Review of Literature
Review of Literature

2.1. History of natural products

Plants remained as the basis for traditional system of medicine for thousands of years, with the first record dating back to about 3000 B.C. However, the fossil details reported that, Neanderthal remains have been found to contain residues of medicinal herbs, which indicate that natural products had been used even before the available evidence (Holt and Chandra, 2002). Later in 2600 B.C. the usage of natural products has been inscribed as cuneiform on clay tablets in Mesopotamia. Certainly many of these products are modified and are still used (Nakanishi, 1999a, 1999b; Cragg and Newman, 2001a, 2001b). Around 2000 B.C. Chinese herbal guide reported the usage of herbaceous plants (Holt and Chandra, 2002). Later, in 1500 B.C. the Egyptians have reported the use of various herbs (Cragg and Newman, 2001a, 2001b; Holt and Chandra, 2002). Ebers Papyrus is accepted as one of the best records among all, which documents around 1000 different substances and formulations most of which are plant-based products (Nakanishi, 1999a, 1999b).

Ayurveda an Indian system of medicine, has its roots in folk medicine and it is presumed that the fundamental and applied principles of Ayurveda became organized and enunciated around 1500 BC. (Ravishankar and Shukla, 2007). From 6th Century BC to 7th Century AD there was a systematic development in Ayurvedic science and it is called the Samhita period. From the knowledge gathered and cherished over these centuries, two major schools evolved. One was the school of physicians called as ‘Dhanvantri Sampradaya’ and the other was the
school of surgeons referred to as ‘Atreya Sampradaya’. These schools had their respective representative compilations in “Charaka Samhita” for the school of Medicine and “Sushruta Samhita” for the school of Surgery (Narayanaswamy, 1981; Ravishankar and Shukla, 2007).

Theophrastus, a philosopher and a natural scientist wrote “De Historia Plantarum” around 300 B.C. in which he has described about the medicinal values of herbs and methods of cultivating them (Biljana, 2012). A compilation of Ayurvedic hymns from 1000 B.C and earlier had described the uses of over 1000 different herbs in India. During eighth century, this literature was translated from Sanskrit and used as the basis for Tibetan system of medicine (Cragg and Newman, 2001a, 2001b). In the middle ages, between fifth to twelfth centuries, monks in monasteries developed several manuscripts about herbs and their uses (Cragg and Newman, 2001a; Holt and Chandra, 2002). In 1596, during Ming dynasty a Chinese drug encyclopedia was created by Li Shih-Chen, which documented 1898 herbal drugs and 8160 prescriptions (Nakanishi, 1999a, 1999b).

Until the early nineteenth century the crude extract of the plant in various forms had been used for treatment. Later with an advent in technology in the field of analytical chemistry and acquiring exhaustive knowledge in science, the isolation and purification of active ingredients from medicinal plants were practiced, which demonstrated their value in medicine (Drews et al., 2000). In 1815, morphine was isolated from opium extract (Serturner, 1817), followed by the isolation of papaverin from opium in 1848 (Sneath, 1985).

In the mid-nineteenth century, the combination of synthetic approach with traditional knowledge has revolutionized pharmaceutical and chemical industries
leading to the development of analogues for existing natural drugs. In late twentieth century, the fields of microbiology, pharmacology and biochemistry collaborated together to discover many novel drugs based on their mode of action (Drews et al., 2000; Yeh and Lim, 2007). Though the pharmaceutical industries have grown to unexpected heights, the current trend is returning back to nature for the search of novel drugs. The reason behind it is that, it is less toxic to the environment and has no side effects. Moreover plants produce molecules with great structural diversity.

2.2. An overview of natural products from various sources

Nature is a treasure that is rich in bioactive molecules with significant therapeutic potential. This wealth can be obtained from every niche of an ecosystem (Clark, 1996; Kelecom, 1999; Grabley et al., 2000; Cragg and Newman, 2001a; Jia et al., 2002). Plants and microbes from different ecosystems can be an affluent source for potential drug leads. (Haefner, 2003; Butler, 2004; Cragg and Newman, 2005; Berdy, 2005; Mishra and Tiwari, 2011).

2.2.1. Natural products from plants

The usage of plants for treating various ailments is as old as humankind. Till date around 35,000 to 70,000 plant extracts have been screened for their medicinal applications (Farnsworth and Soejarto, 1991). These extracts are not only used as direct drugs but are also used as a precursor for the synthesis of new drugs with improved pharmacological properties (Ballabh, 2008). In the last century medicinal plants were considered to be the chief source of natural products because several active compounds have been isolated from different plants (Qin and Xu, 1998; Lee, 1999). Some of these compounds include
vinblastine, vincristine, etoposide, teniposide, paclitaxel, docetaxel, topotecan, and irinotecan that are approved in USA as anticancer agents (Lee, 1999).

Two alkaloids namely vinblastine and vincristine isolated from the plant Catharanthus roseus are used for treating a variety of cancers, including leukemia, lymphoma, breast and lung cancers, (Cragg and Newman, 2005). Similarly a diterpenoid compound, Paclitaxel isolated from the bark of the pacific yew, Taxus brevifolia is significantly active against ovarian cancer, breast cancer and lung cancer (Rowinsky et al., 1992). Camptothecin isolated from the Chinese ornamental tree Camptotheca acuminata is used for the treatment of Ovarian and lung, ovarian, breast, pancreas and stomach cancers (Sriram et al., 2015). Epipodophyllotoxin isolated from the roots of Podophyllum peltatum exhibited strong anti-tumor activity (Stahelin, 1973). Similarly, eight compounds isolated from various extracts of Tinospora cordifolia had high anticancer potential. The compound palmatine was active against KB and HT-29; tinocordiside against KB and CHOK-1; yangambin were active only against KB cell line. Additionally N-formylannonain and 11-hydroxymustakone, exhibited immunomodulatory activity (Manju et al., 2015).

Vandana et al. (2015) studied the antidiabetic and adipogenesis activity of medicinal plants used by Australian Aboriginals and Indian Ayurvedic practitioners. The ethanolic extract of Australian plants (Santalum spicatum and Acacia kempeana) and Indian Ayurvedic plant (Curculigo orchioides) stimulated glucose uptake in adipocytes. Similarly with respect to adipogenesis the Australian plants (Euphorbia drumondii, Acacia tetragonophylla and Beyeria leshnaultii) and the Indian plants (Andrographis paniculata, Pterocarpus
marsupium and Curculigo orchioides) reduced lipid accumulation in differentiated adipocytes. Furthermore the crude extracts of Australian plant (Acacia kempeana and Acacia tetragonophylla) showed effective cytotoxicity against HeLa cells.

Hisayoshi et al. (2014) reported that ethanol and water extracts of Brasenia schreberi inhibited the DNA polymerase activity of HIV-1 RT (HIV-1 reverse transcriptase). Similarly, the aqueous extract of Petasites japonicas and hydroethanolic extracts of Brasenia schreberi significantly inhibited the activity of DNA polymerase to incorporate dTTP into poly(rA)-p(dT)15. Additionally it also inhibited the activity of RNase H to hydrolyze the RNA strand of an RNA/DNA hybrid. The extract also inhibited HIV-1 replication in human cells (Hisayoshi et al., 2015). Two pure compounds galloatechin and epigallocatechin extracted from Mimusops elengi exhibited satisfactory anti HIV-1 activity (Suedeaa et al., 2014).

The ethanolic extract of the whole plant of Couroupita guianensis exhibited wound-healing capacity by decreasing the surface area of the wound and increasing the tensile strength. Furthermore the extract also exhibited antimicrobial activity against Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa and Klebsiella pneumoniae (Umachigi et al., 2007). Similarly, various extracts of flowers and bark of Couroupita guianensis showed that flowers have relatively more analgesic property than bark. Additionally both the extract exhibited significant anti-inflammatory activity against carrageenan-induced inflammation (Geetha et al., 2004).
The leaf and flower extract of *Guazuma ulmifolia* exhibited gastroprotective effects in Wistar rat model of acute gastric ulcer induced by diclofenac. This protection against injury was mainly due to anti-inflammatory and radical-scavenging mechanisms (Berenguer et al., 2007). Similarly the essential oil of *Guazuma ulmifolia* exhibited DPPH radical scavenging activity. Additionally, it also exhibited significant antibacterial activity against *Pseudomonas aeruginosa* and *Staphylococcus aureus* and moderate activity against *Escherichia coli* and *Staphylococcus epidermis* (Boligon et al., 2013).

The aqueous extract of *Ficus religiosa* bark was investigated for its antidiabetic activity in streptozotocin (STZ)-induced diabetic rats. The extract showed significant increase in serum insulin, body weight and glycogen content in liver and skeletal muscle of STZ-induced diabetic rats while there was significant reduction in the levels of serum triglyceride and total cholesterol. This indicated that aqueous extract of *Ficus religiosa* bark possesses significant antidiabetic activity (Pandit et al., 2010).

### 2.2.2. Natural products from microorganisms

Research on microbes for natural products was started only after the discovery of fermentation technology. Among all microorganisms, bacteria and fungi were mainly focused for the production of natural products. Because, these microorganisms have been existing on earth for billions of years and evolution of biosynthetic pathways have been taking place resulting in synthesis of novel secondary metabolites. So far, nearly 22,500 bioactive molecules have been obtained from microbes, in which 45% are produced by actinomycetes, 17% by unicellular bacteria and 38% by fungi (Berdy, 2005).
There are numerous advantages in natural products obtained from microbial sources. Inexhaustible supply of natural products by cultivation of microbes in large-scale fermenter and indefinite storage of microbes, ensure permanent availability of the source and amplification of productivity by optimizing culture conditions (Okami, 1986). Hence, the role of microorganisms in producing various natural products is dramatically increasing, which make microbes as potential candidates for drug discovery (Berdy, 2005).

2.2.2.1. Bacteria as a source for natural products

Bacteria are an inexhaustible source of structurally diverse bioactive metabolites. Endophytic bacteria in particular, represent a hidden ecological system that is not yet completely explored. They live in close association with the plant symbiotically and have the capability of producing bioactive compounds that are highly beneficial to the plant kingdom as well as mankind.

Compounds of medicinal value from *Bacillus cereus* and *Bacillus pumilus* were extracted with various solvents and fractionated by Vijayakumar *et al.* (2014). All the fractions were tested for their cytotoxicity and anticancer property against normal human liver cell lines and 2 cancer cell lines by MTT assay. Two fractions obtained from *B. cereus* showed high cytotoxicity and significant anticancer activity by membrane blebbing and DNA fragmentation. Similarly *Pseudomonas aeruginosa* isolated from oil contaminated soil showed highest biosurfactant production, which effectively controlling the test pathogens. The microbe also suppressed the proliferation of HeLa cells (Vijayakumar and Saravanan, 2015). A new rhizosphere soil bacterium *Pseudomonas putida* produced 5-methyl phenazine-1-carboxylic acid betaine, which exhibits selective
cytotoxicity towards lung and breast cancer cell lines in a dose-dependent manner (Kennedya et al., 2015).

The ethyl acetate extract of Bacillus subtilis sub sp. subtilis RG strongly inhibited the growth of gram positive than gram-negative bacteria. Additionally it also inhibited fungal growth. The extract had DPPH radical scavenging effect and reducing power as well as potent cytotoxic activity against human breast cancer cells (Ramasubburayan et al., 2015). Kamei et al. (1988) reported 710 bacterial isolates from the water and sediment samples of salmon hatcheries. Among all these isolates Pseudomonas, Aeromonas, Vibrio and Coryneforms species predominantly exhibited antiviral activity against infectious hematopoietic necrosis virus.

The exopolysaccharide (EPS) produced by an endophytic bacterium Paenibacillus polymyxa was evaluated for its antioxidant potential by Jun et al. (2009). The crude and purified EPS showed a strong scavenging activity on superoxide and hydroxyl radicals. Cell-free supernatants of four strains of Bacillus subtilis (UMAF6614, UMAF6619, UMAF6639, and UMAF8561) exhibited significant antifungal activity against Podosphaera fusca, the main causal agent of cucurbit powdery mildew. Purification of the crude extract resulted in identification of three lipopeptide antibiotics namely surfactin, fengycin, and iturin A or bacillomycin that are responsible for the inhibitory effect (Diego et al., 2007).

Actinomycetes are filamentous Gram-positive bacteria that are widely distributed in terrestrial and aquatic ecosystems, especially in soil (Ventura et al., 2007; Olano et al., 2009). Among actinomycetes, Streptomyces species alone
produces around 7,600 compounds. Many of these molecules are potent antibiotics, which makes *Streptomyces* the primary source for antibiotics. Moreover, actinomycetes have gained special interest in pharmaceutical industry because of their diverse biological activities.

One novel compound and five known compounds were isolated from novel halophilic actinomycete, *Nocardiopsis terrae*. Among all these compounds, 4-oxo-1,4-dihydroquinoline-3-carboxamide was isolated for the first time from natural sources, that exhibited antimicrobial activity against plant pathogens (Tian *et al*., 2014). The ethyl acetate extract of *Actinomadura spadix* was fractionated and tested for antimicrobial and anti-inflammatory activity. One of the fraction 3,6 di-isobutyl 2,5 piperazinedione reported for the first time in *A. spadix*, showed 100% inhibition against *Candida* sp. and *Enterococcus faecalis*. This compound also exhibited anti-inflammatory activity on carrageenan-induced inflammation in animals (Hairul *et al*., 2014). Naphthacene glycoside, which was originally isolated from Mediterranean sponge (*Dysidea tupha*) has been isolated from *Streptomyces* sp. strain RV15. It showed inhibitory activity against *Chlamydia trachomatis* and was able to inhibit the primary infection in a dose-dependent manner (Reimer *et al*., 2015).

7-Cyano-7-deazaguanine which is the biosynthetic precursor of queuosine-tRNA was isolated for the first time as a novel natural product from a mangrove actinomycete, *Streptomyces qinglanensis*, which exhibited potent anti-cancer activity against HeLa and HepG2 cell lines (Xu *et al*., 2015). The ethyl acetate extract of six actinobacterial strains isolated from sediments of coral reef obtained from Gulf of Mannar Biosphere Reserve, India, were screened for their
antioxidant activity. Among the six strains, *Nocardiopsis* sp, showed strong antioxidant activity (Poongodi *et al*., 2014).

One novel compound and 12 known compounds were isolated from the fermentation broth of the endophytic actinomycetes *Jishengella endophytica*. Compounds namely perlolyrine, 1-hydroxy-β-carboline, lumichrome, 1H-indole-3-carboxaldehyde were active against the influenza A virus subtype H1N1. Results showed that 1-hydroxy-β-carboline could be a promising drug for H1N1 infections (Wang *et al*., 2014). *Streptomyces cyaneofuscatus* and *Streptomyces carnosus* isolated from deep-sea coral reef produced several compounds with various bioactivities, which includes daunomycin, cosmomycin B and galtamycin B (antibacterial and antitumor), maltophilins (antifungal) and lobophorins (anti-inflammatory and antituberculosis) (Afredo *et al*., 2015).

### 2.2.2.2. Natural products from fungi

Fungi are eukaryotic heterotrophs, which obtain nutrients from dead or living matter for their growth. Fungi usually reproduce by spores, which are non-motile except for some groups (James, 1999). About 75,000 species of fungi have been described, but it is estimated that 1 to 1.5 million fungal species exist on earth (Hawksworth *et al*., 1995). Research on natural product from micro fungi started with the discovery of penicillin from *Penicillium notatum* (Fleming, 1929). Penicillin is the most potential antibiotic drug, which is being used even after 80 years of its discovery. However, macro fungi were also used as natural products in China for more than 2000 years, that is reported in ancient manuscripts (Wasser, 2005). Hence, fungi as a potential source for bioactive
natural products possessing antibacterial, antifungal, antioxidant and anticancer activities is well documented (Balde et al., 2010; Doss et al., 2010).

Five polyphenolic compounds, hispidin, hypholomine B, inoscavin A, davallialactone, and phellgridin D were isolated from the ethanolic extract of Phellinus baumii. All these compounds inhibited neuraminidase activity of H1N1, H5N1 and H3N2 and also reduced the virus-induced cytopathic effect (Hwanga et al., 2015). Similarly, Song et al. (2014) isolated one new compound and six known compounds from Phellinus ignarius that were tested against H5N1 influenza A virus. Compound namely eudesm-1β, 6α, 11-triol possessed significant ability in inhibiting influenza virus.

A novel fungus Penicillium verruculosum produced a broad-spectrum antibiotic compound phenazine 1 carboxylic acid, which was reported for the first time in fungi by Shah et al. (2014). This compound exhibited a strong cytotoxicity against cancer cells (KA3IT) and was less toxic towards normal cell lines (NIH3T3, HSCT6, HEK293 and MDCK). From the soil fungus Penicillium citrinum, two new benzopyranones, named coniochaetones E and F, one new xanthone, penicillone C, and one new benzophenone and penicillanone, were isolated together with ten known compounds. All the isolated compounds exhibited cytotoxicity (Trisuwana et al., 2014).

Sohail et al. (2014) studied the antimicrobial activity of acetonitrile and n-hexane extract of Rhizopus stolonifer against fungal pathogens (Aspergillus niger, Aspergillus oryzae, Candida albicans, Penicillium digitatum, Fusarium oxysporum) and bacterial pathogens (Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, Methicillin resistant Staphylococcus aureus, Vancomycin
resistant *Staphylococcus aureus*). Acetonitrile extract proved to be comparatively better than n-hexane. Phytochemical analysis indicated the presence of secondary metabolites like aflotoxin (B1, B2, G1 and G2), which might have been responsible for their antimicrobial potential. Nelson *et al.* (2014) isolated compounds such as aszonapyrone A and sartorypyrone A from the endophytes of marine sponge. Endophytes, namely *Neosartorya paulistensis* and *Neosartorya laciniosa* exhibited significant antibacterial activity against multidrug resistant strain *Staphylococcus aureus*. Similarly, *Penicillium chrysogenum* obtained from the rock of Atacama Desert, produced two compounds, α-linolenic acid and ergosterol endoperoxide, which were active against *Cryptococcus neoformans* and methicillin-resistance *Staphylococcus aureus*, respectively (Vivian *et al.*, 2015).

### 2.3. Exploration of unique resources for natural products

The discoveries of several microbial natural products and their successful clinical application have proved that microbes are rich sources of novel drug leads (Larsen *et al.*, 2005; Lanen and Shen, 2006). Thus, recent research suggests that microbial diversity obtained from diverse habitats and unexplored natural resources offer microbial metabolites with incredibly diversified chemical entities, offering a hopeful alternative for the remedy of many diseases (Lanen and Shen, 2006). Hence, recent bioprospecting attempts are towards exploration of microbial diversity from untapped and unique sources (Zhu *et al.*, 2012). One such exclusive biological niche that promotes the growth of microbes is the intracellular spaces between cells of higher plants. Endophytic fungi are microbes that inhabit such a novel biotope providing several metabolites of biological and ecological relevance (Schulz *et al.*, 2002; Strobel and Daisy, 2003;...
Gunatilaka, 2006; Strobel, 2006; Suryanarayana et al., 2012). Recently, endophytic fungi associated with medicinal plants have drawn much attention from microbiologists, taxonomists, ecologists, agronomists, chemists and evolutionary biologists, as a promising source of secondary metabolites with future prospect in drug discovery (Tan and Zou, 2001; Schulz et al., 2002; Strobel, 2002; Strobel and Daisy, 2003; Strobel et al., 2004; Aly et al., 2011; Kaul et al., 2012).

2.4. Endophytic fungi

Endophytic fungi are hidden components of fungal diversity (Arnold et al., 2003; Arnold, 2007; Rodriguez et al., 2009). They colonize the internal tissues of plants without causing any apparent disease symptoms to the host (Petrini, 1986). It is assumed that nearly 300,000 plant species existing on earth harbor one to several endophytes and a multiple endophytic organisms can also be isolated from a single plant (Hyde and Soytong 2008). Endophytes have been reported from diverse groups of plants including, marine algae (Zhang et al., 2014), bryophytes (Yu et al., 2014), ferns (Raji et al., 2014), conifers (Susan et al., 2014), marine sponges (Ahmed et al., 2012) and angiosperms (Mariana et al., 2014). These fungal components have been reported from plants growing in diverse habitats like, mangroves (Mervat et al., 2014), tropical forests (Higgins et al., 2014), hot desert (Nicholas et al., 2015) and cold deserts (Zhang and Yao, 2015).

2.4.1. Taxonomy of endophytic fungi

The endophytic fungi have been reported from diverse group of plants present in varied environmental conditions. Although endophytes are isolated
from diverse habitats, they belonged only to certain taxonomical groups such as Ascomycetes (comprising Hyphomycetes and Coelomycetes), which constitutes the major endophytic assemblage of plant tissues and Zygomycetes that are rarely isolated as endophytes (Petrini 1986; Puri et al., 2006). The most important feature that have to be observed for morphological identification are, structure of conidia and the process involved in their formation. Additionally, the pigmentation and shape of hyphae, presence or absence of septa and any other specific hyphal element also aid in the taxonomical determination (Bononi and Grandi, 1998).

Beside these, certain non-sporulating forms also occur as endophyte. Identification of endophytic fungi, particularly Coelomycetes, is intricate because they sometimes fail to develop diagnostic features in culture. Moreover, sterile forms are yet another problem faced by mycologists, as they fail to sporulate even after prolonged incubation. Hence, culture characteristics such as colony morphology, pigmentation and growth rate are used to group these sterile forms into ‘morphospecies’ (Bills and Polishook, 1994; Guo et al., 2000). To resolve this problem the mycologists used internal transcribed spacer region of the ribosome sequence in nuclear DNA (ITS rDNA) and nuclear ribosomal large subunit (LSU rDNA) to determine a taxon up to its species level (Unterseher and Schnittler, 2010).

2.4.2. Colonization of endophytic fungi

The composition and distribution of mycobiome in a host species can vary significantly based on multiple factors including biological, environmental and geographical factors (Fisher et al., 1994; Collado et al., 1999; Photita et al., 2001;
Granath et al., 2007; Wang and Guo, 2007; Guo et al., 2008a; Hoffman and Arnold, 2008). Recently, Ashish et al. (2012) carried out a study to determine the influence of various factors involved in endophytic composition. A total of 1,151 fungal isolates representing 29 taxa were isolated from various tissues of Tinospora cordifolia collected from three locations differing in air pollution levels during three seasons. The colonization frequency (CF) varied significantly among tissue types and seasons than their location. CF was maximal during monsoon followed by winter and minimal in summer. Similarly, Guo et al. (2008b) reported that a strong correlation has been observed between colonization rates of endophytes in dry and wet seasons in the leaves of Pinus tabulaeformis.

The colonization frequency of endophytes increases with age of the leaf tissue (Taylor et al., 1999). The increase in species richness of endophytic fungi in mature leaves of Bauhinia brevipes suggests three possible reasons. Firstly, the young leaves are chemically active than mature leaves, to protect it from herbivores attack (Coley, 1983). In contrast, biochemistry of mature leaves encourages more fungal colonization (Fernandes et al., 2011). Secondly, leaf exposure time may also have accounted for increased colonization by endophytic fungi (Fröhlich et al., 2000; Toofanee and Dulymamode, 2002; Arnold and Herre, 2003). In mature leaves, large leaf surface supports endophytic abundance probably by providing more sites for infection (Toofanee and Dulymamode, 2002). Collado et al. (1999) stated that geographical location played a considerable role in the extent of colonization by endophytic fungi, in Quercus ilex growing at four different sites in Madrid, Spain.
2.4.3. Host and tissue specificity in fungal endophytes

Endophytes can be isolated from various plant parts such as roots, leaves, stem and buds. Thus it is clear that a single host plant can be colonized by more than one endophyte (Hyde and Soytong 2008). Although, endophytic fungi are isolated from every tissue, certain species exhibit organ and tissue specificity (Carroll et al., 1977). This nature of the fungi is acquired due to adaptation to certain micro-ecological and physiological conditions present in a particular organ (Petrini et al., 1992). There are some endophytes that appear to be specific to their hosts (Ekanayake et al., 2012). This relationship of endophytes with a single plant is described as host specificity or host preference (Cohen, 2006). Such specificity implies that complex biochemical interaction occur between the host and its associated endophytes (Strobel, 2003; Strobel and Daisy, 2003). This fact has been well documented by Arnold and Herre (2003) in their studies on endophytes. Gond et al. (2007) reported Corynespora sp., Emericella sp. and Chaetomium sp. had tissue specificity for bark and additionally Verticillium sp. was restricted to the leaf tissues while Trichoderma sp. to root tissues of Aegle mermelos. Similarly an endophytic fungus Phialophora sp. exhibited a certain level of tissue specificity towards aerial roots of Ficus benghalensis (Suryanarayanan and Vijaykrishna, 2001).

2.4.4. Role of endophytes in host plants

Endophytes exhibit symbiotic association with their hosts, taking up nutrients from the plant and in turn enhancing resistance of plants to adverse conditions. Comprehensive reports suggest that plant harboring endophyte fungi
have unique advantages against stress (biotic and abiotic) over non-endophytic counterparts (Redman et al., 2002; Bae et al., 2008).

Endophytes show numerous direct and indirect mechanisms to promote plant growth and health. Many endophytes are capable of nitrogen (N\textsubscript{2}) fixation, solubilization of phosphate, enhancing uptake of phosphorus (P), production of siderophores, ACC deaminase and plant hormones such as auxin, abscisins, ethylene and gibberellins, which are important for the regulation of plant growth and development (Singh et al., 2000; Sherameti et al., 2005; Waller et al., 2005; Varma et al., 1999). Endophytes stimulate longer root hairs, increase the surface area for nutrient absorption and enhance exudation of phenolic compounds into the rhizosphere for efficient absorption of soil phosphorus (Malinowski and Belesky, 2000).

*Curvularia protuberata* colonizes all non-embryonic tissues of the geothermal plant *Dichanthelium lanuginosum*. This plant cannot tolerate temperatures above 40°C in the absence of endophyte (Redman et al., 2002; Márquez et al., 2007). Similarly colonization of host by *Neotyphodium coenophialum* leads to the development of extensive root system that enables the host to acquire better soil moisture and absorb nutrients, for circumventing drought and water stress (Malinowski and Belesky, 2000).

Turf grasses associated with the endophyte, *Epichloe festucae* have shown significant resistance over uninfected turf towards two major leaf spot pathogens namely, *Sclerotina homeocarpa* (Clarke et al., 2006) and *Laetisaria fusiformis* (Bonos et al., 2005). Other advantages offered by endophytic fungi include
enhanced resistance to insect, pests (Akello et al., 2007), tolerance to low pH (Lewis, 2004) and high salinity (Waller et al., 2005).

2.4.5. Natural products obtained from endophytic fungi

Discovery of Paclitaxel producing endophytic fungus Taxomyces andreanae, from the yew Taxus brevifolia has evoked research on the pharmaceutical aspect of endophytic fungi (Stierle et al., 1993; Strobel et al., 1996; Shrestha et al., 2001). Fossil evidence suggests that plants and their endophytes have co-evolved (Michael et al., 2012). During the co-evolution, several chemical interactions have occurred between plants and endophytes. Though the endophytic interactions are not clearly understood, the fact that they produce bioactive molecules same as those of their hosts are well established (Strobel, 2002b). They produce a broad range of metabolites with varied structures and biological activities (Tan and Zou 2001; Schulz et al., 2002; Zhang et al., 2006), which are not only involved in the host-endophyte relationship, but have potential pharmacological and agricultural applications (Strobel, 2002a).

The number of secondary metabolites produced by fungal endophytes is greater than that of any other endophytic microorganisms (Zhang et al., 2006). Among these metabolites only a small percentage have been formulated as drugs. In fact, a recent comprehensive study has indicated that 51% of biologically active substances isolated from endophytic fungi were previously unknown, indicating the great chemical diversity and pharmaceutical potential of endophytes (Stierle et al., 1999; Weber et al., 2004; Shen et al., 2006). Therefore, endophytes are considered as one of the prime sources for contributing novel and useful
compounds for industrial applications (Tan and Zou 2001; Zhang et al., 2006; Pupo et al., 2006).

Secondary metabolites from fungal endophytes are known to have antibacterial (Wang et al., 2012), anticancer (Gokulraja et al., 2015), antioxidant (Samanthi et al., 2015), immunosuppressive (Ren et al., 2008), antiviral (Jun-Wei et al., 2012), antituberculosis (Wijeratne et al., 2013), antimalarial (Hemtasin et al., 2011), insecticidal (Abraham et al., 2015) and antidiabetic properties (Pavithra et al., 2014). Though, endophytic fungi possess various bioactive potential, the present research is mainly focused on antibacterial, antioxidant and anticancer activities of the fungal metabolites, as there is an increased demand for fungi-based drugs due to the emergence of multi drug resistant pathogens.

2.4.5.1. Antimicrobial metabolites from endophytic fungi

Paul Vuillemin coined the term antibiotic. Antibiotic-mediated cell death, is a complex process, which starts with the physical interaction of drug molecule with its specific target modifying the invading bacterium at the ultrastructural and molecular levels. Each antimicrobial drug has a specific target and unique mechanism of action. Inappropriate usage of antibiotics results has led to antibiotic resistance that has created a necessity for the search of better antimicrobial agents. At this juncture, natural products obtained from endophytes come to our rescue as excellent resources for antibiotics (Wang et al., 2012).

Li et al. (2014) reported new compounds containing the decalin moiety, eupenicinicol A and B, sirenin derivatives, eupenicisirenin A and B, and known compounds, (2S)-butylitaconic acid, (2S)-hexylitaconic acid, xanthomegnin, and viridicatumtoxin that have been extracted from the endophytic fungus,
Eupenicillium sp. isolated from the roots of Xanthium sibiricum. Among these metabolites (2S)-Butylitaconic acid and (2S)-hexylitaconic acid exhibited noticeable activity against Acinetobacter sp. Similarly, a new lanostanoid, 19-norlanosta-5(10),6,8,24-tetraene-1α,3β,12β,22S-tetraol, isolated from an endophytic fungus, Diaporthe sp. (LG23), inhabiting the leaves of Mahonia fortune exhibited pronounced antibacterial efficacy against both Gram-positive and Gram-negative bacteria, especially against the clinical isolates of Streptococcus pyogenes and Pseudomonas aeruginosa as well as a human pathogenic strain of Staphylococcus aureus (Li et al., 2015). A novel bioactive compound 6,7-(2'E) dibutenyl-5,8-dihydroxy-(Z)-cyclooct-2-ene-1,4-dione was extracted from the endophytic fungus Penicillium sp. M-01 isolated from Sophora flavescens. The compound showed strong antifungal activity against a group of fungi that includes Botryosphaeria berengriana, Physalospora piricola, Fusarium oxysporum and Fusarium moniliforme (Yua et al., 2014).

Bioassay guided fractionation of the organic extract of an endophytic fungus Xylaria sp. isolated from leaves of Anoectichilus setaceus, led to the isolation of the known antibacterial compound, helvolic acid. This compound is active against the Gram-positive bacteria, Bacillus subtilis and methicillin-resistant Staphylococcus aureus (Pamoda et al., 2014). Similarly, Penicillium sp an endophytic fungus isolated from the leaves of Garcinia nobilis, produced three new polyketides named penialidins A–C, along with one known compound, citromycetin. The antibacterial efficacies of the new compounds were tested against the clinically important risk group 2 (RG2) strains of Staphylococcus aureus and Escherichia coli. The strains of E. coli (RG1), Bacillus subtilis and Acinetobacter sp. BD4 were also included in the assay. Compound penialidins C
showed better and promising activity against the clinical target *S. aureus* as well as against *B. subtilis* when compared with the reference antibiotic streptomycin. Penialidins B was also highly active against *S. aureus* (Jean-Bosco *et al*., 2014).

An endophytic fungus *Phoma* sp. isolated from *Arisaema erubescens*, produced a new compound 3,6,7- trihydroxy-a-tetralone together with known compounds cercosporamide, b-sitosterol and trichodermin. These compounds exhibited strong antibacterial and antifungal activity (Wang *et al*., 2012). In another study, *Phoma* sp. isolated from *Cinnamomum mollissimum* produced 5-hydroxyra-mulosin that exhibited antifungal activity against *Aspergillus niger* (Santiago *et al*., 2012). Similarly, Phomodine a known compound obtained from *Phoma* sp. isolated from *Saurauia scaberrinae* also exhibited antibacterial activity against *Staphylococcus aureus* (Hoffman *et al*., 2008). Ethyl acetate extract of *Phomopsis* sp isolated from *Plumeria acutifolia* produced terpenoids, which exhibited antibacterial activity (Nithya and Muthumary, 2010).

Out of a total of 40 endophytic fungi isolated from *Ocimum sanctum*, only six endophytic fungi exhibited antibacterial and anticandidal activity (Pavithra *et al*., 2012). *Colletotrichum* sp. isolated from *Calophyllum inophyllum* exhibited antibacterial activity against *Staphylococcus aureus* and *Bacillus subtilis* (Raju and Victoria, 2015). Similarly Nameirakpam and Femina (2012) reported that ethyl acetate extract of *Penicillium* sp. isolated from *Camellia sinensis* exhibited antibacterial activity. *Fusarium* sp. an endophytic fungi from *Mirabilis jalappa* also exhibited strong antimicrobial activity (Devaraju and Sathish, 2011).
2.4.5.2. Antioxidant property of endophytic fungal metabolites

Free radicals are atoms or groups of atoms with an unpaired number of electrons and can be formed when oxygen interacts with certain molecules. Free radicals are developed in cells from several exogenous and endogenous sources. They are involved in various physiological functions of the cell (DeCoursey and Ligeti, 2005; Liu et al., 2008). Furthermore, free radicals can also be harmful to our immune system and cause damage to body cells leading to many degenerative diseases. This kind of pathophysiological condition emerges when homeostasis of free radicals in the cells are disturbed (Valko et al., 2006; Halliwell and Gutteridge, 1999; Dhallal et al., 2000). Antioxidants donates electron to free radicals converting them into harmless molecules, thus protecting cells from oxidative damage, aging and various diseases.

Antioxidants are habitually produced by many endophytes. Discovery of pestacin and isopestacin, potential antioxidant compounds obtained from Pestalotiopsis microspore, isolated as endophyte from Terminalia morobensis has led to the exploration of antioxidant potential of endophytic fungi. Pestacin C$_{15}$H$_{14}$O$_{4}$, 1, 3-dihydro isobenzofuran appears naturally as a racemic mixture and acts by cleaving reactive C–H bond and through O–H abstraction to a lesser extent (Harper et al., 2003). Isopestacin (C$_{15}$H$_{12}$O$_{5}$) an isobenzofuranone, scavenges superoxide and hydroxyl free radicals (Strobel et al., 2002c). Two new fungal polyketides were isolated from the ethyl acetate extract of Penicillium citrinum, an endolichenic fungal strain isolated from a Parmotrema species in Sri Lanka. The two compounds were demonstrated to show radical scavenging
activity using 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay (Samanthi et al., 2015).

Xiao et al. (2014) isolated 15 known compounds along with two novel compounds namely, 3-hydroxy-2-methoxy-5-methylpyridin-2(1H)-one and 3-hydroxy-N-(1-hydroxy-3-methylpentan-2-yl)-5-oxohexanamide, from the endophytic fungus Botryosphaeria dothidea, collected from the stems of white cedar (Melia azedarach). Among all the 17 compounds, altenusin and djalonensone showed better DPPH radical scavenging activities. The antioxidant potential of various extracts of Phomopsis liquidambari, isolated from Artemisia annua revealed that phenolic content was high in methanol extract and lowest in hexane extract (Yixin et al., 2014). Antioxidant potential of the hydroethanolic extract of 13 strains of endophytic fungi isolated from the leaves of Costus spiralis was estimated by DPPH and Ferric reducing ability of plasma (FRAP) assay. Extracts obtained from all the fungi exhibited promising activity in both the assays (Poliana et al., 2014).

Ethyl acetate extract of Nigrospora sp. isolated from Ginkgo biloba showed potential DPPH radical scavenging activity. Additionally phytochemical analysis of the extract revealed the presence of phenolic compounds and anthraquinones (Pawle et al., 2014). Afra et al. (2015) reported 21 endophytic fungi from Calotropis procera, Catharanthus roseus, Euphorbia prostrata, Vernonia amygdalina and Trigonella foenum-graecum. All the fungal strains were evaluated for their total phenolic content and total antioxidant capacity. However, only Aspergillus sp. from Trigonella foenum-graecum seeds had highest total phenolic content and antioxidant activity in DPPH radical scavenging assay.
Similarly, ethyl acetate fraction of the *Aspergillus fumigatus* showed best antioxidant activity in DPPH, hydroxylradical scavenging, reducing power, lipid peroxidation and Superoxide dismutase (SOD) inhibitory effect assays (Jin *et al*., 2014).

Various endophytic fungi namely *Aspergillus flavus*, *Fusarium oxysporum*, *Fusarium moniliforme*, *Trichothecium* sp were isolated from *Viscum album* and evaluated for their antioxidant potential using various assays. Among all, *Fusarium oxysporum* exhibited total antioxidant activity equivalent to ascorbic acid in FRAP assay. Similarly, *Aspergillus flavus* reduced DPPH free radicals and FRAP value of (133.22 µg/ml) ascorbic acid equivalent of Fe$^{2+}$/mg. *Fusarium moniliforme* showed H$_2$O$_2$ radical scavenging activity (Govindappa *et al*., 2014). Alkaloids, phenols, flavonoids, saponins, and terpenes were the main phytochemicals present in all the 21 fungal endophytic extracts obtained from *Eugenia jambolana*. Thirty six percent of the fungal extracts having high phenolic content exhibited potent antioxidant activity. *Chaetomium* sp., *Aspergillus* sp., *Aspergillus peyronelii* and *Aspergillus niger* strain showed the highest antioxidant activity ranging from 50% to 80% (Yadav *et al*., 2014).

**2.4.5.3. Metabolites from endophytic fungi as anticancer agents**

Cancer is a pathological condition characterized by unregulated cell proliferation, spread of abnormal cells and lack of apoptosis that result in death if not controlled (Sasikumar *et al*., 1999; Wyllie *et al*., 1999; Reed, 1999). Cancer occurs as a result of repeated mutations in specific regions of DNA and escape of the mutated genes from destruction in cell cycle regulation (Gibbs, 2003). For cancer, chemotherapy is the preferred strategy to treat the disease with some
limitations due to its side effects. The natural compounds have least side effects. Hence, several anticancer compounds isolated from endophytic fungal species can be considered as a safe alternative for synthetic drugs.

Paclitaxel (taxol) is a renowned tetracyclic diterpenoid bioactive compound, which was originally discovered from the bark of *Taxus brevifolia* (Wani *et al*., 1971). Paclitaxel has the unique mode of action of binding specifically to tubulin and preventing their depolymerization during the processes of cell division (Wang *et al*., 1999; Schiff *et al*., 1979). Further examination of the endophytes of *T. wallichiana* revealed *Pestalotiopsis microspora*, which also produced taxol. Many other endophytic fungi such as *P. microspora* (Strobel *et al*., 1996) and *Periconia* sp. (Li *et al*., 1998), residing in plants other than *Taxus* species also produced taxol. Hence, several endophytic fungi from different plants were screened for taxol production.

Unfortunately, taxol production upon fermentation by endophytes is in the range of picograms to micrograms per liter. Hence, considerable efforts are being made to determine the feasibility of taxol production by fermentation, which would effectively reduce its market price (Strobel, 2002a; Strobel and Daisy, 2003). For increased taxol production by *Fusarium redolens*, the growth conditions were optimized by altering one factor at a time. In the optimized medium, taxol production was three times higher than the original medium (Garyali *et al*., 2014). *Phoma betae* an endophytic fungus isolated from *Ginkgo biloba* was cultured for the production of taxol on a modified liquid medium. It produced 795µg/l, which is 15,900 fold more than the first report of taxol-producing fungus, *Taxomyces andreanae* (Kumaran *et al*., 2012).
As endophytic fungi residing in plants other than *Taxus* sp. also produced taxol, molecular techniques have been employed for fast and precised screening. Sixty fungal endophytes isolated from the inner bark of *Taxus baccata* and *Taxus wallichiana* were screened for taxol production using two key genes, DBAT (10-deacetylbaccatin III-10-O-acetyl transferase) and BAPT (C-13 phenylpropanoid side chain-CoA acyltransferase) that are involved in taxol biosynthesis. Among 60 isolates, *Fusarium redolens* produced taxol with the yield of 66.25 µg/l (Garyali *et al.*, 2013). Similarly, Gokulraja *et al.* (2015) evaluated *Cladosporium oxysporum* for taxol production using DBAT gene. The taxol produced by this fungus suppressed proliferation of human colon cancer cell line HCT15.

Another cytotoxic plant alkaloid Camptothecin (CPT), was originally isolated from *Camptotheca acuminata* (Wall *et al.*, 1966). Camptothecin has unique mode of action that inhibits the nuclear enzyme topoisomerase-I, which is essential for DNA replication (Hsiang *et al.*, 1985). Hycamtin (topotecan) and Camtostar (irinotecan) are CPT semi-synthetic drugs, which are clinically used against ovarian, small lung and refractory ovarian cancers (Sirikantaramas *et al.*, 2007). The discovery of camptothecin from endophytic fungus *Entrophospora infrequens* isolated from plant *Nothapodytes foetida* has opened a new vista in the field endophytic fungi to substitute the anticancer drug camptothecin (Puri *et al.*, 2005).

Ethanolic extract of *Catharanthus roseus*, when added to the suspension culture of endophyte *Fusarium solani* produced 10.6 fold higher camptothecin compared to control (2.8µg/L). Interestingly, addition of pure ethanol 5%v/v to the suspension culture resulted in camptothecin production upto15.5 fold higher
than control. This study indicated the dual role of ethanol apparently as an energy source and also as an elicitor for camptothecin production (Aarthi and Smita, 2015). Two isolates of *Aspergillus* LY341 and LY355, and *Trichoderma atroviride* LY357, isolated from *Camptotheca acuminate* produced 7.93, 42.92, and 197.82µg l⁻¹ of CPT respectively (Pu *et al*., 2013). There was a 50–75 fold increase of CPT yield, when optimized conditions, elicitor and adsorbent resin were used.

Podophyllotoxin (PDT) is a well-known aryltetralin lignan with potent anticancer properties, mainly occurring in the genera *Diphyllleia, Dysosma, Juniperus* and *Sinopodophyllum* (Yang *et al*., 2003; Lu *et al*., 2006; Cao *et al*., 2007; Kusari *et al*., 2009). It has been used as a precursor for chemical synthesis of the anticancer drugs like etoposide, teniposide, and etopophose phosphate, which act as topoisomerase inhibitors (Amardeep *et al*., 2008; Zeng *et al*., 2004; Guo *et al*., 2004; Eyberger *et al*., 2006). *Fusarium solani* an endophytic fungus isolated from roots of *Podophyllum hexandrum* has been reported to produce podophyllotoxin. The presence of podophyllotoxin in dry fungal biomass was confirmed and quantified by HPLC and mass spectrometry (Nadeem *et al*., 2012).

A total of 92 strains of endophytic fungi were isolated from *Sinopodophyllum hexandrum, Diphyllleia sinensis* and *Dysosma veitchii*. Fermented broth of all the strains were screened for podophyllotoxin. Six strains had the ability to produce podophyllotoxin (Yang *et al*., 2003). Similarly two strains of endophytic fungi *Phialocephala fortinii* isolated from rhizomes of *Podophyllum peltatum* (Eyberger *et al*., 2006), *Fusarium oxysporum* isolated from
Juniperus recurva (Amardeep et al., 2008) and Alternaria sp. isolated from Sabina vulgaris (Lu et al., 2006), yielded various quantities of podophyllotoxin.

Vinblastine was first reported from the endophytic fungus Alternaria sp. isolated from the phloem of Catharanthus roseus (Guo et al., 1998). Later, an endophytic fungus Fusarium oxysporum isolated form the phloem of C. roseus also produced vincristine (Zhang et al., 2000). An unidentified endophytic fungus isolated from Catharanthus roseus also produced vincristine (Yang et al., 2004). In another study, the endophytic fungus Fusarium oxysporum isolated from Catharanthus roseus were reported to produce vinblastine and vincristine (Ashutosh et al., 2013). Vinblastine and vincristine are the terpenoid indole alkaloids derived by coupling of two well-known anticancer monomers vindoline and catharanthine (Perez et al., 2002; Wang et al., 2010). The primary mode of action of vincristine is by interfering with microtubule formation and mitotic spindle dynamics, disrupting intracellular transport and decreasing tumour blood flow (Perez et al., 2002; Moore and Pinkerton, 2009).

2.5. Rationale for selection of host plants of the present study

Fossil evidence suggested that plants and their endophytes have co-evolved (Michael et al., 2012). During co-evolution endophytes must have undergone several genetic changes by acquiring certain genes or DNA fragments from host. This gene transfer is believed to have driven the endophytes to adapt host microenvironments (Germaine et al., 2004). This unique relationship gives endophytic fungi an ability to synthesize some bioactive molecules originally associated with the host plants (Stierle et al., 1993; Strobel et al., 1996; Shrestha et al., 2001). Hence, selection of host should be towards the plants that are rich in
bioactive metabolites or the medicinal plants that served the indigenous population for several years to treat diseases.

2.6. Bioactive potential of selected host plants

In *Azadirachta indica* (Meliaceae), the most important bioactive constituents present in the leaves include, β-Sitosterol, Hyperoside, Nimbandiol, Nimbolide, Quercetin, Quercitrin and Rutin. Leaves of this plant is known to possess a variety of pharmacological properties such as anticancer (Wu et al., 2014), antibacterial and antiviral (Bharitkar et al., 2014), antifilarial (Niladri et al., 2014), anti-inflammatory and antinociceptive (Soares et al., 2014), insecticidal (Anirban et al., 2014) and antidiabetic activity (Rosa et al., 2012).

Leaves of *Cascabela thevetia* (Apocynaceae) are known to possess most important bioactive constituents like aucubin, ursolic acid and β–amyrin–acetate. These leaves are known to have a wide range of pharmacological properties such as anti inflammatory (Thilagavathi et al., 2010), antitermite (Thilagavathi et al., 2010), diuretic (Harborne and Baxter, 1983), laxative (Williamson and Evans, 1988), antiedemic (Bruneton, 1999), RNA inhibitor, antidote (Chang et al., 1985).

*Couroupita guianensis* (Lecythidaceae) is known to exhibit a variety of pharmacological properties such as antimicrobial, ovicidal (Baskar et al., 2013), antiulcer (Elumalai et al., 2012), immunomodulatory activity (Pradhan et al., 2008), antimycobacterial and antibiofilm (Al-Dhabi et al., 2012).

*Ficus religiosa* (Moraceae) has various pharmacological properties such as anticancer activity (Vishal and Thangavel, 2011), anticonvulsant (Patil et al., 2011), ulcer protectant (Saha and Goswami, 2010), antidiabetic (Pandit et al., 2010) and antiasthmatic activity (Megha et al., 2011). Some of the important
medicinal properties of *Guazuma ulmifolia* (Sterculiaceae) includes wound-healing capacity (Senthil *et al*., 2011), anti microbial and antioxidant (Aline *et al*., 2013), antiviral (Adriana *et al*., 2006), gastroprotective (Berenguer *et al*., 2007) and antiobesity (Iswantini *et al*., 2011). Similarly *Madhuca longifolia* (Sapotaceae) also exhibited many pharmacological properties such as wound healing capacity (Smita *et al*., 2010), anti-inflammatory, analgesic and antipyretic (Shekhawat and Vijayvergia, 2010) activities. It is also used to treat hydrocele (Ayyanar and Ignacimuthu, 2005). *Plumeria rubra* (Apocynaceae) is found to have wide range of pharmacological activities such as antibacterial (Egwaikhide *et al*., 2009), anticancer (Leonardus *et al*., 1990), anxiolytic (Manavi *et al*., 2013), antioxidant and hypolipidemic (Merina *et al*., 2010), antihypertensive and vasodilator (Socorro *et al*., 2013).

*Milletia pinnata* (Fabaceae) exhibited various medicinal properties such as antidiarrheal (Shoba and Thomas, 2001), antifungal (Misra and Sahu, 1977), antiplasmodial (Simonsen *et al*., 2001), antiinflammatory (Srinivasan and Muruganandan, 2001) and analgesic (Dahanukar *et al*., 2000). Pharmacological properties of *Albizia saman* (Caesalpiniaceae) are antimicrobial (Raymond *et al*., 2011), anti-ulcer (Arumugam *et al*., 2011), antioxidant (Arulpriya *et al*., 2010), analgesic (Muzammil *et al*., 2013) and insecticidal (Iqbal *et al*., 2009). Similarly *Tabebuia rosea* (Bignoniaceae) also has various bioactivity, which includes anticancer (Sichaema *et al*., 2013), antioxidant, antibacterial and antiinflammatory (Franco *et al*., 2013), antidiabetic (Maria *et al*., 2012), antidote (Oteroa *et al*., 2000) and antimalarial activity (Compadre *et al*., 1982). Diverse bioactive potential of these plants has motivated to select them as a host for isolation of endophytic fungi to carry out this study.