6.1 Introduction

Microbial secondary metabolites are emerging as alternatives for synthetic compounds. Fungal endophytes have been shown to produce various bioactive compounds like artemisinin, morphine, cannabinoids and others (Kusari and Spiteller, 2011). The possibility of endophytes synthesizing the same compounds as that of the host plant was reported. The discovery that the endophyte \textit{Taxomyces andreanae} isolated from the Pacific yew tree \textit{Taxus brevifolia} produces the anticancer compound Taxol (Stierle \textit{et al}., 1993). Many reported that Taxol was isolated from the endophytic fungi \textit{viz.}, \textit{Pestalotiopsis microspora} from \textit{Taxodium distichurn} (Jia-yao \textit{et al}., 1996), \textit{Pestalotiopsis quepinii} from \textit{Wollemia nobilis} (Strobel \textit{et al}., 1997), \textit{Periconia} sp. from \textit{Torreya grandifolia} (Li \textit{et al}., 1998), \textit{Tubercularia} sp. from \textit{Taxus mairei} (Wang \textit{et al}., 2000), \textit{Bartalinia robillardoides} from \textit{Aegle marmelos} (Gangadevi and Muthumary, 2008), \textit{Pestalotiopsis pauciseta} from \textit{Cardiospermum helicacabum} (Gangadevi \textit{et al}., 2008), \textit{Fusarium solani} from \textit{Taxus celebica} (Chakravarthi \textit{et al}., 2008), \textit{Phyllosticta citricarpa} from \textit{Citrus medica} (Senthil \textit{et al}., 2008), \textit{Botryodiplodia theobromae} from \textit{Taxus baccata} (Raja \textit{et al}., 2008), \textit{Fusarium oxysporum} from \textit{Rhizophora annamalayana} (Elavarasi \textit{et al}., 2012), \textit{Fusarium redolens} from Himalayan yew (Garyali \textit{et al}., 2013). These reports have inspired several efforts to recognize endophytes as sources of associated plant natural products. “Endophytes producing anti-neoplastic camptothecin (CPT) and its structural analogs were reported (Puri \textit{et al}., 2006; Kusari \textit{et al}., 2009, Shweta \textit{et al}., 2010). Anticancer pro-drugs podophyllotoxin (Eyberger \textit{et al}., 2006) and deoxypodophyllotoxin (Kusari \textit{et al}., 2008), and natural insecticides azadirachtin A and B (Kusari \textit{et al}., 2011c) are also reported. Endophytes are capable of producing other associated plant secondary metabolites of therapeutic importance remains to be discovered”.

Chromatographic fingerprint technique is a quick, sensitive and accurate analytical tool for the analysis of a large number of plant samples for secondary metabolites (Zhao \textit{et al}., 2005). The chromatographic fingerprints recognized by TLC (Thin layer chromatography), HPLC (High Performance Liquid Chromatograpy), HPTLC (High Performance Thin Layer Chromatography) and GC (Gas
Chromatography) have been known as rapid and reliable means for quantification and identification of herbal medicines (Liang et al., 2009).

High-throughput analysis was allowed by the invention of multi-channel mass spectrometry interface (Eldridge et al., 2002). LC-MS (Liquid chromatography-mass spectrometry) is the most sensitive technique for attaining information about a compound. (Cremin and Zeng, 2002). Liquid chromatography-nuclear magnetic resonance (LC-NMR) allows the online identification of organic molecules (Bobzin et al., 2000). LC-NMR, despite its lower sensitivity compared to LC-MS, offers a powerful device for rapid detection as well as characterization of compounds/structure classes of novel compounds. LC-NMR is particularly useful in cases where the data from LC-MS are partial or do not permit the accurate identification of the active components of a sample.

This chapter describes the identification of active metabolites in the extract of endophytic fungi *Pithomyces* sp. and *Cheatomium globosum* isolates from *B. serrata* and *V. negundo* respectively. Extracts from these isolates have been subjected to preparative-TLC, HPLC and LC-MS for identification of bioactive compounds with boswellic acid and catechin reference standards.

### 6.2 Materials and Methods

#### 6.2.1 Preparation of crude extract from endophyte culture broth

The endophytic fungal isolates, *Pithomyces* sp. and *C. gloeosporioides* from *B. serrata* and *C. globosum* and *A. alternata* isolated from *V. negundo* were grown on potato dextrose agar at 27°C for 5 days. The preparation of the crude extracts from endophyte culture broth has been described earlier in the materials and method section of chapter 5. These extracts were used for further characterization of the bioactive compounds.

Solvents used for extractions, solubilisation and chromatography were of technical grade and were distilled twice before use. HPLC-grade solvents *viz.*, methanol, acetonitrile and water were purchased from Fisher Scientific, India.
6.2.2 Thin Layer Chromatography (TLC) analysis of the extracts

Thin Layer Chromatography was performed for the endophyte extracts of *Pithomyces* sp., *C. gloeosporioides*, *C. globosum* and *A. alternata* and boswellic acid, catechin, caffeic acid and ursolic acid were used as reference standards.

6.2.3 HPLC analysis

Metabolite profiling of the endophyte extracts *viz.* *Pithomyces* sp., *C. gloeosporioides*, *C. globosum* and *A. alternata* was performed by Reverse Phase-HPLC (Waters, Milford, USA) equipped with UV-vis detector (Waters, 2489). The stationary phase was C18 column (Symmetry ®, Waters, 4.6 x 250 mm, 5 µm). An isocratic mobile phase consisting of acetonitrile: water: acetic acid: 18:82:2 (v/v) was delivered at a flow rate of 0.5 ml/min and the elution profiles were read at 260 nm”. Standard calibration curve was prepared using standard Boswellic acid and catechin at a concentration of 200 ppm (injection volume 10 µl) as described by Shen *et al.* (2004).

6.2.4 LC-MS Analysis

Liquid chromatography Mass spectrometry (LC-MS) was performed using Water Synapt G2 connected to a UPLC system (Acquity, Waters, Milford, MA, USA) to analyze the presence of different metabolites of diverse mass in the crude extracts of *Pithomyces* sp., *C. gloeosporioides*, *C. globosum* and *A. alternata*. Mass spectra data were acquired by electrospray ionization (ESI) in negative ion/positive ion mode. ESI was carried within a range of mass to charge 100-1,000 (m/z).

6.3 Result

6.3.1 Thin Layer Chromatography

Five bands were observed in *Pithomyces* sp., six bands in *C. gloeosporioides*, seven bands *C. globosum* and five bands in *A. alternata* (Fig. 6.1).
Characterization of Bioactive compounds

Fig. 6.1 TLC profile of the crude endophytic extracts visualized under UV light. P: Pithomyces sp.; C.g: Colletotrichum gloeosporioides; C. gl: Cheatomium globosum; A.a: Alternaria alternata; B: Boswellic acid; U: Ursolic acid; C: Catechin; Ca: Caffeic acid

6.3.2 HPLC analysis

All the four endophytic extracts were subjected for HPLC, in that the HPLC chromatogram Pithomyces sp. extract matched with the standard boswellic acid which showed the peak at retention time 3.12 and 3.2 respectively (Figs. 6.2a & 6.2b), whereas the chromatograms of the C. globosum matched with the standard catechin which showed the peak at retention time 3.773 and 3.707 min respectively (Fig. 6.3a & 6.3b).

Fig. 6.2a HPLC chromatogram of standard boswellic acid
Characterization of Bioactive compounds

**Fig. 6.2b** HPLC chromatogram of *Pithomyces* sp. extract

**Fig. 6.3a** HPLC chromatogram of standard catechin

**Fig. 6.3b** HPLC chromatogram of *Cheatomium globosum* extract
6.3.3 LC-MS analysis

The endophytic extracts were subjected to LC-MS. The mass spectrometry had shown the presence of different metabolites of diverse mass in the crude extracts of *Pithomyces* sp., *C. gloeosporioidis*, *A. alternata* and *C. globosum* and compared with the standards *viz.* boswellic acid, caffeic acid, catechin and ursolic acid. The mass of *Pithomyces* sp. endophytic extract (453.1047) had a metabolite matching with the standard boswellic acid (455.2301) (Fig. 6.5b and 6.4) and *C. globosum* extract (290.9737) had a metabolite matching with the standard catechin (290.9737) (Fig. 6.7b and 6.6).

![LC-MS spectra of boswellic acid standard](image-url)
Characterization of Bioactive compounds

Fig. 6.5a LC profile of *Pithomyces* sp. extract

Fig. 6.5b MS profile of *Pithomyces* sp. extract
Characterization of Bioactive compounds

Fig. 6.6   LC-MS spectra of catechin standard

Fig. 6.7a LC profile of Cheatomium globosum extract
6.4 Discussion

“Plant endophytic fungi are an important and novel source of natural bioactive compounds (Schulz et al., 2002, Strobel, 2003) with their potential applications in medicine, agriculture and food industry. In the past decades, many valuable bioactive compounds with cytotoxic, insecticidal, antimicrobial, and anticancer activities have been successfully discovered from the endophytic fungi”. During the “long period of co-evolution, a friendly relationship was formed between each endophyte and its host plant”. Some endophytes have the ability to produce the same or similar bioactive compounds as those originated from their host plants (Zhao, 2010). They are reported to provide a broad range of bioactives with distinct structure, which includes alkaloids, benzopyranones, chinones, flavonoids, phenolic acids, quinines, steroids, terpenoids, tetralones, xanthones and others (Tan and Zou, 2001).

Thin layer chromatography (TLC) has been used for the analysis of natural and synthetic products such as pharmaceuticals, plant and biological samples. Many samples can be analyzed concurrently and rapidly at comparatively low cost. TLC
enables analysts to separate and determine compounds in complex mixtures, including various environmental samples (Bhawani et al., 2010).

HPLC profile of the extract of *Pithomyces* sp., *C. gloeosporioides*, *C. globosum*, *A. alternata* were compared with the standards Boswellic acid, Ursolic acid, Catechin and Caffeic acid. It is expected that the activity observed by the extracts could be due to the presences of these molecules. Kasuri et al. (2011c) identified Campothecin by HPLC coupled with multi component high resolution tandem mass spectrometry (LC-HRMS) from *F. solani*, also Liu et al. (2010) identified *Xylaria* sp. endophytic fungus from *C. accuminata* plant and was evaluated for metabolite profiling. Catechin, a phenol compound was reported with biological activities *viz.*, lipoxygenase inhibition (Maqsood and Benjakul, 2010), antibacterial activity (Shimamura et al., 2007) and anticancer properties (Demeule et al., 2002).

Boswellic acids are pentacyclic triterpene molecules that are produced by genus *Boswellia*. It is estimated that they make up 30% of the resin in *B. serrata*. It contains alpha-boswellic acid, beta-boswellic acid, 3-acetyl-beta-boswellic acid, 11-keto-beta-boswellic acid and 11-keto-beta-acetyl- boswellic acid (Wang et al., 2011).

“Discoveries of podophyllotoxin produced by endophytic fungi *Phialocephata fortinii* isolated from *P. peltatum* (Eyberger et al., 2006) and *Trametes hirsute* isolated from *P. hexandrum* (Puri et al., 2006)” were subjected to metabolomics analysis using HPLC system. Amna et al. (2006) reported that endophytic *Entophospora inferequens* and *Neurospora crassa* (Rehman et al., 2008) isolated from *N. nimmoniana* reported to produce a pentacyclic quinoline alkaloid campothecin (CPT) a potent antineoplastic agent.

Kumar et al. (2013) identified the extracts of *C. globosum* EF18, an endophyte isolated from *Withania somnifera*, effective against *Sclerotinia sclerotiorum*. The NMR analysis and mass showed that compound ‘A’ that is similar to Antibiotic Sch210971 (m/z 445 and λ max 290), previously isolated from *C. globosum* by Yang et al. (2006).

In conclusion, the bioactive metabolites boswellic acid from endophyte isolate of *Pithomyces* sp. from *B. serrata* and catechin from endophyte *C. globosum* isolate of *V. negundo* were identified using reference standards.