pathogens.

Similarly, to observe the effect of pH on enhanced metabolite production the isolates were grown in three different pH, i.e., acidic (pH 5), neutral (pH 7) and alkaline





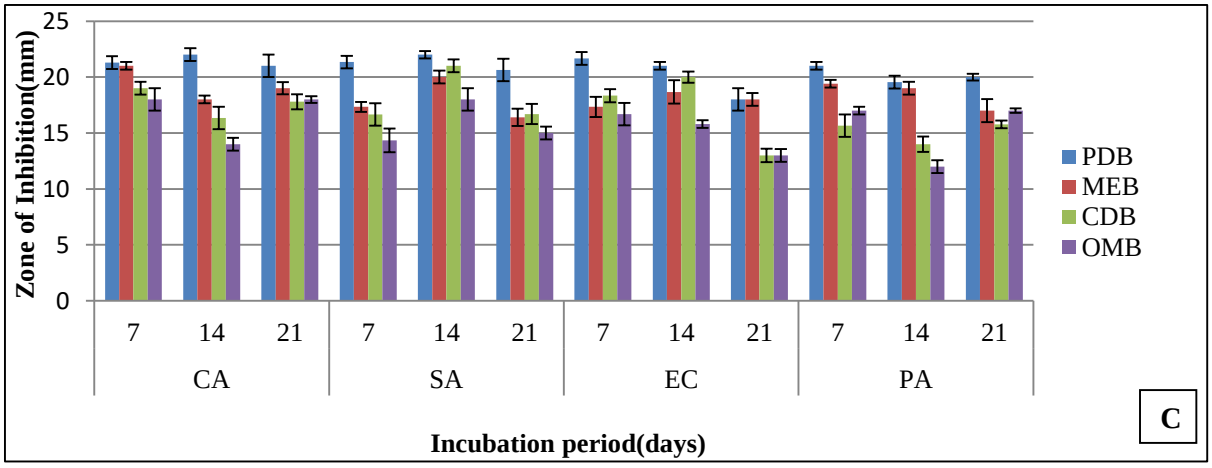
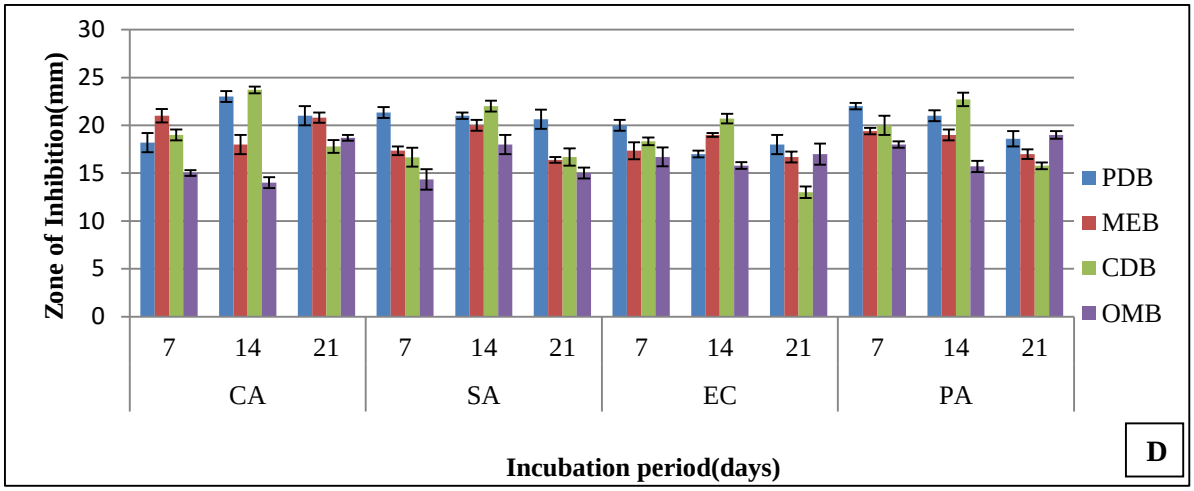
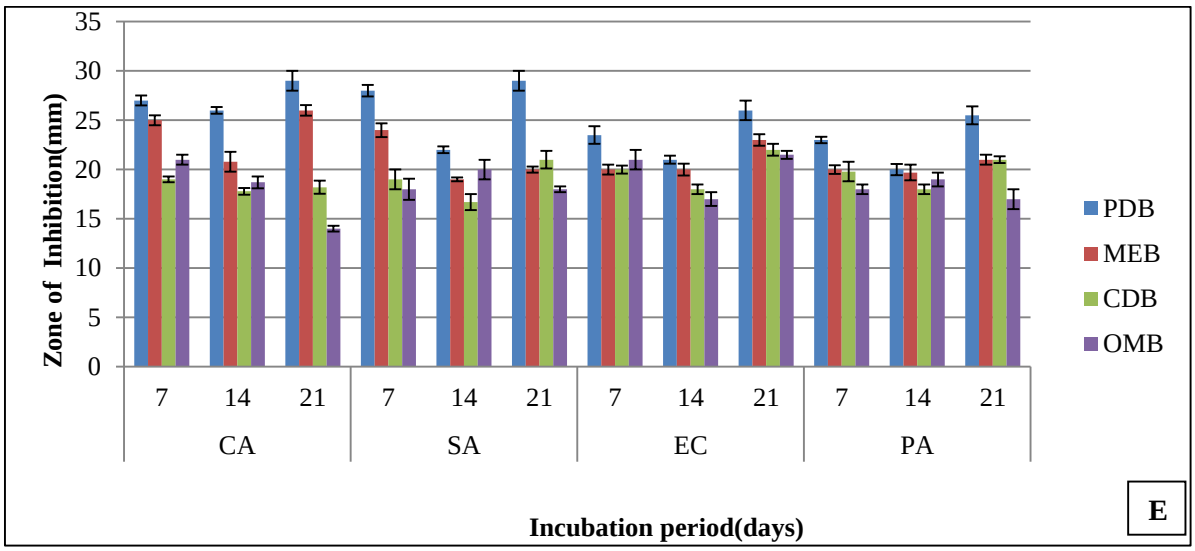


Figure 4.12 (A-C) Effect of different media and incubation periods on the antimicrobial activity of crude secondary metabolites of endophytic fungal isolates HCS3, HCS6 and EF1 respectively against the test pathogens: CA= *C. albicans*, SA= *S. aureus*, EC= *E. coli* and PA= *P. aeruginosa.*





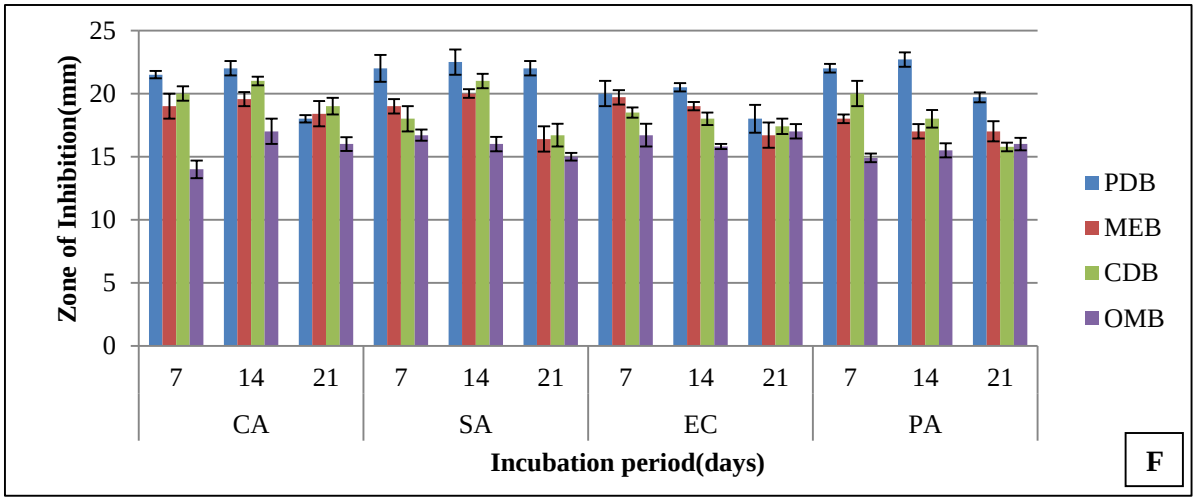


Figure 4.12 (D-F) Effect of different media and incubation periods on the antimicrobial activity of crude secondary metabolites of endophytic fungal isolates EF6, EFB9 and ZOB3 respectively against the test pathogens: CA= *C. albicans*, SA= *S. aureus*, EC= *E. coli* and PA= *P. aeruginosa.*

(pH 9) keeping the media and incubation period optimum. The result indicated that four isolates namely, *Colletotrichum* sp. (HCS3), *Scopulariopsis brevicaulis* (EF1), *Penicillium chrysogenum* (EFB9) and *Colletotrichum gloeosporioides* (ZOB3) showed enhanced antimicrobial activity in neutral pH (pH 7) (**Fig. 4.13 A, C, E & F**). Whereas, the isolates, Morphotype (HCS6) and *Purpureocillium lilacinum* (EF6) showed enhanced activity in alkaline pH (pH 9) against all the test pathogens (**Fig. 4.13 B & D**). Further, endophytic isolates were also grown in their respective growth medium at different temperatures ranging from 25-40^{o}C to observe the effect of temperature on metabolite production. The result indicated that all of the isolates showed maximum activity at temperature of 30^{o}C. None of the isolates showed better activity when cultivated in higher temperatures i.e, at 35^{o}C and 40^{o}C respectively.

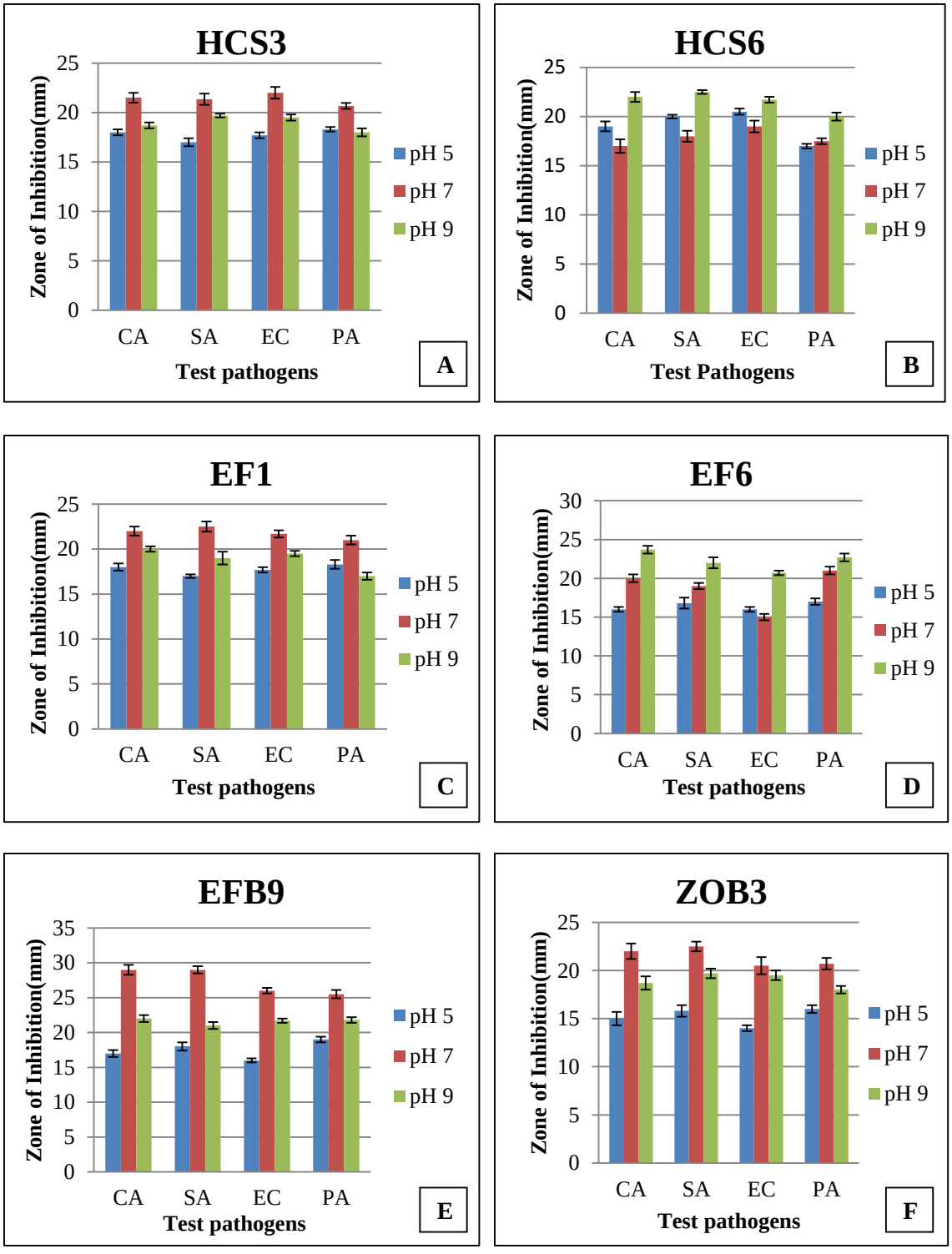


Figure 4.13 (A-F) Effect of different pH on the antimicrobial activity of crude secondary metabolites of endophytic fungal isolates HCS3, HCS6, EF1, EF6, EFB9 and ZOB3 against the test pathogens: CA= *C. albicans*, SA= *S. aureus*, EC= *E. coli* and PA= *P. aeruginosa*.

4.5 Molecular identification and phylogeny study

Out of the six endophytic fungi that showed inhibitory effect against the test organisms, two isolates with promising antimicrobial activity were further characterized by molecular techniques using ITS rDNA sequence analysis. The sequences were confirmed for closest homologues and phylogenetic relationships were determined by ITS2 sequences and RNA secondary structure analyses.

4.5.1 Molecular characterization and phylogeny of *Colletotrichum* sp. (HCS3)

The microscopical and colonial morphology of the isolate HCS3 resembled to that of *Colletotrichum* sp. when grown on both PDA and MEA media (**Fig. 4.14**). However, the species level identification was further achieved by isolating the genomic DNA and sequencing the ITS region. The region for ITS rDNA was amplified and sequenced using universal primers ITS4 and ITS5. The contig rDNA sequence so obtained was annotated and deposited to the GenBank with accession number MN128230. A BLAST homology search of ITS rDNA revealed that the isolate HCS3 showed closest homology to *Colletotrichum coccodes* (MF076580.1) with Maximum Identity of 98.25%; Query Coverage 91% and E-value 0.0. Based on this, a total of 995 ITS rDNA sequences belonging to different *Colletotrichum* spp., were randomly retrieved from Genbank and screened for the presence of complete ITS rDNA region (18S rDNA-ITS1-5.8S-ITS2-rDNA-28S rDNA). A total of 83 sequences with complete ITS rDNA regions were finally selected for generating phylogenetic tree. A tree generated by Maximum Parsimony (MP) method showed that the isolate HCS3 (MN128230) clustered within the clades of *C. coccodes*. (**Fig. 4.15 A**). The sequences were further trimmed for the ITS2 region and to confirm its endophytic nature, another phylogenetic tree was constructed using 30 ITS2

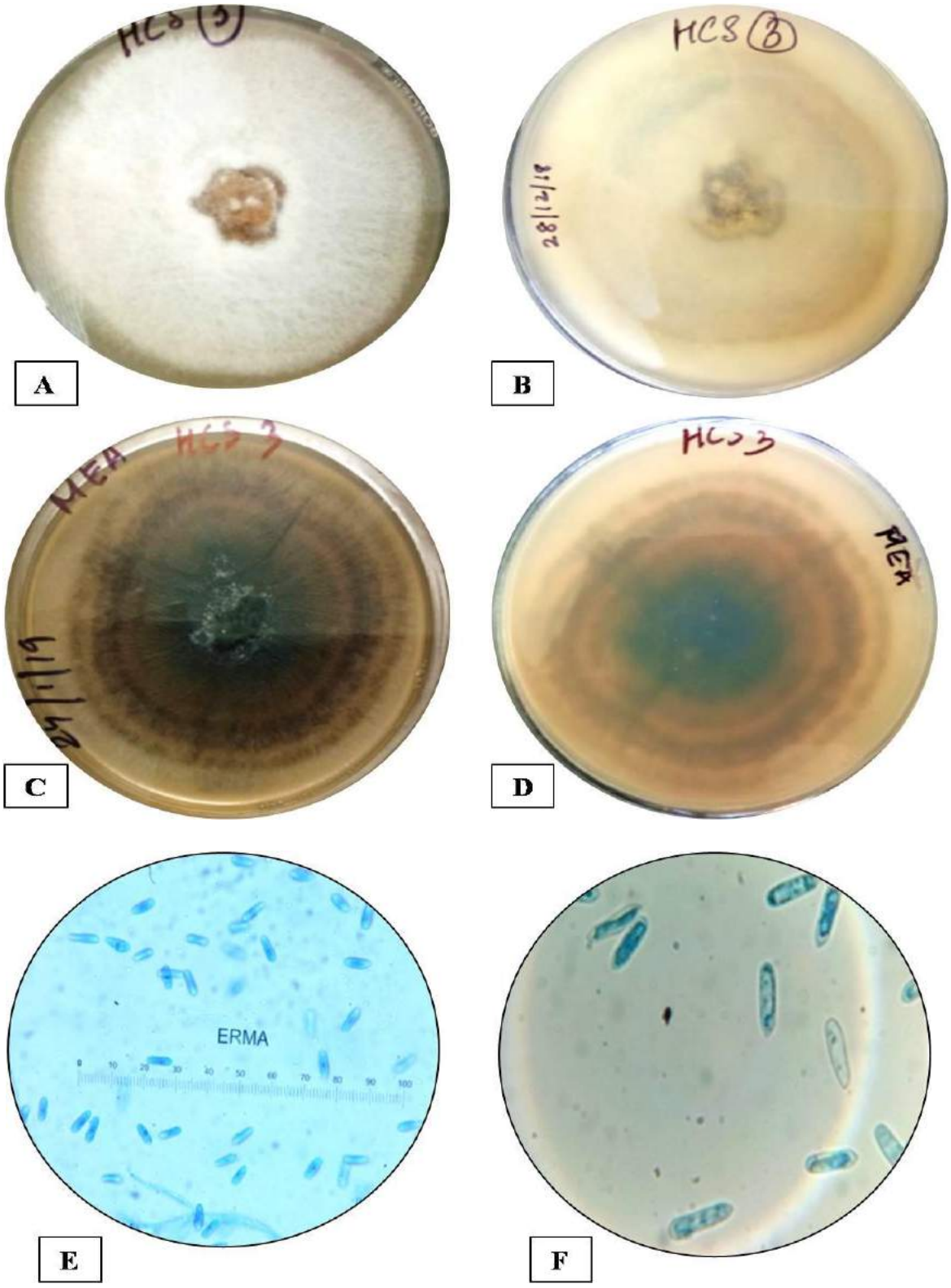


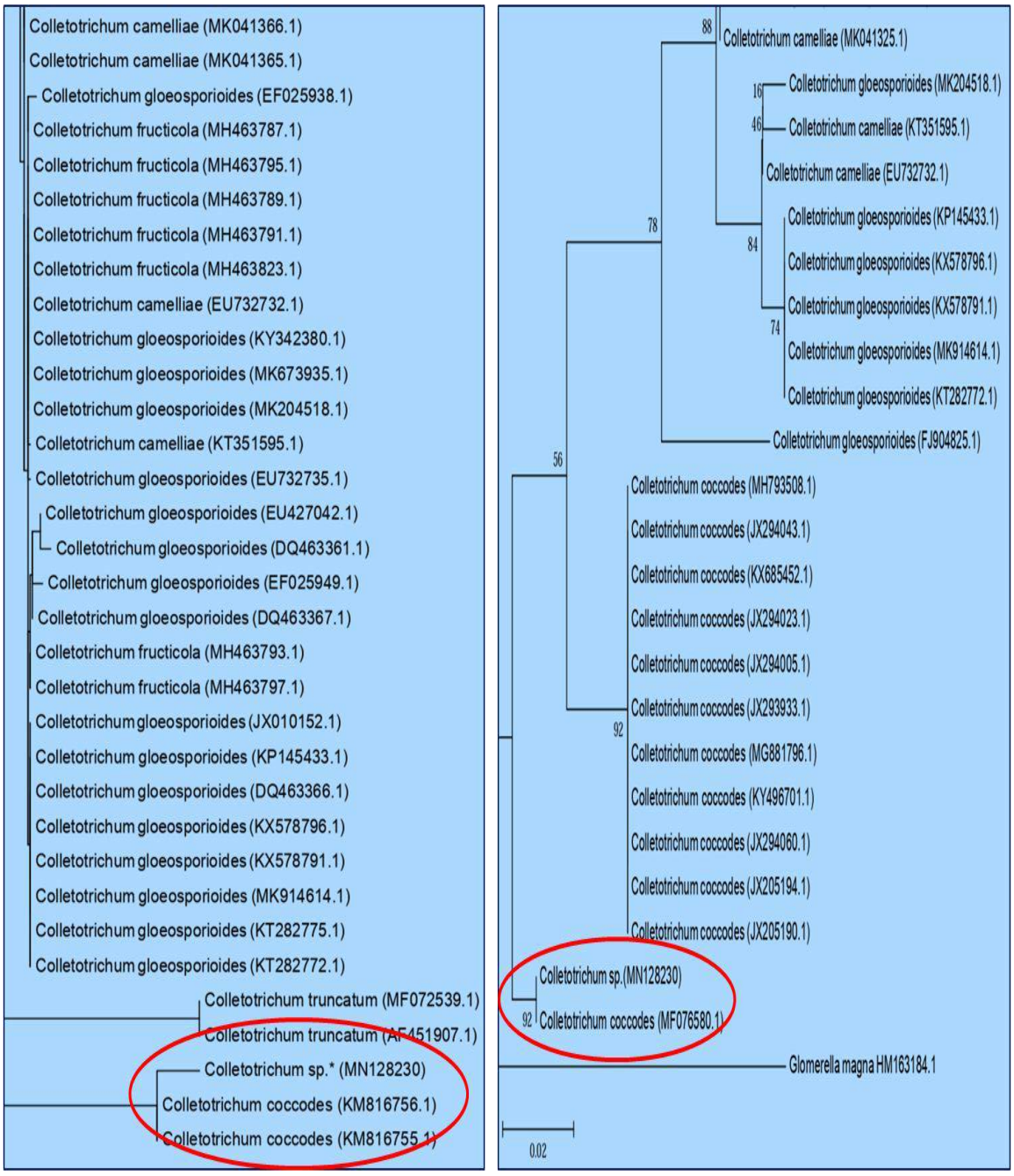
Figure 4.14 Colonial morphology of *Colletotrichum* sp. (HCS3) on PDA medium: A) Front view, B) Reverse view and MEA medium, C) Front view D) Reverse view, Spores (E and F) (under 40x and 100x respectively).

sequences. The phylogenetic tree generated by MP showed that the isolate shares a close affinity with an endophytic *C. coccodes* (accession no.-MF076580) which is supported by a bootstrap value of 92 (**Fig. 4.15 B**). To further validate and predict its endophytic nature, RNA secondary structure analysis were carried out using 12 ITS2 sequences of *C. coccodes* considering pathogenic and endophytic lifestyle and were compared with the RNA secondary structure of the isolate HCS3. The consensus RNA secondary structure of the pathogenic *C. coccodes* consisted of a conserved core bulge radiating into 3 major helices (H1, H2 and H3) while RNA secondary structure of endophytic *C. coccodes* comprised of one incomplete (H1) and four complete helices (H2, H3, H4 and H5). In the present investigation the RNA secondary structure of the isolate HCS3 (MN128230) showed structural similarity with that of endophytic *C. coccodes* (MF076580) (**Fig. 4.16**). The result further showed that the pathogenic and endophytic ITS2 RNA secondary structures of *C. coccodes* exhibited different folding patterns and dissimilar structural motifs. The finding indicated that isolate HCS3 shared close similarity with the endophytic lifestyle both in terms of ITS2 folding pattern and identical structural motifs. The details of different structural features of ITS2 RNA secondary structures of different lifestyles of *C. coccodes* are presented in **Table 4.12**.

Table 4.12 Comparison of ITS2 secondary RNA motif features among *C. coccodes* of different life styles

***C. coccodes* Lifestyles**	**Hairpin loop (s)**	**Internal loop (s)**		**Helices**	**Bulges**	
		Symmetric	**Asymmetric**		**Single bulge**	**Multi bulge**
Pathogenic	3	0	3	3	5	1
Endophytic (MF076580)	4	1	1	5	4	1
Endophytic isolate HCS3 (MN128230)*	4	1	1	5	4	1

*indicates own isolate



A B

Figure 4.15 Phylogenetic tree generated using Maximum Parsimony showing clustering of the isolate HCS3 under the clade *Colletotrichun coccodes*. Trees were constructed using ITS rDNA (A) and ITS2 (B) sequences.

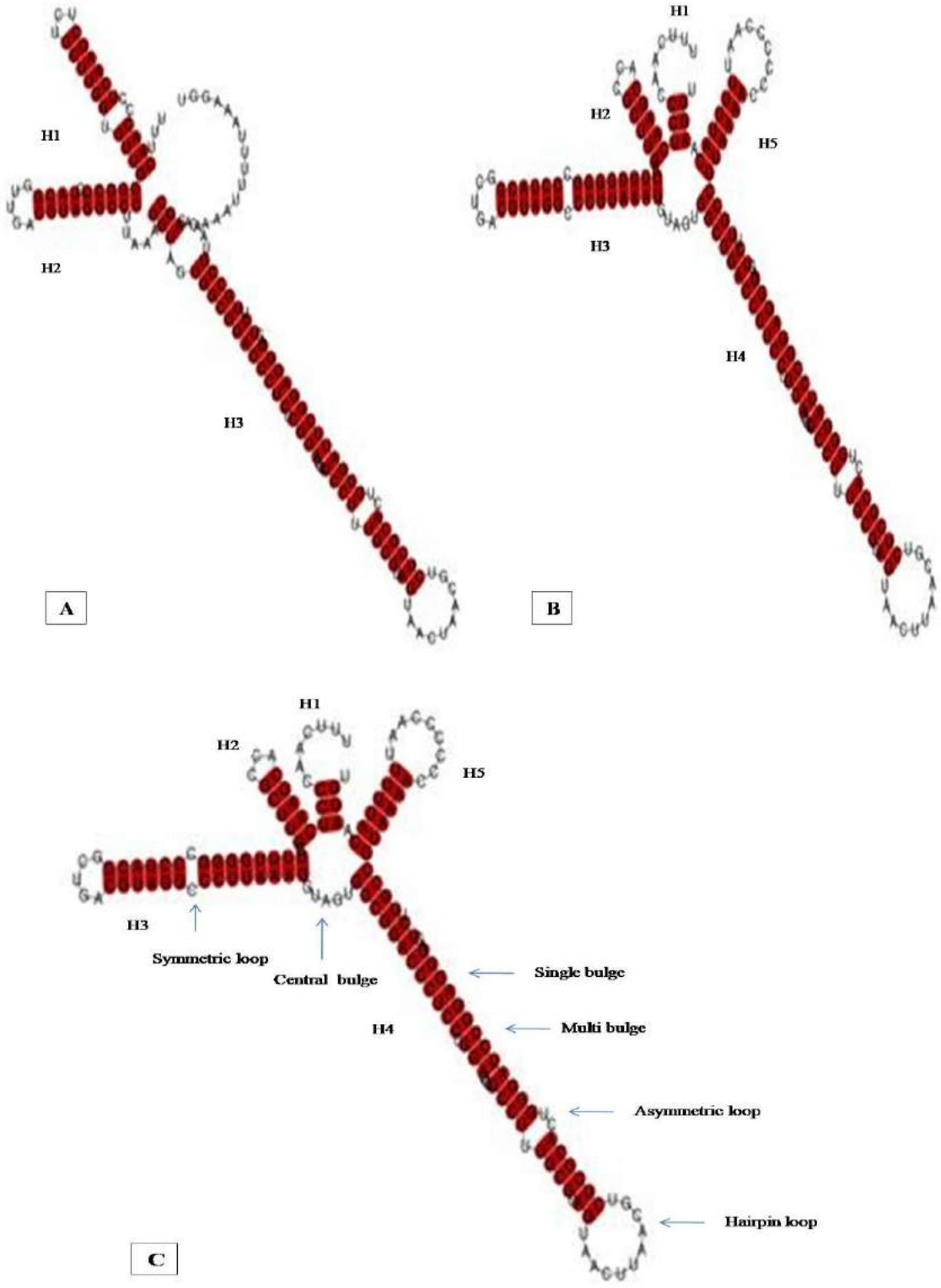


Figure 4.16 ITS2 secondary RNA structures showing comparison between different lifestyles of pathogenic (A) *Colletotrichun coccodes*, (B) endophytic *C. coccodes* (MF076580) and (C) isolate HCS3 *C. coccodes* (MN128230).

4.5.2 Molecular characterization and phylogeny of Morphotype (HCS6)

The isolate designated as Morphotype HCS6 showed similar colonial morphology and growth patterns to that of *Phyllosticta* sp. (**Fig. 4.17**) However, due to nonappearance of distinct reproductive structures or spores the identity of the isolate could not be confirmed. Therefore, to achieve proper identification the genomic DNA was isolated and sequencing of the ITS region was done. The region for ITS rDNA was amplified and sequenced using universal primers ITS4 and ITS5. The contig rDNA sequence so obtained was annotated and deposited to the GenBank with accession number MN170572. A BLAST homology search of ITS rDNA revealed that the isolate HCS6 (accession no. MN170572) showed closest homology with *Phyllosticta capitalensis* (MK105811.1) with Maximum Identity 98.91%, Query Coverage 89% and E-value 0.0. On this basis, a total of 2856 ITS rDNA sequences belonging to different *Phyllosticta* spp. were randomly retrieved from Genbank and screened for the presence of complete ITS rDNA region (18S rDNA-ITS1-5.8S-ITS2-rDNA-28S rDNA). To generate the phylogenetic tree, a total of 27 sequences with complete ITS rDNA regions were finally selected. A tree generated by MP method showed clustering of the isolate HCS6 with the clades of *Phyllosticta capitalensis* (**Fig. 4.18A**). The sequences were further trimmed for the ITS2 region and to validate the endophytic nature of the isolate, another phylogenetic tree was constructed using 19 ITS2 sequences. The MP phylogenetic tree showed that the isolate shared a close affinity with an endophytic *Phyllosticta capitalensis* (MK105811), supported by a bootstrap value of 100 (**Fig. 4.18B**). For further validation and prediction of the endophytic nature of the isolate, RNA secondary structure analysis was carried out using ITS2 sequences of endophytic *P. capitalensis* and was compared with the RNA secondary structure of the isolate HCS6. Due to unavailability of complete ITS2

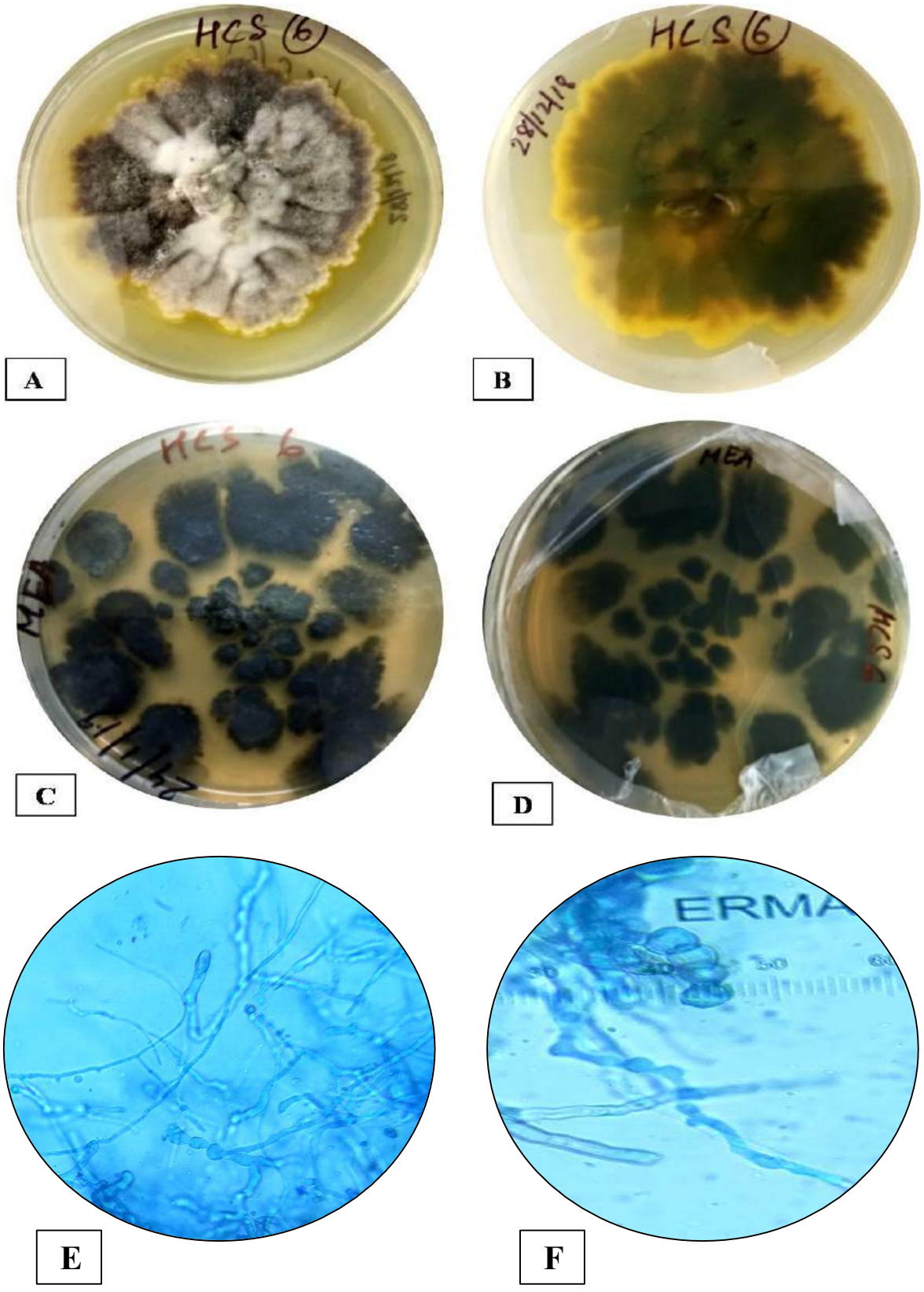


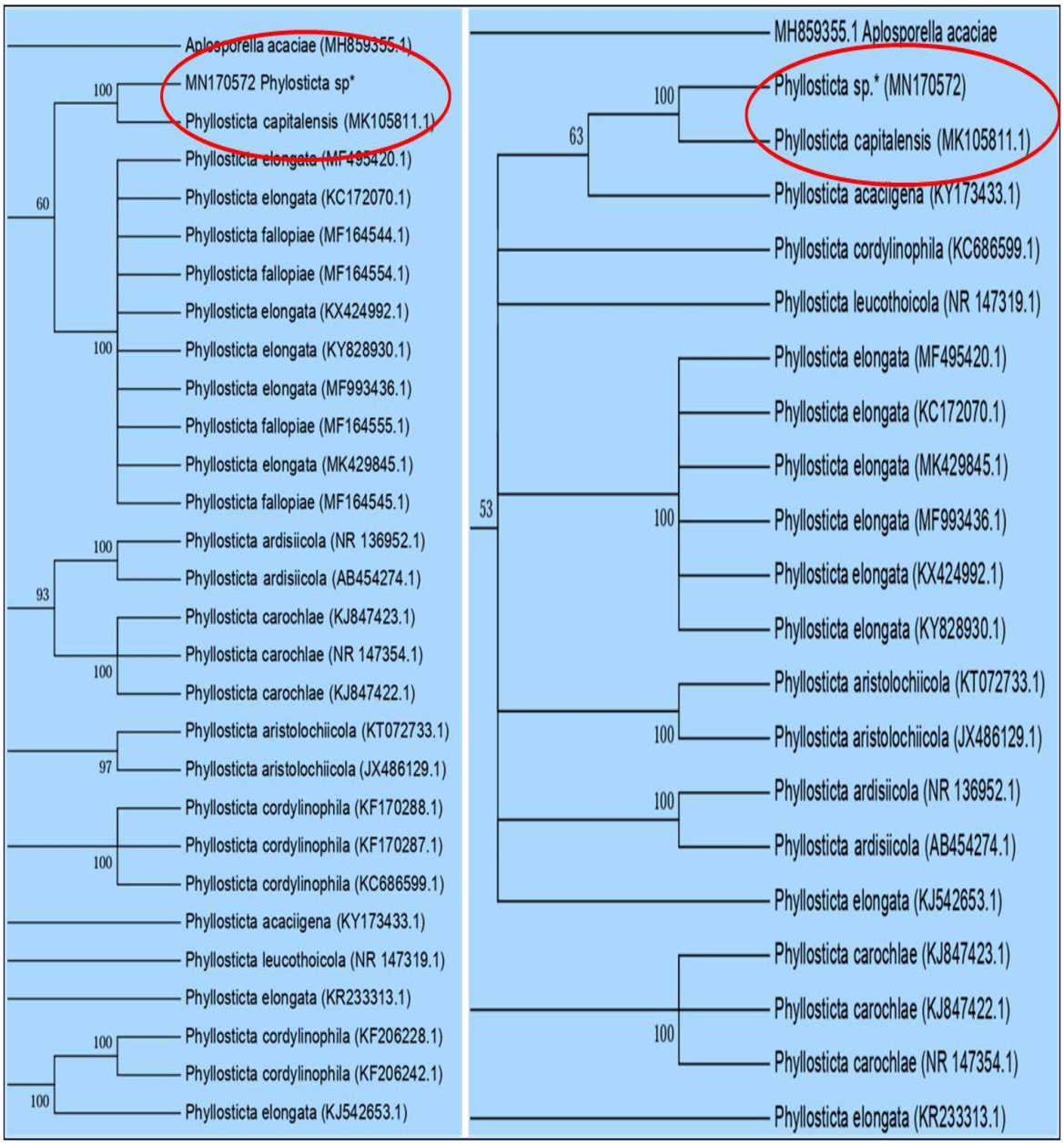
Figure 4.17 Colonial morphology of Morphotype (HCS6) on PDA medium: A) Front view, B) Reverse view and MEA: C) Front view D) Reverse view; Sterile Hyphae (E) and Conidia like structure (F) (under 40x).

sequences of pathogenic *P. capitalensis*, comparison could not be drawn with their pathogenic lifestyle. The consensus structure for endophytic *P. capitalensis* MK105811 consisted of a central bulge with 4 radiating major helices (H1, H2, H3 and H4), 2 symmetric loops, 2 asymmetric loops and 4 hairpin loops. Similar RNA secondary structure was also observed for isolate HCS6 (MN170572) with minor differences (**Fig. 4.19**). This revealed that the isolate shared extreme similarity with that of endophytic *P. capitalensis* both in terms of ITS2 folding pattern as well as identical structural motifs. The details about different structural features among the ITS2 RNA secondary structures of endophytic *P. capitalensis* are presented in the **Table 4.13**.

Table 4.13 Comparison of ITS2 secondary RNA motif features of *P. capitalensis* different life styles

***P. capitalensis* lifestyles**	**Hairpin loop (s)**	**Internal loop (s)**		**Helices**	**Bulges**	
		Symmetric	**Asymmetric**		**Single bulge**	**Multi bulge**
Endophytic isolate HCS6 (MN170572.1)*	4	2	2	4	4	1
Endophytic (MK105811.1)	4	2	2	4	3	1

*indicates own isolate



A B

Figure 4.18 Phylogenetic tree generated using Maximum Parsimony showing clustering of isolate HCS6 under the clade *Phyllosticta capitalensis*. Trees were constructed using ITS rDNA (A) and ITS2 (B) sequences.

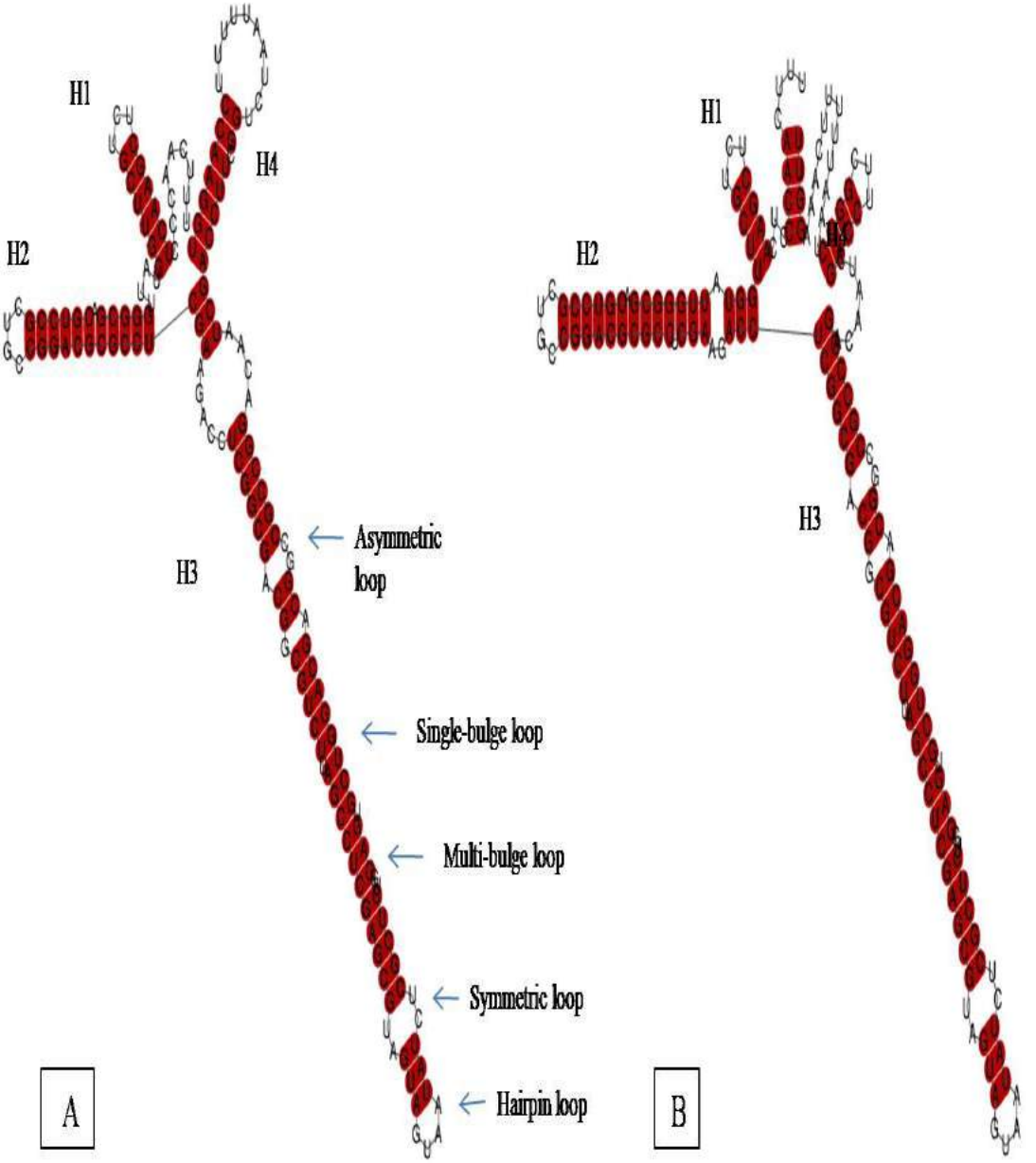


Figure 4.19 ITS2 secondary RNA structures showing comparison of different motif features between (A) isolate HCS6 *Phyllosticta* sp. (MN170572.1) and (B) endophytic *Phyllosticta capitalensis* (MK105811.1).

4.6 Characterization of bioactive secondary metabolites of potent fungal isolates

The crude organic extracts obtained from the six endophytic fungal isolates that show prominent antimicrobial activity were characterized by FTIR and GCMS analyses.

4.6.1 Metabolite profiling of endophytic fungus *Colletotrichum coccodes* (HCS3)

The ethyl acetate extract obtained from *C. coccodes* (HCS3) was characterized by FTIR spectroscopy analysis. **Figure 4.20** represents the FTIR spectra of ethyl acetate extract of the endophytic fungus *C. coccodes*. The FTIR spectroscopic analyses revealed the presence of different functional groups with bioactive properties which is presented in the **Table 4.14**. The wave number 668.97 cm^{-1} indicates presence of Halo compounds and the wave number 1746cm^{-1} indicates the presence of ester compounds and δ-lactones. Wave numbers 1166.90 cm^{-1} indicates the presence of Sulphonic acids and Sulphonamides while 1377.56 cm^{-1} indicates presence of Nitro compounds. The wave numbers of 1465.65 cm^{-1}, 2924.73 cm^{-1} and 3448.01cm^{-1} indicates the presence of Alkanes (containing methylene groups), Carboxylic acids and Primary Amines respectively.

The analysis of the GCMS chromatogram of the crude extract of *C. coccodes* HCS3 (**Fig. 4.21**) indicated the presence of several compounds which are reported to be bioactive. Analysis of the peaks revealed the presence of several compounds with bioactive properties which is listed in the **Table 4.15**. At peak value 53.121, compounds such as Geranyl geraniol, Farnesol and a phytosterol namely Squalene were detected. Peak value 40.241 indicated presence of Undecanoic acid and N-hexadecanoic acid. Other compounds like Phenol, 2, 4-Bis (1, 1-Dimethyl ethyl), Oxirane Phenyl, Hexacosanol Acetate and Oleic Acid were detected at peak values 30.04, 20.84, 42.47 and 44.28 respectively.

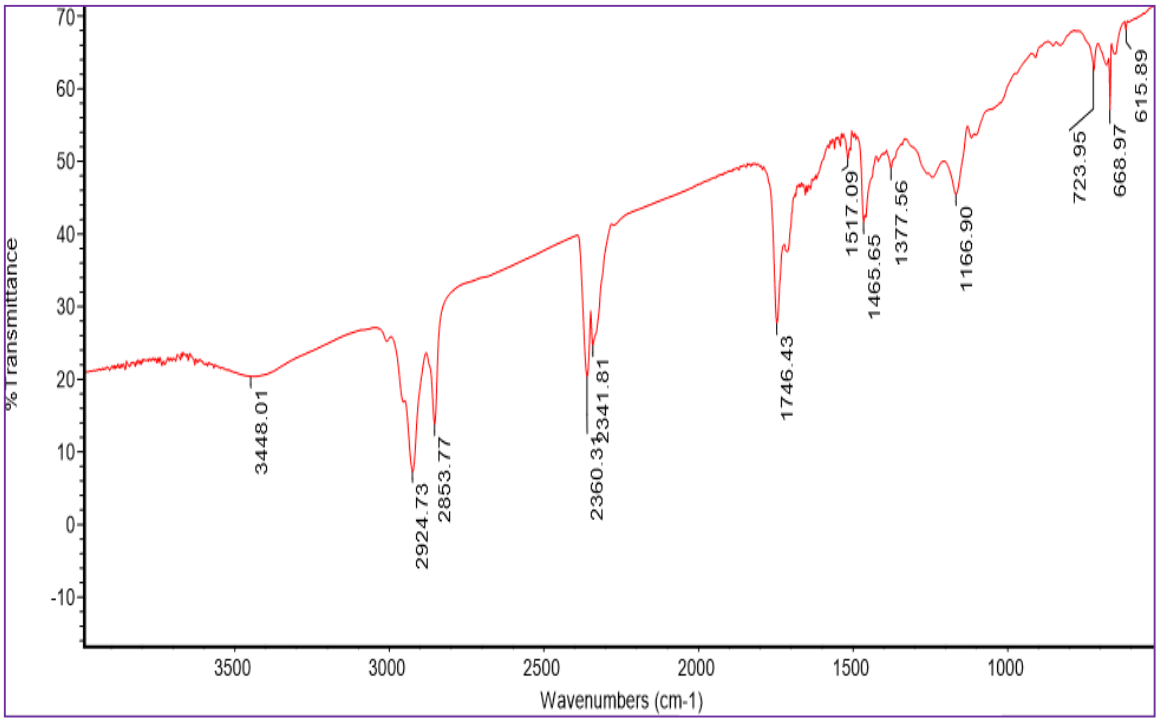


Figure 4.20 FTIR spectra of the ethyl acetate extract of endophytic fungus isolate HCS3 (*Colletotrichum coccodes*)

Table 4.14 Functional groups and compound classes present in the ethyl acetate extract obtained from endophytic fungus *C. coccodes*

Sl. No.	Wave number (cm^{-1})	Functional Group	Compound class
1	668.97	C-Br stretching	Halo compound
2	1166.90	S=O stretching	Sulfonic acid, Sulphonamide
3	1377.56	C-F stretching	Fluoro compound
4	1465.65	C-H bending	Alkane (Methylene group)
5	1517.09	N-O stretching	Nitro compound
6	1746.43	Ester	δ- lactones
7	2924.73	O-H stretching	Carboxylic acid
8	3448.01	N-H stretching	Primary amine

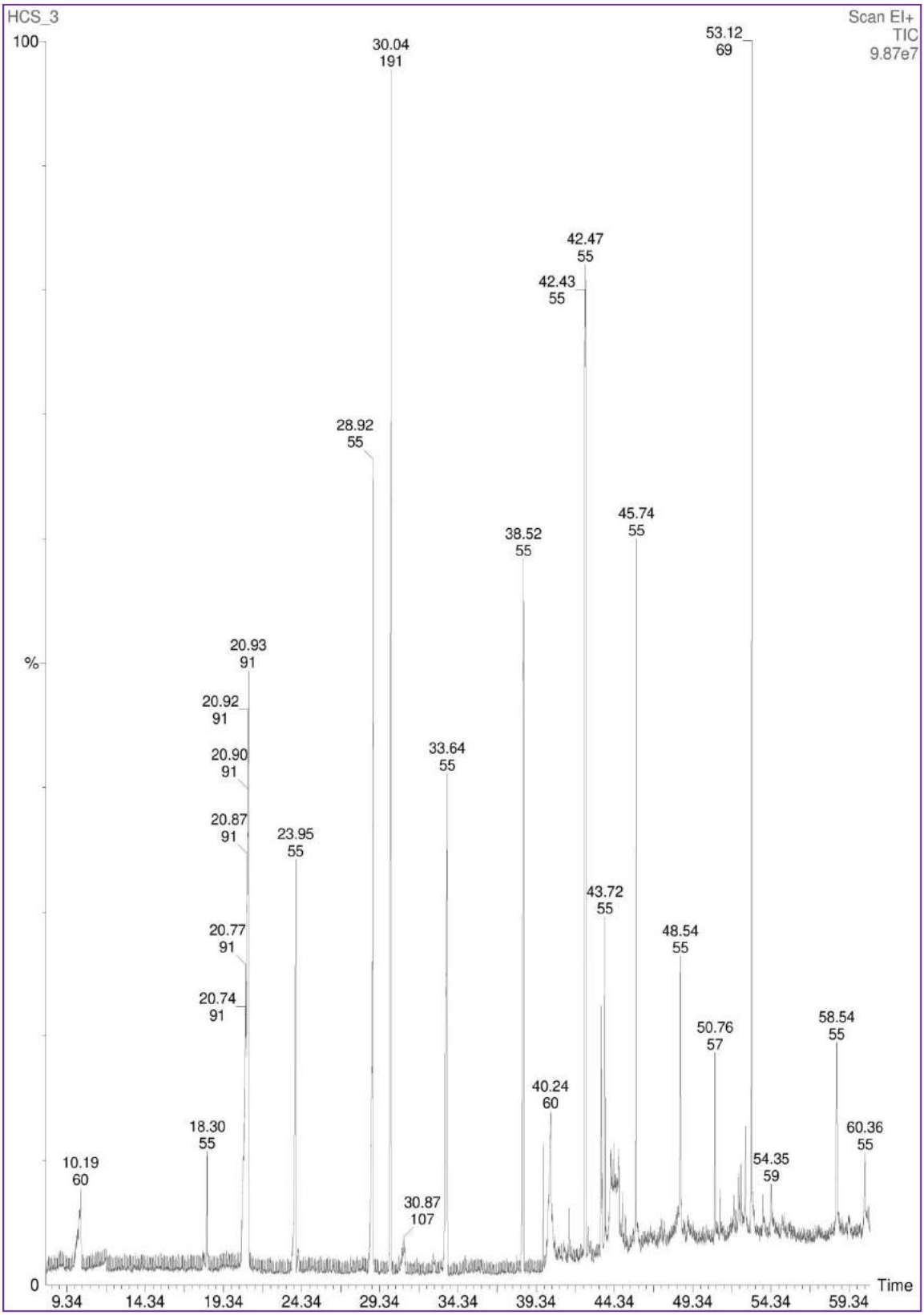


Figure 4.21 GCMS chromatogram of the ethyl acetate extract of endophytic fungus *C. coccodes*

Table 4.15 Major identified compounds present in the ethyl acetate extract of endophytic fungus *C. coccodes*

Compound	Retention Time	MW	Formula	Properties
Geranyl geraniol	53.12	290	C20H34O	Antimicrobial against *S. aureus* (Kobayashi *et al*., 2005)
Farnesol		236	C16H28O	Anti-quorum sensing molecule and virulence factor of *C. albicans* (Derengowski *et al*., 2009)
Squalene		410	C30H50	Used in the treatment of wounds and skin problems, antioxidant, cytotoxic activities against human cancer cell lines (De Los Reyes *et al*., 2015)
Phenol, 2, 4-Bis (1, 1-Dimethyl ethyl)	30.04	206	C14H22O	Antioxidant activity (Padmavathi, 2014)
Oxirane Phenyl	20.84	120	C8H8O	Anti-proliferative activity towards two cell lines (Montana *et al*., 2014)
Undecanoic acid	40.24	186	C11H22O2	Antifungal agent, inhibits biofilm formation (Shi *et al*., 2016; Liang, 2005)
N-Hexadecanoic acid		256	C16H32O2	Anti-inflammatory (Aparna *et al*., 2012)
Hexacosanol Acetate	42.47	424	C28H56O2	Antitumor properties (Mbosso *et al*., 2010)
Oleic Acid	44.28	282	C18H34O2	Anticancer (Jiang *et al*., 2017)

4.6.2 Metabolites profiling of endophytic fungus *Phyllosticta capitalensis* (HCS6)

The FTIR spectroscopic analysis of the ethyl acetate extract obtained from the endophytic fungus *Phyllosticta capitalensis* (HCS6) is presented in **Figure 4.22**. The FTIR spectroscopic analysis of ethyl acetate extracts of endophytic fungus *P. capitalensis* (HCS6) showed presence of different functional groups with varied range of compounds which is presented in the **Table 4.16**. The FTIR spectrum shows different peak values. Peak value 1262.64 cm^{-1} indicates presence of aromatic esters and alkyl aryl ether. The peaks at 1508.71 cm^{-1} and 1560.59 cm^{-1} indicate presence of nitro compounds and 1079.97 cm^{-1} indicates presence of amines. Peak value of 1718.45 cm^{-1} showed presence of compounds such as α, β-unsaturated esters and esters while and peak value of 1739.33 cm^{-1} indicated presence of δ-lactones. Alkenes were indicated by peaks at 965.17 $cm^{-1}$and 1654.96 cm^{-1}. Peak values of 2853.91cm^{-1}, 2924.92cm^{-1} and 2960.92 cm^{-1} indicated the presence of alkanes.

GCMS analysis obtained from *P. capitalensis* HCS6 indicated the presence of various compounds (**Fig. 4.23**). The identified compounds with their bioactive properties are listed in the **Table 4.17**. A major compound Phenol, 2, 4-Bis (1, 1-Dimethyl ethyl) was identified with peak value 30.02. Peak value 45.769 indicated presence of 1-Octacosanol. Further, peak values 44.10 and 48.57 showed the presence of Oleic Acid and Hexacosanol Acetate. Other compounds that were present include Phenol,3,5-Bis (1,1-Dimethylethyl), 7-Hexadecene, 7-Tetradecene, Undec-10-Ynoic Acid, 2-Decanynoic Acid, 9-Tricosene, 8-Heptadecene, Acetic Acid, Hexadecyl Ester and 5-Eicosene.

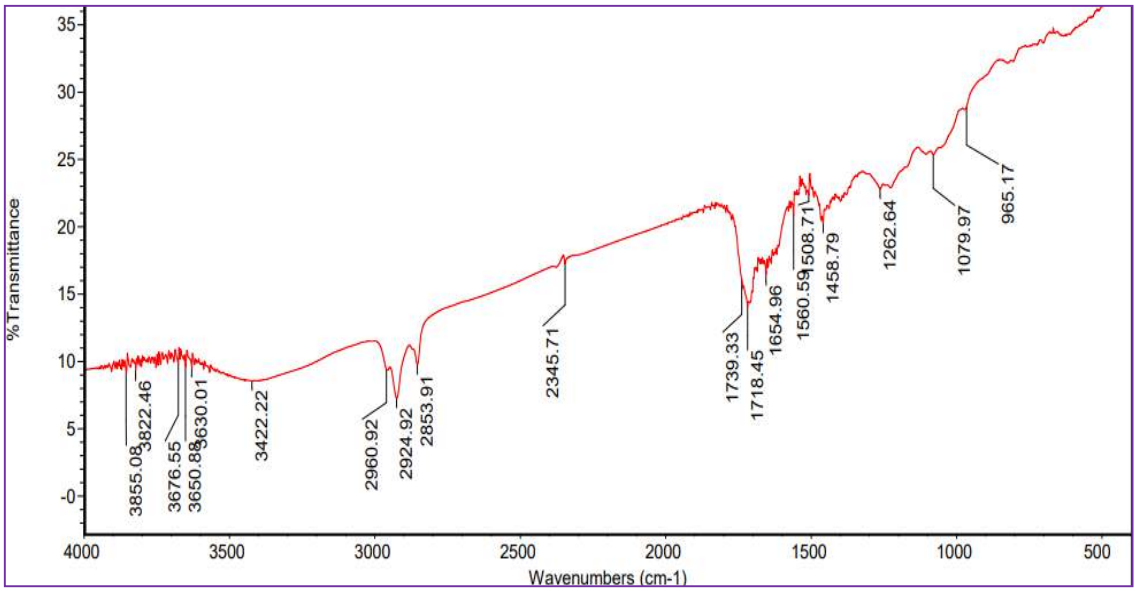


Figure 4.22 FTIR spectra of the ethyl acetate extract of endophytic fungus isolate HCS6 (*Phyllosticta capitalensis*)

Table 4.16 Functional groups and compound classes present in the ethyl acetate extract obtained from endophytic fungus *P. capitalensis*

Sl. No.	Wave number (cm^{-1})	Functional Group	Compound class
1	965.17	C=C bonding	Alkene
2	1079.97	C-N stretching	Amine
3	1262.64	C=O stretching	Aromatic ester; Alkyl aryl ether
4	1508.71	N=O stretching	Nitro compound
5	1560.59	N=O stretching	Nitro compound
6	1654.96	C=C stretching	Alkene
7	1718.45	C=O stretching	α, β-unsaturated esters
8	1739.33	C=O stretching	Esters; δ-lactone
9	2853.91	C-H stretching	Alkane
10	2924.92	C-H stretching	Alkane
11	2960.92	C-H stretching	Alkane

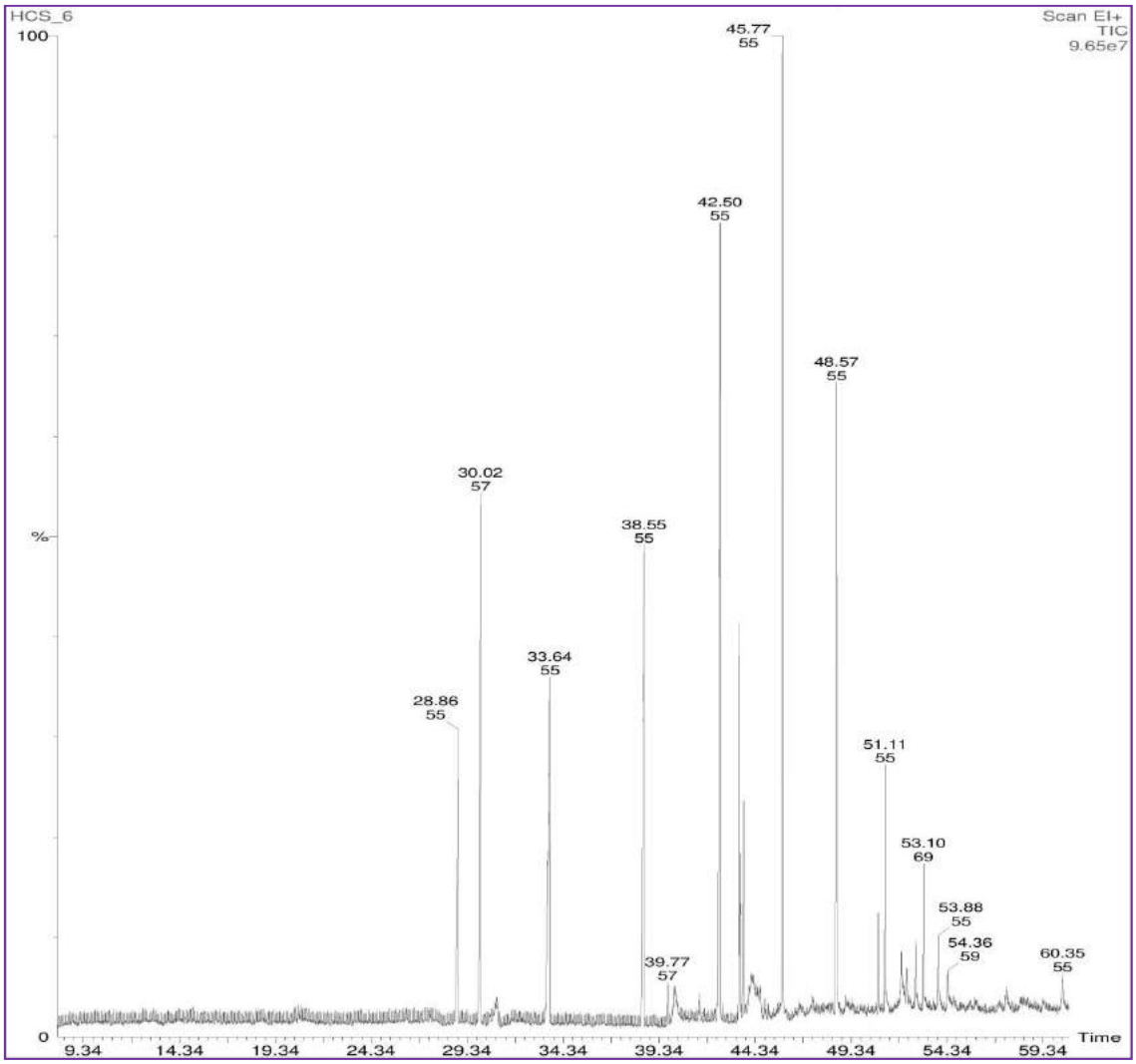


Figure 4.23 GCMS chromatogram of the ethyl acetate extract of endophytic fungus *P. capitalensis*

Table 4.17 Major identified compounds present in the ethyl acetate extract of endophytic fungus *P. capitalensis*

Compound	Retention Time	MW	Formula	Properties
Phenol, 2, 4-Bis (1, 1-Dimethyl ethyl)	30.02	206	C14H22O	Antioxidant activity (Padmavathi, 2014)
Oleic Acid	44.10	282	C18H34O2	Anticancer (Jiang *et al*., 2017)
1-Octacosanol	45.76	410	C28H58O	Anti-oxidant and anti-bacterial activity (Sengupta *et al*., 2018)
Hexacosanol Acetate	48.57	424	C28H56O2	Antitumor properties (Mbosso *et al*., 2010)

4.6.3 Metabolite profiling of endophytic fungus *Scopulariosis brevicaulis* (EF1)

FTIR spectroscopic analysis of ethyl acetate extracts of endophytic fungus *Scopulariopsis brevicaulis* (EF1) also showed peaks that revealed the presence of different functional groups as revealed from the spectrum is shown in **Figure 4.24**. Wave numbers at different peaks indicated presence different classes of compounds with various functional groups which are presented in the **Table 4.18**. The wave number 1261 cm^{-1} indicated presence of aromatic esters and alkyl aryl esters. Presence of alkanes was indicated in the wave numbers 1465.04 cm^{-1}, 2853.98 cm^{-1} and 2925.10 cm^{-1}. Similarly, wave number 1742.54 cm^{-1} showed presence of esters and δ-lactones. Presence of alcohol and primary amines were detected in wave number 3447.56 cm^{-1}.

GCMS chromatogram analysis of the crude extracts of the fungus, *S. brevicaulis* (EF1) revealed the presence of various compounds (**Fig. 4.25**). The major identified with different peak values are presented in **Table 4.19.** The peak value 29.89 indicated the presence of the compounds Phenol, 2, 4-Bis (1, 1-Dimethyl ethyl) and Oxirane. Another compound namely, Acety tributyl citrate was also detected in the peak 49.41. All the compounds showed wide range biological activities like antimicrobial and antioxidant properties.

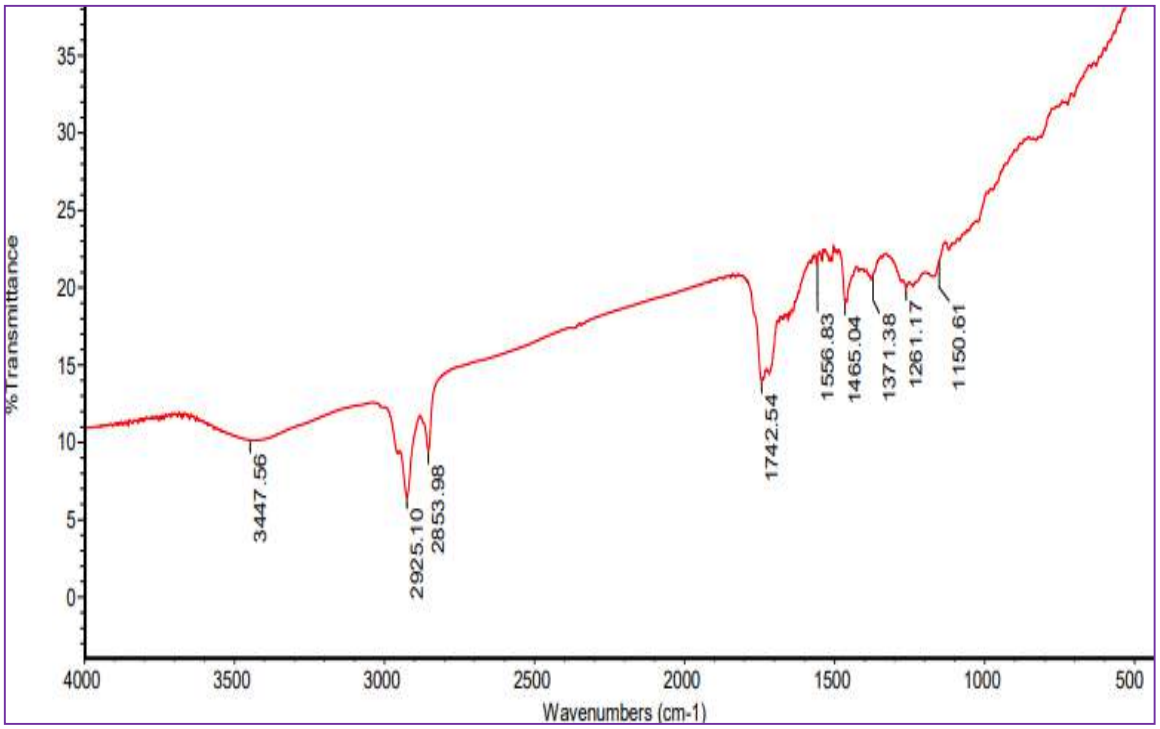


Figure 4.24 FTIR spectra of the ethyl acetate extract of endophytic fungus isolate EF1 (*Scopulariopsis brevicaulis*)

Table 4.18 Functional groups and compound classes present in the ethyl acetate extract obtained from endophytic fungus *S. brevicaulis*

Sl. No.	Wave number (cm^{-1})	Functional Group	Compound class
1	1261.17	C-O stretching	Aromatic esters; alkyl aryl ether
2	1465.04	C-H bonding	Alkane
3	1742.54	C=O stretching	Esters; δ-lactone
4	2853.98	C-H stretching	Alkane
5	2925.10	C-H stretching	Alkane
6	3447.56	O-H stretching N-H stretching	Alcohol Primary amine

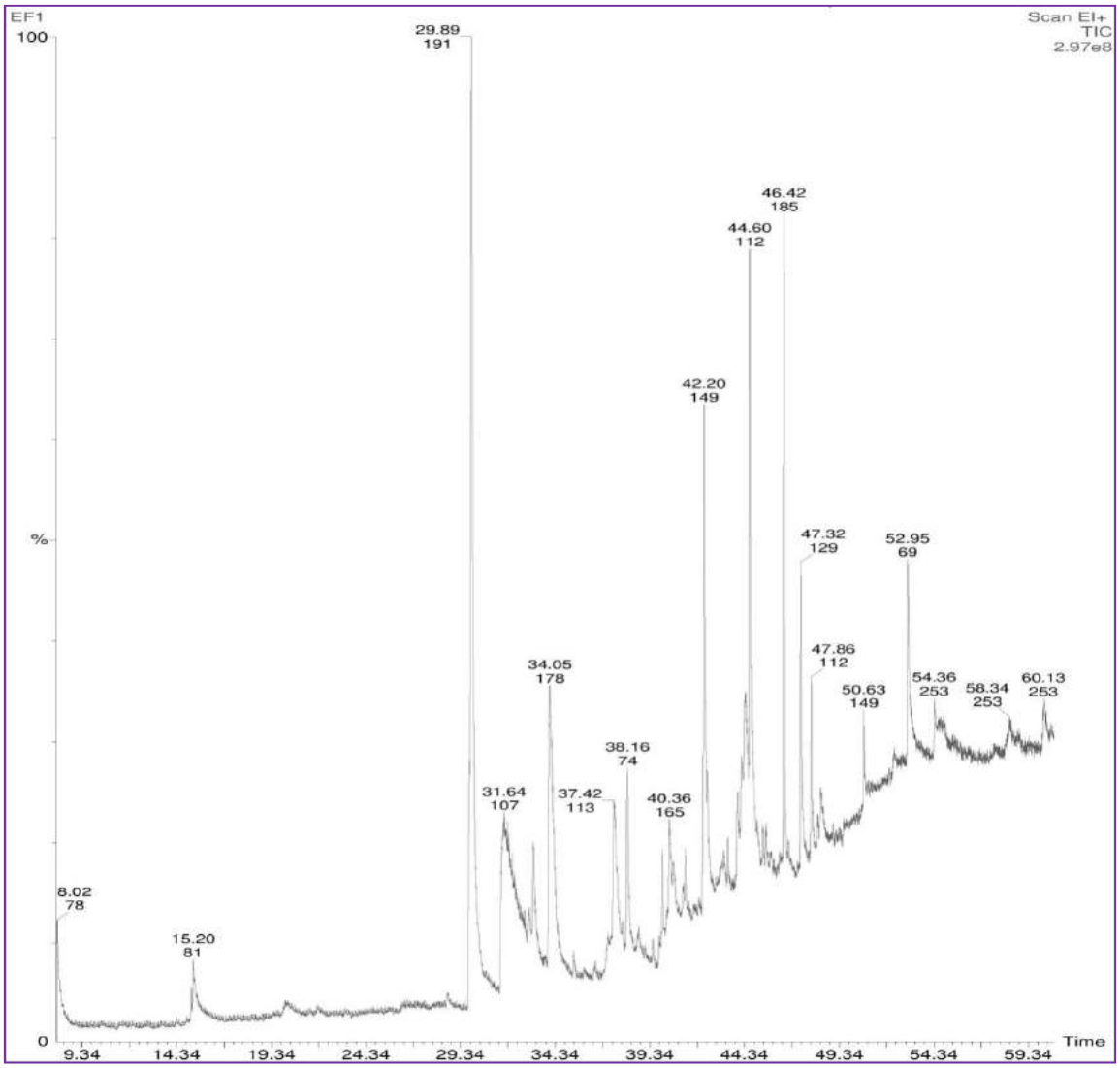


Figure 4.25 GCMS chromatogram of the ethyl acetate extract of endophytic fungus *S. brevicaulis*

Table 4.19 Major identified compounds present in the ethyl acetate extract of endophytic fungus *S. brevicaulis*

Compound	Retention Time	MW	Formula	Properties
Phenol, 2, 4-Bis (1, 1-Dimethyl ethyl)	29.89	206	C14H22O	Antioxidant activity (Padmavathi, 2014)
Oxirane		206	C8H8O	Anti-proliferative activity towards two cell lines (Montana *et al*., 2014)
Acety Tributyl citrate	46.41	402	C20H34O8	Pharmaceutical excipient (Kim *et al*., 2018)

4.6.4 Metabolite profiling of endophytic fungus *Purpureocillium lilacinum* (EF6)

FTIR spectroscopic analysis of ethyl acetate extracts of endophytic fungus *Purpureocillium lilacinum* (EF6) revealed the presence of different compound classes belonging to various functional groups as depicted from the spectrum shown in **Figure 4.26**. Wave numbers at different peaks indicated the presence of different functional group is presented in the **Table 4.20**. The wave number 722.26 cm^{-1} indicates the presence of mono-substituted benzene derivatives. Presence of compounds belonging to esters and tertiary alcohol groups were detected in the wave number 1166.98 cm^{-1}. Wave numbers 1261.15 cm^{-1} and 1464.70 cm^{-1} indicated presence of alkyl aryl ether and alkanes respectively. Compound classes like δ-lactones were detected in wave number 1746.70 cm^{-1}.

Further the chromatogram obtained from GCMS analysis of the crude extract of the fungal isolate *P. lilacinum* (EF6) (**Fig. 4.27**) revealed the presence of major compounds like Di-N-Octyl phthalate, Bis (2-ethylhexyl) phthalate and 1, 2-Benzene dicarboxylic acid at peak value 50.61. The occurrence of various compounds in the crude extract of *P. lilacinum* (EF6) is presented in **Table 4.21**. Similar to other endophytic extracts, the metabolites also showed antimicrobial activities against wide range of pathogens.

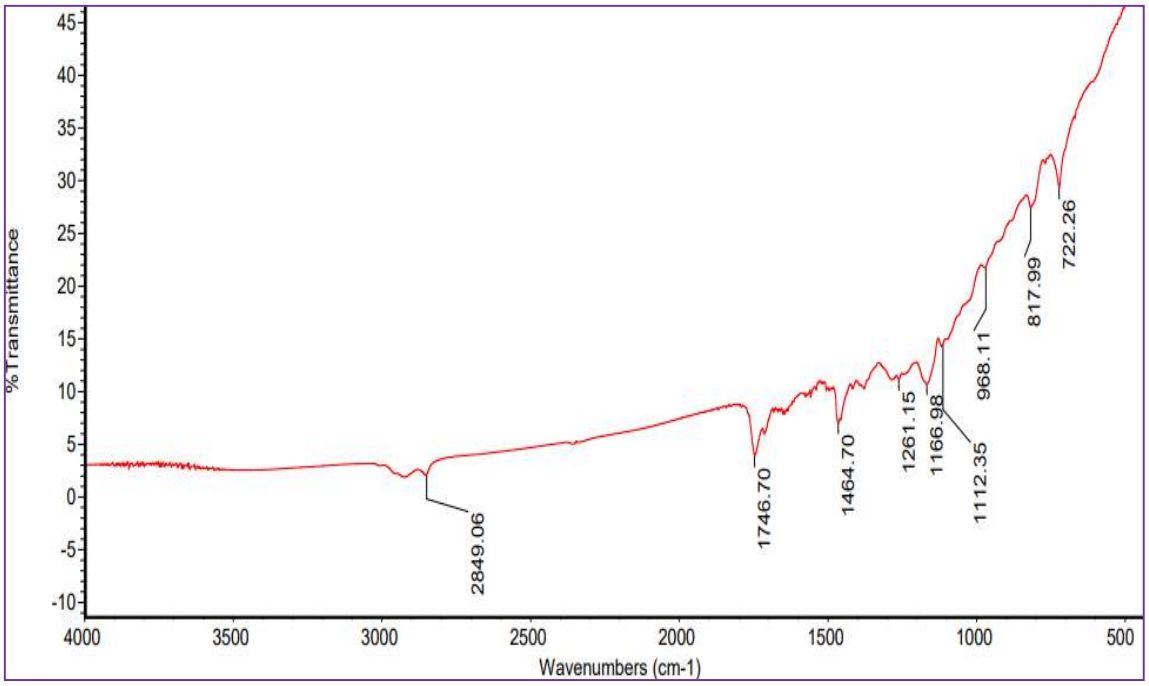


Figure 4.26 FTIR spectra of the ethyl acetate extract of endophytic fungus isolate EF6 (*Purpureocillium lilacinum*)

Table 4.20 Functional groups and compound classes present in the ethyl acetate extract obtained from endophytic fungus *P. lilacinum*

Sl. No.	Wave number (cm^{-1})	Functional Group	Compound class
1	722.26	C-H bonding	Mono-substituted benzene derivative
2	1166.98	C- O stretching	Ester; Tertiary alcohol
3	1261.15	C-O stretching	Alkyl aryl ether
4	1464.70	C-H bending	Alkane (methyl group)
5	1746.70	C=O stretching	δ-lactone

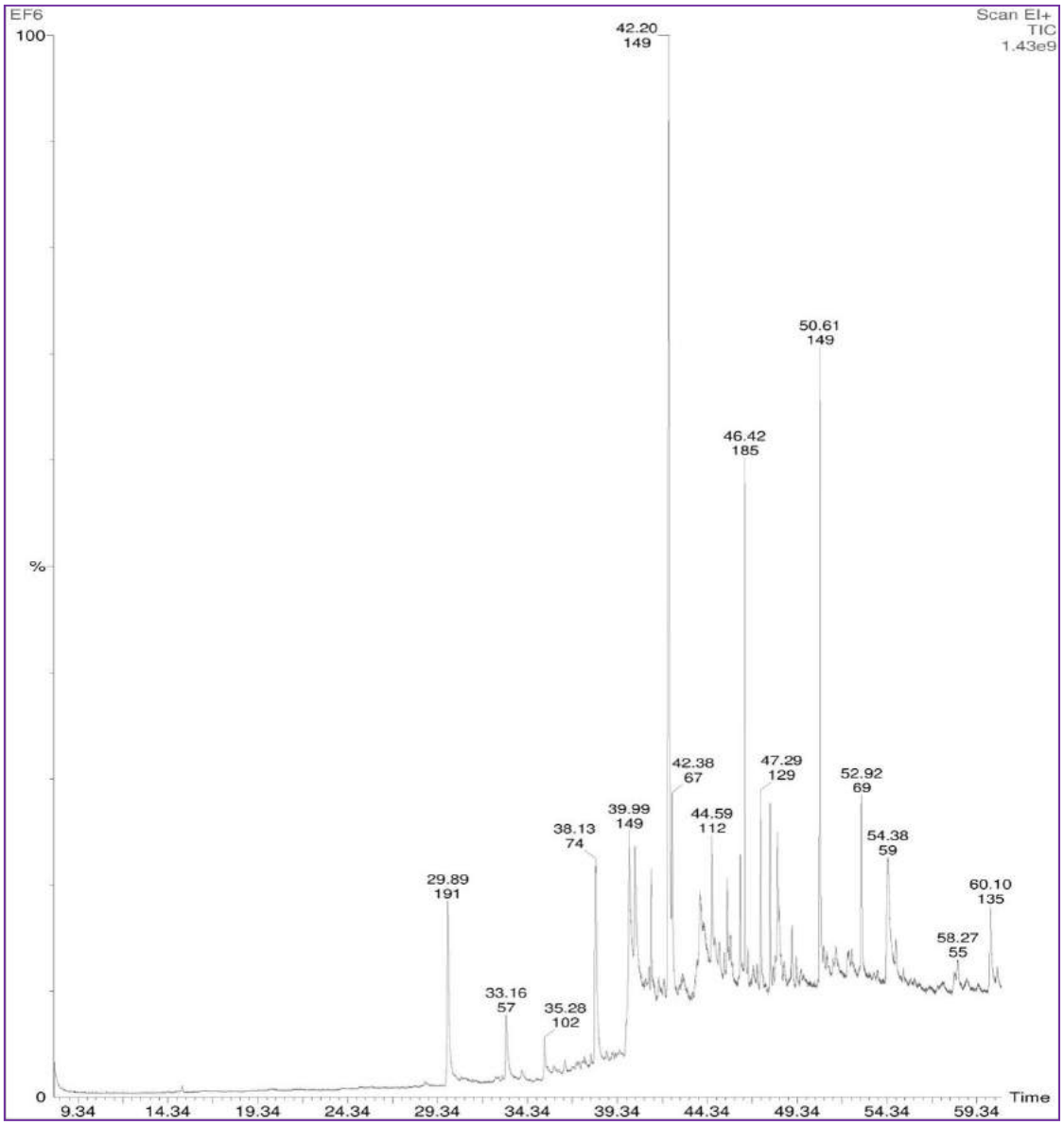


Figure 4.27 GCMS chromatogram of the ethyl acetate extract of endophytic fungus *P. lilacinum*

Table 4.21 Major identified compounds present in the ethyl acetate extract of endophytic fungus *P. lilacinum*

Compound	Retention Time	MW	Formula	Properties
Dibutyl Phthalate	42.19	278	C16H22O4	Antimicrobial compound (Roy *et al*., 2006)
Tributyl acetyl citrate	46.41	402	C20H34O8	Pharmaceutical excipient (Kim *et al*., 2018)
1, 2-Benzene dicarboxylic Acid	50.61	278	C16H22O4	Antimicrobial agent (Roy *et al*., 2016)

4.6.5 Metabolite profiling of endophytic fungus *Penicillium chrysogenum* (EFB9)

FTIR spectrum of the ethyl acetate extract of endophytic fungus, *Penicillium chrysogenum* EFB9 (**Fig. 4.28**) also revealed presence of various compound classes with different functional groups at different wave numbers. The functional groups with their compound classes are presented in **Table 4.22**. Wave number 1165.34cm^{-1} indicated presence of esters and tertiary alcohols. Presence of fluoro compounds and phenols were detected in the wave number 1376.85 cm^{-1}. Wave numbers 1458.11 cm^{-1} and 2853.44 cm^{-1} revealed presence of alkanes (containing methyl groups) while wave number 1646.77 cm^{-1} showed the presence of alkenes. Similarly, the presence of alcohols and primary amines in the extract were indicated in the wave number 3447.84cm^{-1}.

Analysis of the crude extract by GCMS showed a chromatogram (**Fig. 4.29**) that revealed the presence of different compounds which is presented in the **Table 4.23**. The extract also showed presence of compounds like Geranyl geraniol and Squalene were also found to be present in the extract as detected from the peak value 52.93. Another excipient compound, Tributyl acetyl citrate was also revealed from peak value 46.41.

4.6.6 Metabolite profiling of endophytic fungus *Colletotrichum gleosporoides* (ZOB3)

FTIR spectrum of the crude ethyl acetate extract of endophytic fungus, *Colletotrichum gleosporoides* (ZOB3) revealed presence of various functional groups with different compound classes (**Fig. 4.30**). The compound classes like esters and tertiary alcohols were indicated by wave number 1165.34 cm^{-1}. Presence of fluoro compounds and nitro compounds were revealed by the wave number 1376.85 cm^{-1}.

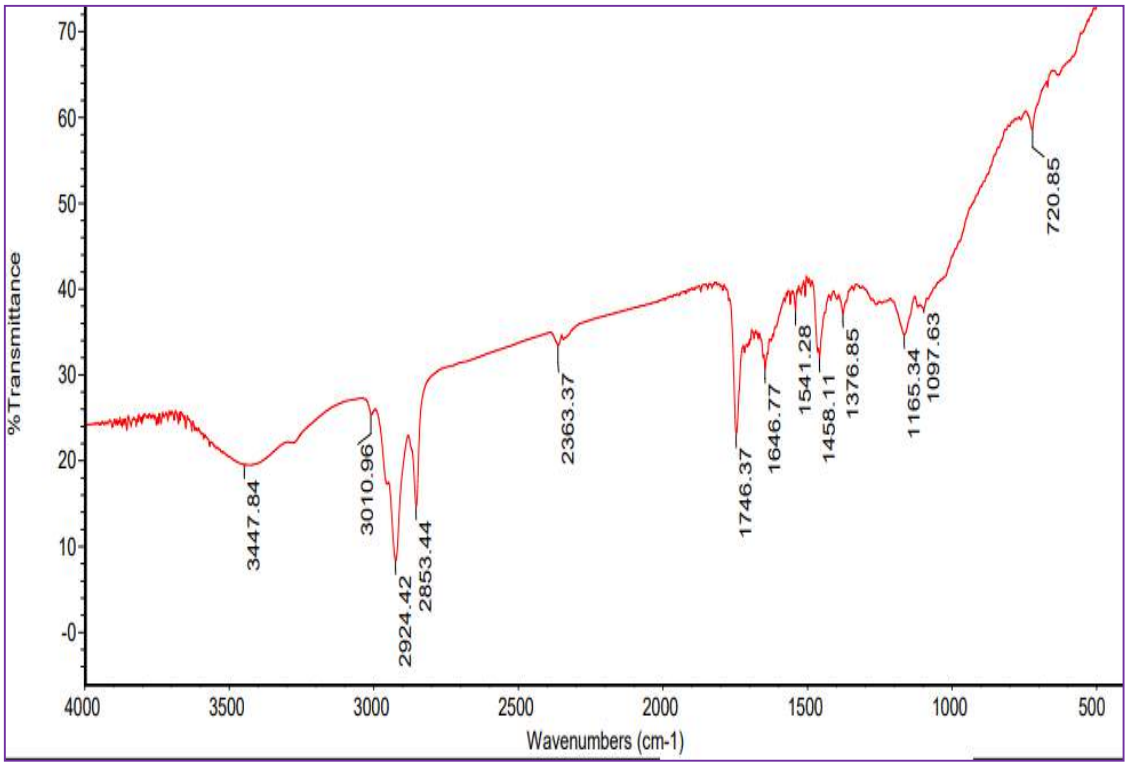


Figure 4.28 FTIR spectra of the ethyl acetate extract of endophytic fungus isolate EFB9 (*Penicillium chrysogenum*)

Table 4.22 Functional groups and compound classes present in the ethyl acetate extract obtained from endophytic fungus *P. chrysogenum*

Sl. No.	Wave number (cm^{-1})	Functional Group	Compound class
1	1165.34	C-O stretching	Ester; Tertiary alcohol
2	1376.85	C-F stretching O-H bending	Fluoro compound Phenol
3	1458.11	C-H bending	Alkane(methyl group)
4	1646.77	C=C stretching	Alkene
5	2853.44	C-H stretching	Alkane
6	3447.84	O-H stretching N-H stretching	Alcohol Primary amine

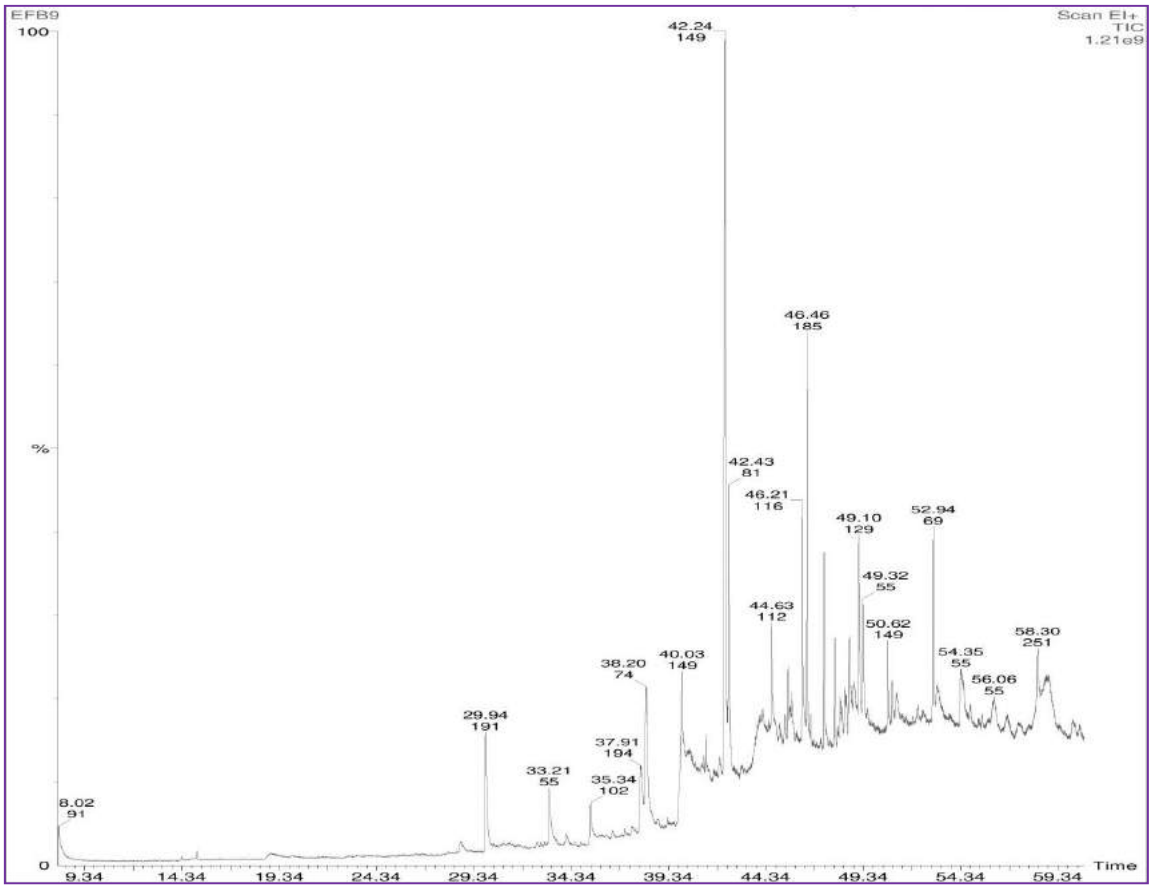


Figure 4.29 GCMS chromatogram of the ethyl acetate extract of endophytic fungus *Penicillium chrysogenum*

Table 4.23 Major identified compounds present in the ethyl acetate extract of endophytic fungus *P. chrysogenum*

Compound	Retention Time	MW	Formula	Properties
Hexadecanoic Acid, 1-(Hydroxymethyl)-1,2-Ethanediy	49.10	568	C35H68O5	Antifungal, Antioxidant and Cancer preventive (Devi and Serfoji, 2018)
Tetradecenal (Z)		210	C14H26O	Antioxidant (Mujeeb *et al*., 2014)
Squalene	52.93	410	C30H50	Treatment of wounds and skin problems, antioxidant, cytotoxic activities against human cancer cell lines (De Los Reyes *et al*., 2015)
Geranyl geraniol		290	C20H34O	Antimicrobial against *S. aureus* (Kobayashi *et al*., 2005)

Wave numbers 1458.11cm^{-1}, 2853.44cm^{-1} and 2924.42cm^{-1} indicated the presence of alkanes and the wave number 1646.77cm^{-1} showed the presence of alkenes. The extract also showed presence of alcohol and primary amines as indicated by the wave number 3447.84 cm^{-1}. The different compound classes present in the extract are presented in **Table 4.24**.

Chromatogram obtained by GCMS analysis of the crude extract of *C. gleosporoides* (ZOB3) (**Fig. 4.31**) revealed the presence of various compounds at different retention time as shown in **Table 4.25**. The peak value 22.18 indicated the presence of 2-Allylphenol. Presence of compounds like Oxirane and 3-Methyl-2-(2-Oxopropyl) Furan were detected in peak value 27.01. Peak value at 32.38 indicated presence of 3-Benzylsulfanyl-3-Fluoro-2-Trifluoromethyl-Acrylonitrile and Tributyl acetyl citrate.

The FTIR and GCMS analysis therefore provided an insight to the variety of compounds produced by the endophytic fungi with wide range of biological activities. Some of the compounds here reported to exhibit antimicrobial, antioxidant, anti-inflammatory and cytotoxic activities like Geranyl geraniol, Squalene, Phenol, 2, 4-Bis (1, 1-Dimethyl ethyl), Oleic Acid, 1-Octacosanol, Hexacosanol Acetate were commonly revealed from most of the isolates. Thus, GCMS analysis of the crude extracts obtained from endophytic fungi showed wide range of compounds. However, metabolites obtained from *C. coccodes* HCS3 showed strong biological activities with maximum number of compounds followed by *P. capitalensis* HCS6.

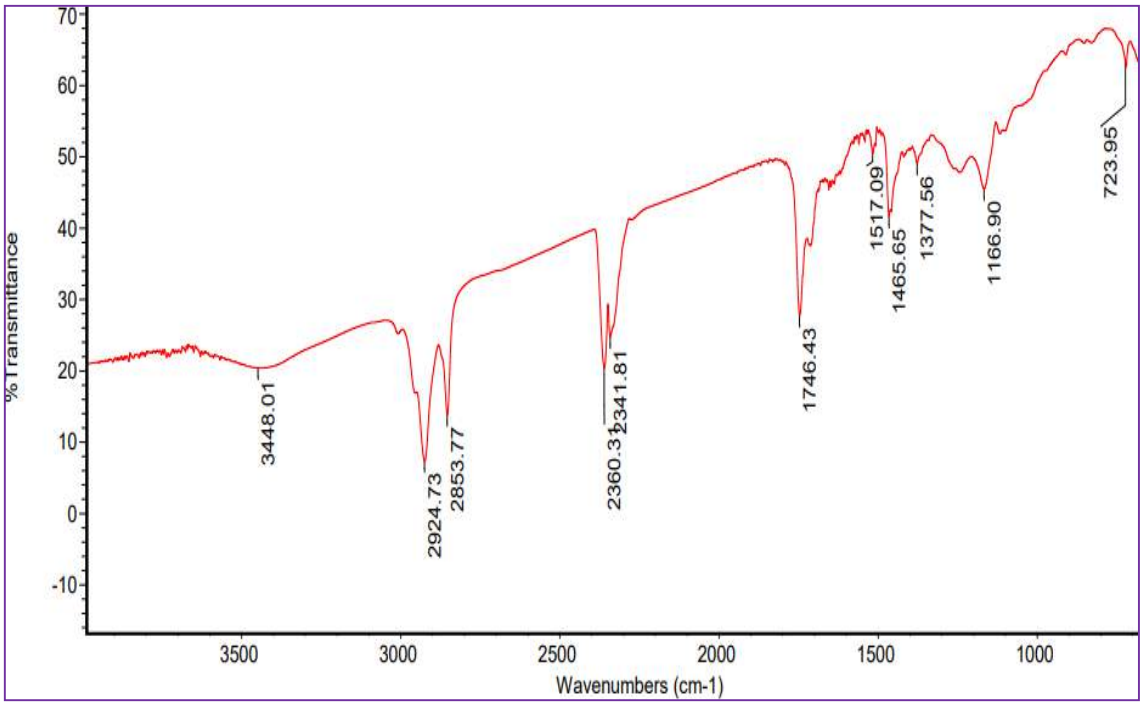


Figure 4.30 FTIR spectra of the ethyl acetate extract of endophytic fungus isolate ZOB3 (*Colletotrichum gleosporoides*)

Table 4.24 Functional groups and compound classes present in the ethyl acetate extract obtained from endophytic fungus *C. gleosporoides*

Sl. No.	Wave number (cm^{-1})	Functional Group	Compound class
1	1165.34	C-O stretching	Ester; Tertiary slcohol
2	1376.85	C-F stretching O-H bending	Fluoro compound Phenol
3	1458.11	C-H bending	Alkane(methyl group)
4	1541.28	N-O stretching	Nitro compound
5	1646.77	C=C stretching	Alkene
6	2853.44	C-H stretching	Alkane
7	2924.42	C-H stretching	Alkane
8	3447.84	O-H stretching N-H stretching	Alcohol Primary amine

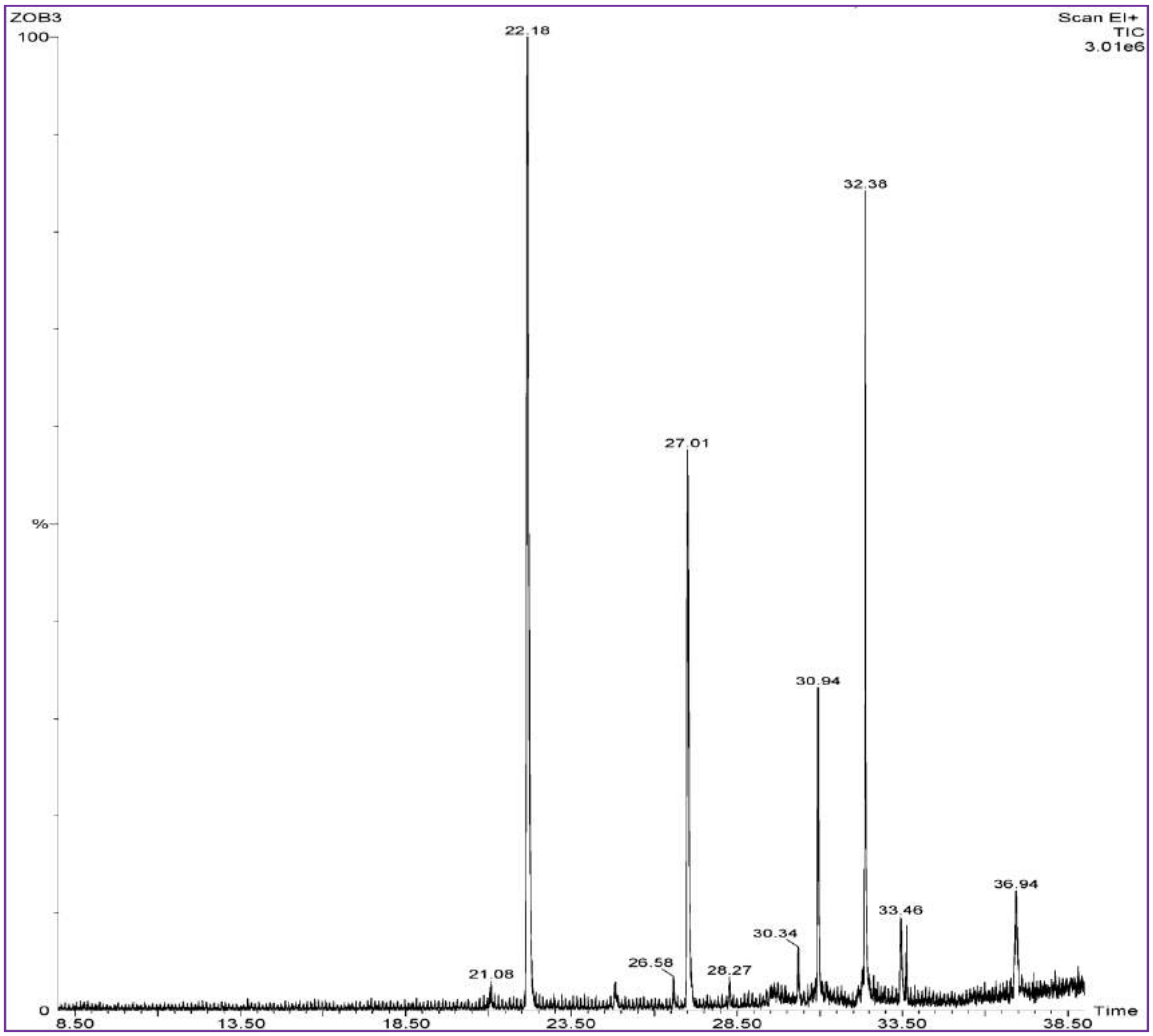


Figure 4.31 GCMS chromatogram of the ethyl acetate extract of endophytic fungus *C. gleosporoides*

Table 4.25 Major identified compounds present in the ethyl acetate extract of endophytic fungus *C. gleosporoides*

Compound	Retention Time	MW	Formula	Properties
2-Allylphenol	22.18	366	C9H10O	Antioxidant, antimicrobial, anti-proliferative, anti-inflammatory (Neto *et al*., 2019)
Oxirane	27.01	156	C8H8O	Anti-proliferative activity towards two cell lines (Montana *et al*., 2014)
3-Methyl-2-(2-Oxopropyl)Furan		138	C8H10O2	Antimicrobial (Al-Wathnani *et al*., 2012; Biswal, 2020)

4.7 Purification and identification of metabolites of *C. coccodes* HCS3

Upon FTIR and GCMS analyses, it was found that the metabolites obtained from the endophytic fungus, *C. coccodes* HCS3 showed the presence of several bioactive compounds and hence was further analyzed to purify and identify the pure compound that might have a contribution to its bioactive properties. The organic extract obtained from *C. coccodes* HCS3 (99.9 mg) was purified on a silica gel column eluted with $CHCl_3$-*i*-PrOH (9:1) affording 7 groups of homogeneous fractions. The residue (7.60 mg) of the third fraction was further purified by analytical TLC eluted with $CHCl_3$-*i*-PrOH (9:1) yielding an amorphous solid. ^{1}H NMR spectra were recorded at 500 MHz, in $CDCl_3$. The same solvent was used as an internal standard. Electro spray Ionization/Mass Spectrometry (ESI/MS) and liquid chromatography (LC)/MS analyses were performed using the LC/ MS time-of- light (TOF) system. Analytical and preparative thin-layer chromatography (TLC) was carried out on silica gel and reversed-phase plates. Column chromatography was performed using silica gel. The pure compound was identified as tyrosol by comparing its spectroscopic properties with the data reported in literatures. Tyrosol (**Fig. 4.33**) had: ^{1}H NMR (500 MHz), δ: 7.10 (d, *J* = 8.2 Hz, H-2 and H-6), 6.79 (d, *J* = 8.2 Hz, H-3 and H-5), 3.82 (t, *J* = 6.6 Hz, H_2-8), 2.80 (t, *J* = 6.6 Hz, H_2-7) and ESIMS (+), *m/z*: 299 $[2M + Na]^+$, 161 $[M + Na]^+$.

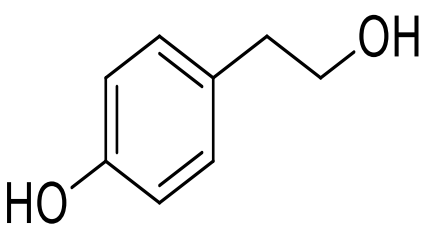


Figure 4.32 Chemical structure of Tyrosol isolated from *Colletotrichum coccodes*.