CHAPTER-III

LITERATURE REVIEW
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LITERATURE REVIEW

3.1 Need of new natural substances
There is increasing demand of safe, natural bioactive compounds to provide aid to living beings. In last few decades, the world has faced severe new diseases and re-emerging conditions, such as AIDS, SARS, Swine flu (H1N1), cancer and also infections caused by multi-drug resistant pathogens (Morgan et al., 2004) (Fig. 3.1). At the same time, new compounds are necessary to treat parasitic infections such as Leishmaniosis, Malaria etc. (Morgan et al., 2008).
Agriculture field also demands new natural products which can function to treat the diseases or act as pesticides (Demain, 2000). Many synthetic agricultural products are posing problems related to their safety and environmental issues, hence need to find alternate way to control pest and pathogens. This situation led the exploration of diverse natural sources for the finding of safe and potent agents. There are currently over 10 million compounds available to myriad aspects of human life (Schulz et al., 2002). However, there is an unlimited demand for new substances due to the emergence of drug resistant pathogens as well as the continued presence of untreatable diseases.
Novel natural substances are regarded as a potential source for the innovation in the field of medicine as well as agro chemistry, as these compounds provides greater ecological safety and diverse chemical structures, which is more beneficial over the synthetic chemical compounds (Baker et al., 2012).

3.2 Natural products as a potential source of new metabolites
Metabolites are naturally derived products and by products from plants, animals and microorganisms (Baker, 2000). Pasteur’s discovery of novel fermentation process attracted the interest of various other research groups for the synthesis or isolation of bioactive components from plant source (Strobel, 2004).
Natural products are obtained from different sources such as medicinal plants, marine organisms, terrestrial micro-organisms, vertebrates and invertebrates (Newman et al., 2000).
Natural products have performed and continue to demonstrate a key role in the drug discovery process and also in treating various human diseases throughout the world. Natural products have remained the important source for new drug discovery (Strobel and Daisy, 2003). These compounds are helpful in treating and preventing variety of acute and chronic human diseases.

The novel metabolites of natural products provide a new chemical entity of diverse structures, which acts as a template for the synthesis of new drug. Natural secondary metabolites from living systems are showing more “drug-likeliness and biological friendliness” than totally synthetic molecules (Koenh et al., 2005) which make them a good choice for the discovery and development of new drug.

Study of all approved agents during the time period of more than 25 years reveals the use of natural products as a source of novel structures (Newman et al., 2007).

Though the natural compounds comprises only one tenth of the total compounds, the compounds obtained from the micro-organisms seems to be more potent than the synthetic ones (Kulda et al., 2006). Hence, natural products can serve as a potent and promising source of novel metabolites.
### Table 3.1 Approximate numbers of known natural products (Kuldau et al., 2006)

<table>
<thead>
<tr>
<th>Sources of Natural products</th>
<th>All known Over 500,000</th>
<th>All bioactive 80-100,000</th>
<th>Antibiotic 30-40,000</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Microbial metabolites</strong></td>
<td>60-80,000</td>
<td>32-34,000</td>
<td>25-27,000</td>
</tr>
<tr>
<td>Actinobacteria</td>
<td>~20,000</td>
<td>~12,000</td>
<td>~10,000</td>
</tr>
<tr>
<td>Fungi</td>
<td>~30,000</td>
<td>~15,000</td>
<td>~10,000</td>
</tr>
<tr>
<td>Unicellular bacteria</td>
<td>~20,000</td>
<td>~7,000</td>
<td>~5,000</td>
</tr>
<tr>
<td><strong>Plant kingdom</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lower plants (algae, etc.)</td>
<td>50-60,000</td>
<td>~6,000</td>
<td>~100-200</td>
</tr>
<tr>
<td>Higher plants</td>
<td>300-350,000</td>
<td>~25,000</td>
<td>~15,000</td>
</tr>
<tr>
<td><strong>Animal kingdom</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Marine invertebrates</td>
<td>25-30,000</td>
<td>~10,000</td>
<td>~5,000</td>
</tr>
<tr>
<td>Terrestrial animals</td>
<td>4-5,000</td>
<td>~200</td>
<td>~100</td>
</tr>
</tbody>
</table>

In the early years of metabolite development, Actinobacteria, especially *Streptomyces* was the major source of secondary metabolites, which approximately counted about 62%, while bacterial sources counted 15% and that of fungi to about 23%. In the later ages from 80’s onward, the ratio of secondary metabolites produced by fungi changed drastically and it is continuously increasing. From 80’s onward, the fungi are accounting for almost 61% of secondary metabolites, while Actinobacteria accounts around 28.5%, which is less than one third of fungal metabolites (Table 3.2).

### 3.3 Medicinal plants

Plants have been a part of human life throughout the history of mankind. Different products are obtained from plant source having different benefits to the humans. Plants have the potential to synthesize a wide variety of chemical compounds having biological properties (Tapsell et al., 2006).
### Table 3.2 Approximate numbers of bioactive microbial metabolites (Kuldau et al., 2008)

The use of plants for treating many infectious diseases is probably as old as humankind. The knowledge regarding medicinal values of the plants and their use as medicines, go back to the time of earliest settlers. The most primitive literary source of information regarding healing practices by using plants was given by the Vedic hymns of the migrant Aryan tribes (Zysk, 1996). Most ancient healthcare systems followed by Indians have typed the medicinal use of plant source in ancient classical Indian text such as Ayurveda, Rig-Veda, Charaksamhita and Sushruta. According to World Health Organization (WHO) report, 80% population of the world depends on herbal medicines for primary healthcare necessities (Azaizen, 2003). The use of natural compounds for medicinal purposes is documented in the western world document “De Materia Medica”, written by Dioscorides, which gives a description of many medicinal plants (Tyler et al., 1988) that remains important in modern

<table>
<thead>
<tr>
<th>Species</th>
<th>1940-1974</th>
<th>%</th>
<th>1975-2000</th>
<th>%</th>
<th>2001 onwards</th>
<th>%</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actinobacteria</td>
<td>3400</td>
<td>62</td>
<td>7200</td>
<td>42</td>
<td>3100</td>
<td>28.5</td>
<td>13700</td>
</tr>
<tr>
<td><em>Streptomyces</em> sp.</td>
<td>2900</td>
<td></td>
<td>5100</td>
<td></td>
<td>2400</td>
<td></td>
<td>10400</td>
</tr>
<tr>
<td>Other Actinobacteria</td>
<td>500</td>
<td></td>
<td>2100</td>
<td></td>
<td>700</td>
<td></td>
<td>3300</td>
</tr>
<tr>
<td><em>All microscopic bacteria</em></td>
<td>800</td>
<td>15</td>
<td>2300</td>
<td>13</td>
<td>1100</td>
<td>10</td>
<td>4200</td>
</tr>
<tr>
<td>Myxobacteriales</td>
<td>25</td>
<td></td>
<td>400</td>
<td></td>
<td>210</td>
<td></td>
<td>635</td>
</tr>
<tr>
<td>Cyanobacteria</td>
<td>10</td>
<td></td>
<td>30</td>
<td></td>
<td>1250</td>
<td></td>
<td>1290</td>
</tr>
<tr>
<td><em>All fungi</em></td>
<td>1300</td>
<td>23</td>
<td>7700</td>
<td>45</td>
<td>6600</td>
<td>61</td>
<td>15600</td>
</tr>
<tr>
<td>Microscopic fungi</td>
<td>950</td>
<td></td>
<td>5400</td>
<td></td>
<td>4900</td>
<td></td>
<td>11250</td>
</tr>
<tr>
<td>Basidiomycetes</td>
<td>300</td>
<td></td>
<td>1800</td>
<td></td>
<td>1500</td>
<td></td>
<td>3600</td>
</tr>
<tr>
<td>Other fungi</td>
<td>20</td>
<td></td>
<td>200</td>
<td></td>
<td>160</td>
<td></td>
<td>380</td>
</tr>
<tr>
<td>Total per year</td>
<td>5500</td>
<td>100</td>
<td>17000</td>
<td>100</td>
<td>10800</td>
<td>100</td>
<td>33500</td>
</tr>
</tbody>
</table>
medicines, as they serve as a source of important pure chemical components which play a vital role in modern medicinal system. Hence, the use of natural plant products for the discovery of new drugs proved to be the most successful approach, as the plants have an ability to synthesize a diverse chemical compounds, used to perform important biological functions (Tapsell et al., 2006).

![Fig.3.2 Proportion of biologically active isolates from different sources tested for antimicrobial activities (Hongsheng, 2010).]

### 3.4 Endophytes

Endophyte refers to the bacterial or fungal micro-organisms which colonize in the interior plant tissues. Different scientists have addressed them in different ways based on their presence in the plant body:

- Tervet and Hollis (1948): Endophytes are micro-organisms which live inside the plants without causing disease symptoms.
- Quispel (1992): Endophytic bacteria or fungi colonize the host tissue internally, sometimes in high number, without damaging the host or eliciting symptoms of plant disease (Schulz, 1993).
- Wilson (1993): Micro-organisms living within tissues for all or part of their life cycle without causing any visible symptoms of their presence.
Hallman et al (1997): Endophytic bacteria reside inside surface sterilized plant tissues or extracted from plants and have no visibly harmful effect on the host plant (Morens, 2004).

Schulz and Boyle (2006): Bacteria that colonize the internal tissue of the plants showing no external signs of infection or negative effect on their host. The endophytes attracted the attention due to their ability to protect their host against pathogens, insects and pests (Borse et al., 1990). Almost all the plant species harbour one or more endophytic organism (Petrini, 1986). These are ubiquitous in all the plant species (Strobel, 2003).

De Bary (1866) was first to introduce a term ‘epiphyte’ referring to fungi living on the surface of their host and ‘endophytes’ for those living inside the host plant (Tan and Zou, 2001). Endophytes constitute different group of micro-organisms which includes bacteria, fungi, yeast and cyanobacteria colonizing in various parts of the host plants, such as leaves, stems, roots, fruits etc.

The first fungal endophyte was reported in 1904 by Freeman, a German scientist, in Persian darnel which is an annual grass (Petrini, 1991) and the first bacterial endophyte within the tissue of the plant was identified in 1926 (Hallman et al., 1997). Endophytes have been reported to produce plenty of bioactive compounds as their secondary metabolite, which shows structural diversity. ‘Secondary metabolites are low molecular weight compounds, not necessary for the growth but are produced to perform specific function in nature’ (Demain, 1988; Schulz, 2005).

These metabolites provide many benefits to their host plants including protection, ultimately giving survival value to the plants (Strobel, 2004). Secondary metabolites of various endophytes show broad antimicrobial activity which plays a valuable role in treating the drug-resistant micro-organisms which can be a major threat in the future (Liu et al., 2001). Thus, currently endophytes are viewed as a promising source of bioactive natural compounds (Morens, 2008).

3.4.1 Endophyte –Plant Interaction

There are approximately 3, 00,000 plant species exists on the earth and each plant harbours one or more endophytes of different species (Strobel and Daisy, 2003; Huang et al., 2007). Endophyte shows symbiotic association with its host plant without causing any harm to the host.

The studies of endophytic fungi and their relationships with host plants shed light on the evolution of endophyte plant symbiosis and the ecological factors that influence
the endophyte host plant interaction (Saikkonen, 1998). Endophyte-plant interactions is different from pathogen-plant interaction as neither disease symptoms develop on the plant host nor the fungus is eliminated by the plant host (Suryanarayanan, 2009). The gene of an endophyte and plant controls the type of interaction between them along with environmental factors (Moricca and Ragazzi, 2008). Initial stages of interactions include recognition of hosts, penetration of endophyte in host etc. Before entry, the spores of the fungi recognize the host plant by lectin like molecules, followed by the penetration of endophyte inside the tissue of host plant which is achieved by softening the cuticle and the wall of the epidermal cells or breaking the cuticle by mechanical force. Once the penetration in the host plant is achieved the endophyte shifts to the silent state due to which the defence mechanism is not activated in the host (Sieber, 2007; Chapela, 1993).

Endophytes develop a compromising relation with host plant, maintaining antagonism and mutualism. This results in a symbiotic association with the host plant, offering benefits to both (Sapp, 1994; Feath, 2002). Endophyte utilizes certain plant metabolites to retrieve essential nutrients and energy for their growth and survival in the host plants. In turn, the endophytes produces certain metabolic compounds which promote the growth of host plants along with additional benefits such as drought tolerance, resistance to pathogens, insect protection, growth enhancement etc., which supports the balanced living environment for both.

3.4.2 Factors affecting endophytic community in the plant

Endophytes are associated with a host plant, to sustain dynamic environment for their habitat. Many factors such as plant tissues, soil type, rainfall and interaction with other micro-organisms may affect the type of endophytic community in the host plant (kuklinsky et al., 2005). Many studies have revealed that the important factors for diversity of endophytes in plant includes the enhancement of primary productivity, nutrient retention, (Tilman et al., 1997), nutrient flow (Cardinale et al., 2002), water availability and resistance to pathogen invasion by host (Levine et al., 1999).

Factors associated with host plant affecting the endophytic communities may include plant genotype, age of the plant, and nutrient supply by the host plant, environmental conditions, and physical parameters such as temperature, UV radiations etc. (Kulkarni et al., 2014). Soil characters such as pH, salinity and texture may show the effect on endophytic community in the host plant (Kulkarni et al., 2014).
3.5 Endophytic fungi

This fungus colonizes the inter-cellular spaces and inside of xylem and phloem cells of healthy plant tissues (Hallman et al., 1997). Endophytic fungi are ubiquitous in nature. The ubiquity of these fungi has been estimated with 1 million species of endophytic fungi residing in plants (Dreyfuss et al., 1994). A highly specific endophytic community is developed in each plant (Petrini, 1996), which includes Ascomycetes, Basidiomycetes, Oomycetes and Deuteromycetes (Arnold et al., 2000). Endophytic fungi are known to affect plant community, diversity and structure (Krings et al., 2007). This group of fungi can have an impact on host plant which can increase fitness of plant by various means such as increasing biomass and decreasing water consumption or decreasing fitness by altering resource allocation (Rodriguez et al., 2009).

Medicinal plants acts as a repository of fungal endophytes associated with novel metabolites of pharmaceutical importance (Kumar et al., 2005; Wiyakrutta, 2004). Endophytic fungi are classified into four different classes. The classification is based on host range, colonizing tissue type, colonization in plant and diversity of endophytes in plant, transmission and fitness benefits of endophytes (Rodriguez et al., 2009).

- **Class I endophytes (Clavicipitaecous)** are phylogenetically related *Clavicipitaecous* species, fastidious in nature and are restricted to some cool and warm season grasses (Stone et al., 2004; Bischoff et al., 2005). These primarily transmit the fungi vertically through maternal plants to offspring via seed infections (Saikkonen et al., 2002). The host species, host genotype and environmental conditions determine the benefits contributed by these fungi to their host plants (Faeth et al., 2006).

- **Class II endophytes** comprise of members of the *Dikarya* (*Ascomycota* or *Basidiomycota*) which benefits the host plants in stress tolerance (Rodriguez et al., 2008).

- **Class III endophytes** are distinguished depending on their transmission and occurrence. They shows diversity within host tissues, plants and population. This includes vascular and nonvascular plants, woody and herbaceous angiosperms in tropical forests of Antarctic communities (Davis and Shaw, 2008; Higgins et al., 2007; Murali et al., 2007).
Class IV endophytes are generally Ascomycetous fungi forming melanised structures like microsclerotia and inter and intracellular hyphae in the roots. These endophytes are found in nonmycorrizal plants from Antarctic, alpine, sub alpine temperate zones (Jumpponen, 2001).

![Diagram showing the location of different classes of endophytes](image.jpg)

Fig. 3.3 Location of different classes of endophytes (Rodriguez et al., 2009)

### 3.5.1 Taxonomy of endophytic fungi

Fungi of class Ascomycetes, Basidiomycetes and Deuteromycetes are mostly reported as endophytic fungi (Petrini, 1986). Endophytic population in the tissue of the plant is highly variable and it changes with the location within the plant (leaves, stem, and roots). The population density of endophytes within the plant tissue mainly depends on the type of endophytic organisms, genotype of the host plant and environmental conditions.

### 3.5.2 Host specificity of endophytic fungi

Endophytic fungi have a wide range of host and colonize several taxonomically unrelated plant hosts, suggesting no host specificity endophytic fungi (Cohen, 2006). They can be isolated from plants of different families, growing under diverse ecological and geographical conditions (Petrini, 1986).
Organ specificity has been observed in some endophytic fungi. Some strains of fungi isolated from different parts of the single plant differ in their utilization of various substances (Corell and Petrini, 1983).

Some specificity with respect to the location of endophytic species within the needles (petiole or blade) of conifers has been observed (Carroll and Carroll, 1978). Factors associated with the host of the endophytes such as age of the host, season and environment of host plant may influence the biology of endophytes. Host plant plays very important role in the general metabolism of endophytes. Hence, endophytes can be isolated from plants belonging to the diverse families and classes and growing under different geographical and ecological conditions (Petrini et al., 1986).

3.6 Secondary metabolites

The hunt for new antimicrobial compound is important due to increase in the resistance of pathogenic micro-organisms towards the antibiotics. Secondary metabolites from microbes have received an increased attention as useful agent for development of medicines (Cragg and Newman, 2005).

Metabolic processes forms the base of life. These processes are mainly divided into catabolic reactions and anabolic reactions. Catabolic reactions yields energy by various breakdown processes, whereas anabolic reactions uses this energy for the formation of many cell components such as proteins.

In micro-organisms, the metabolism is mainly of two types- primary and secondary. Kliebenstein (2004) have reported that primary metabolism involves the anabolic and catabolic processes required for various cell functions such as growth and development, nutrient assimilation, cell maintenance and proliferation, whereas secondary metabolism refers to the production of compounds by microbes that are not necessary for cell growth. Secondary metabolites are generally produced by an intermediate products formed during primary metabolism. Secondary metabolites were first recognized by Sach in 1873 and were referred as natural products (Berdy, 2005).
Endophytic micro-organisms isolated from traditionally used medicinal plants are a good source of bioactive secondary metabolites (Tejasvi et al., 2007; Huang et al., 2008). Secondary metabolites are produced due to the interaction between endophytes and plants which is controlled by the genes of endophytes and environmental conditions of plant.

Endophytic fungi of all higher plants are of growing interest as promising sources of biologically active agents (Selim et al., 2012). Various natural products of endophytic fungi possess unique structures and bioactivities, which offers an enormous potential for its exploitation in medicinal, agricultural and industrial field (Zhang et al., 2006). Therefore, endophytes may be “treasure trove” for new bio activeness due to their wide biochemical diversity and hence they are called as ‘chemical factories inside plants’ (Kulkarni et al., 2014).

### 3.6.1 Factors affecting secondary metabolites production

Studies have revealed various factors influencing secondary metabolites production in endophytic fungi including environmental as well as genetic factors. Bills et al. (2002) studied the metabolite distinction between tropical and temperate endophytic microbes. In this study number of bioactive natural products from tropical as well as temperate region was compared statistically. It was observed that the endophytes of tropical region produced more number of bioactive secondary metabolites as

<table>
<thead>
<tr>
<th>Primary metabolites</th>
<th>Secondary metabolites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Essential for growth and development</td>
<td>Not essential for growth and development</td>
</tr>
<tr>
<td>Not produced as an adaptation</td>
<td>Produced as an adaptation for specific function</td>
</tr>
<tr>
<td>Uniform</td>
<td>Variable</td>
</tr>
<tr>
<td>Conservative</td>
<td>Diverse</td>
</tr>
<tr>
<td>Relatively simple structure</td>
<td>Highly complex structure</td>
</tr>
<tr>
<td>Produced by every micro-organism</td>
<td>specifically produced by selected few microorganisms</td>
</tr>
<tr>
<td>produced during the growth phase of cell</td>
<td>produced during the non-growth phase of cell</td>
</tr>
</tbody>
</table>

Table 3.3 Comparison between primary and secondary metabolites
compared to endophytes of temperate region, suggesting the influence of environmental conditions in metabolite production.

Some endophytic micro-organisms may become pathogenic to the host plant in response to some kind of environmental conditions. The pathogenesis may be induced by the production of certain compounds by endophytes, suggesting the impact of environmental conditions on the metabolite production by endophytes (Hendry et al., 2002).

Faeth and Fagem (2002) suggested that environmental conditions along with the genotypic combination of endophyte and host show variation in endophyte – host interaction. Such variations may affect the metabolic profile of endophytes.

3.6.2 Characteristics of secondary metabolites:

- Specifically produced by selected microorganisms.
- Not essential for the growth and reproduction of organisms from which they are produced.
- Influenced by environmental factors.
- Some microorganisms produce secondary metabolites as a group of compounds (usually structurally related) instead of a single one. e.g. about 35 anthracyclines are produced by a single strain of Streptomyces.
- The biosynthetic pathways for most secondary metabolites are anabolic.
- The enzymes involved in the formation of secondary metabolites are more complex and differs from enzymes involved in the formation of primary metabolites.
- Growth conditions, especially media composition extremely influence the production of secondary metabolites.

3.6.3 Pathways for secondary metabolite production:

Three main pathways involved in secondary metabolites production by fungi are

a. Shikimate-Chorismate pathway (Romagni, 2004)
b. Mevalonic acid pathway (Romagni, 2004)
c. Polyketide pathway (Deacon, 2005)
a. Shikimate-Chorismate pathway:

Fig 3.4 Shikimate- Chorismate pathway (Source-google.com)

The shikimate pathway is a metabolic pathway used by bacteria, fungi, and plants for the biosynthesis of aromatic amino acids. The enzyme shikimate kinase, catalyses the ATP-dependent phosphorylation of shikimate to form shikimate 3 phosphate. Shikimate 3-phosphate is then coupled with phosphoenol pyruvate to give 5-enol pyruvyl shikimate-3-phosphate via the enzyme 5-enol pyruvyl shikimate-3-phosphate
(EPSP) synthase. Then 5-enolpyruvyl shikimate-3-phosphate is transformed into chorismate by chorismate synthase. Prephenic acid is synthesized by a Claisen rearrangement of chorismate by Chorismate mutase. Prephenate is oxidatively decarboxylated with retention of the hydroxyl group to give $p$-hydroxyphenyl pyruvate, which is transmitted using glutamate as the nitrogen source to give tyrosine and $\alpha$-ketoglutarate. (Fig. 3.4)

<table>
<thead>
<tr>
<th>EC Code</th>
<th>Enzyme Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC1.1.1.24</td>
<td>Quinate dehydrogenase</td>
</tr>
<tr>
<td>EC1.1.1.25</td>
<td>Shikimate dehydrogenase</td>
</tr>
<tr>
<td>EC1.1.1.282</td>
<td>Quinate/shikimate dehydrogenase</td>
</tr>
<tr>
<td>EC1.1.99.25</td>
<td>Quinate dehydrogenase (pyrroloquinoline-quinone)</td>
</tr>
<tr>
<td>EC 2.5.1.19</td>
<td>3-phosphoshikimate 1-carboxyvinyltransferase</td>
</tr>
<tr>
<td>EC2.1.54</td>
<td>3-deoxy-7-phosphoheptulonate synthase</td>
</tr>
<tr>
<td>EC2.7.1.71</td>
<td>Shikimate kinase</td>
</tr>
<tr>
<td>EC3.3.2.13</td>
<td>Chorismatase</td>
</tr>
<tr>
<td>EC4.1.3.45</td>
<td>3-hydroxybenzoate synthase</td>
</tr>
<tr>
<td>EC4.2.1.10</td>
<td>3-dehydroquinate dehydratase</td>
</tr>
<tr>
<td>EC4.2.3.4</td>
<td>3-dehydroquinate synthase</td>
</tr>
<tr>
<td>EC4.2.3.5</td>
<td>Chorismatesynthase</td>
</tr>
<tr>
<td>EC 5.4.4.2</td>
<td>Isochorismate synthase</td>
</tr>
</tbody>
</table>

b. Mevalonic acid pathway:

Mevalonic acid pathway, also known as the isoprenoid pathway or HMG-CoA reductase pathway is an essential metabolic pathway present in eukaryotes (all mammals), a few eubacteria archea, cytosol and mitochondria of plants and fungi. This pathway produces two five-carbon compounds, acting as a building blocks called isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), which are further used to synthesize different isoprenoids such as cholesterol, heme, sterols etc. (Holstein et al., 2004).

This pathway begins with production of acetoacetyl-CoA, involving Claisen-like condensation mechanism, catalyzed by thiolase enzyme. The third acetyl-CoA is then condensed with a acetoacetyl-CoA to form 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) through aldol reaction. This reaction is carried out by enzyme HMG-CoA synthase. The CoA derivative is then converted to Mevalonic acid (MVA) by NADPH-dependent HMG-CoA reductase. (Fig. 3.5)
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Fig 3.5 Mevalonic acid pathway (Source- Wikipedia)

Acetyl-CoA + Acetyl-CoA
CoA-SH
Acetoacetyl-CoA

Thiolase (Acetoacetyl-CoA transferase ACAT)
HMG-CoA synthase

3-hydroxy-3-methylglutaryl-CoA (HMG-CoA)

STATINS (HMG-CoA Inhibitors)
HMG-CoA reductase
Mevalonic acid
Mevalonate kinase

ATP
Mevalonate-5-phosphate
ATP
Phosphomevalonate kinase

Mevalonate-5-pyrophosphate
Mevalonate-5-pyrophosphate decarboxylase

CO₂
Isopentenyl-5-pyrophosphate (PP)
Isopentenyl-PP isomerase

Dimethylallyl-PP
Biphosphonates
Farnesyl diphosphate synthase (FPPS)
Geranyl-PP

Biphosphonates
Farnesyl diphosphate synthase (FPPS)

Farnesyl-PP
Geranyl/Geranyl diphosphate synthase (GGPPS)
GeranylGeranylPP
Squalen
Ubiquiones
Sterol
Dolichols
Prenylated proteins

Cholesterol
Heme A
Further, Mevalonic acid is phosphorylated to Mevalonate 5-diphosphate sequently by mevalonate kinase and diphosphomevalonate kinase. The decarboxylation of diphosphate by mevalonate diphosphate decarboxylase subsequently yields C5 building blocks isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). IPP is isomerized to DMAPP by the enzyme isopentenyl pyrophosphate isomerase. Both IPP and DMAPP are the universal precursor of natural product called isoprenoid.

c. Polyketide pathway:

Fig 3.6 Polyketide pathway (Source- Google.com)
The polyketide pathway leads to synthesise of some quinines, mainly napthoquinones and anthraquinones. Some flavonoids are also synthesized by this pathway. The polyketide pathway is also involved in the production of some plant phenolics. For the production of quinines, polyketide pathway is mostly found in the micro-organisms such as bacteria and fungi.

Acetyl CoA is the starting material in the polyketide pathway. The initial step involves the addition of 3 malonyl-CoA to acetyl CoA, yielding a linear tetraketide chain. This linear chain can cyclize via Orsellinic acid type mechanism or Claisen condensation through hydrolysis of thioester group. This yields a polyketide precursor such as Orsellinic acid and 6-Methylsalisylic acid. Both reactions lead to the formation of phenyl ring. The opening and rearrangement of phenyl ring then gives various polyketide such as patulin. (Fig. 3.6)

3.7 Endophytic fungi as a source of bioactive secondary metabolites

Endophytic fungi are well known to produce valuable bioactive compounds, which has generated an interest to study deeply about them. The discovery of Taxol, an anti-cancer compound from endophytic fungi, made them a promising source for the isolation of novel potent bioactive compounds of significant importance.

Endophytic fungi demonstrated successful production of valuable bioactive products by fermentation which significantly impacted the pharmaceutical arena (Lewis et al., 1997). Secondary metabolite production by fungal endophytes is larger than those other endophytic organisms (Zhang et al., 2006). About 51% of bioactive compounds from fungi were unknown (Strobel, 2002). Bioactive compounds produced by endophytic fungi includes alkaloids, flavonoids, phenolic compounds, aromatic compounds, steroids, terpenoids, quinines and other functionally bioactive compounds, which has a wide range of applications in various fields such as agro-chemistry, immunology, oncology, agriculture etc. (Bacon & White, 2000). Hence, endophytic fungi prove to be a promising group of micro-organisms for the exploration of new compounds.

3.7.1 Antimicrobial compounds

Antimicrobial compounds are low molecular weight organic compounds and are active at low concentrations against pathogenic micro-organisms (Strobel, 2003). Natural metabolites from endophytic fungi have been proved to show antimicrobial properties such as antifungal, antibacterial and antiviral, against many pathogens.
Bicas (2009) showed that antimicrobial compound known as “Guanacastepene” was produced by an endophytic fungi, isolated from Daphnopsis, which shows an antibacterial activity against methicillin-resistant *Staphylococcus aureus* and vancomycin- resistant *Enterococcus faecium*.

Another metabolite-ambuic acid is isolated from endophytic fungi *Pestalotiopsis microspora*, which shows an antifungal activity (Li, 2001).

Horn (1995) reported that an endophytic *Phomopsis* sp. produces a metabolite called Phomopsichalasin, which shows antibacterial activity, mainly against organisms such as *Staphylococcus aureus*, *Bacillus subtilis*, and *Salmonella enterica*, also shows moderate activity against *Candida tropicalis* - a fungi.

Bioactive metabolite “Phomopsilactone” and “Ethyl 2,4- dihydroxy-5,6-dimethylbenzoate” produced by *Phomopsis cassiae* shows antifungal activity mainly against *Cladosporium cladosporioides* and *C. sphaerospermum*, a phytopathogenic fungi (Gunatilaka, 2006).

Strobel (2004) reported a metabolic compound “Pestalothel C” from Endophytic fungi *Pestalotiopsis theae* which retains anti-HIV properties.

![Chemical structures of Guanacastepene, Ambuic acid and Pestalotheol](source-google.com)

### 3.7.2 Anticancer Compounds

GuribFakim (2006) reported that 60% of drug approved for the treatment of cancer are of natural origin. With the discovery of Taxol, a billion dollar anti-cancer drug,
microbes has upsurge for drug discovery in recent years. Taxol and few of its derivatives represent the first major group of anticancer agents produced by endophytes. Taxol is a highly functionalized diterpenoid found in few (Taxus) species (Suffness, 1995).

Taxol producing endophytic fungi *Taxus andreaceae* was discovered in yew *T. Brevifolia* (Strobel, 1993). Taxol is used to treat mainly breast and ovarian cancers, but now it has been used for the treatment of other tissue-proliferating diseases as well. This compound stabilizes tubulin molecules and disturbs their dynamic equilibrium during the process of cell-multiplication (Liu, 2009).

Another fungus *Fusarium solani* isolated from *Camptotheca acuminata*, was found to produce important anti-cancer compound “Camptothecin”(C_{20}H_{16}N_{2}O_{4}) which is a alkaloid and a potent antineoplastic agent (Wani, 1971).


![Chemical structures of Paclitaxel and Camptothecin](source google.com)

**Fig 3.8 Chemical structures of Paclitaxel and Camptothecin**

**Source- google.com**

### 3.7.3 Anti-oxidant Compound

Antioxidants are the compounds which act against the Reactive Oxygen Species (ROS) and free radicals. Almost all the plant species in nature shows anti-oxidant activity contributed by the polysaccharides from the plants.

The micro-organisms are also proved to be a potent source of anti-oxidant compounds, useful in the treatment of ROS-linked diseases such as diabetes mellitus, cancer, arthritis and neurodegenerative diseases (Castilo, 2002). Many studies revealed that endophytic fungi are able to produce bioactive compounds which show a significant anti-oxidant activity.
A compound “Graphislactone A” isolated from endophytic fungi *Cephalosporium* sp. shows an effective anti-oxidant activity by scavenging free radicals (Liu, 2007). Guan (2005) reported that fungus “*Phomopsis* sp. PSU-D15” produces a compound “Phomoenamide” which shows a potent anti-oxidant activity. “Isopestacin” is an anti-oxidant compound scavenges superoxide and hydroxyl free radicals, isolated from endophytic fungi ’*Pestalotopsis microspora*’ (Seifried, 2007).

![Fig 3.9 Chemical structures of Phomoenamid (Source-pubchem.com)](image)

### 3.7.4 Insecticidal Compounds

The interest in the field of bio-insecticides is increasing day by day. Few studies reflect some endophytic fungi retaining promising anti-insect activity. A novel indole diterpene, ‘Nodulisporic acid’ shows insecticidal activity against the larvae of blowfly, isolated from endophytic *Nodulisporium* sp. from plant *Bontia daphnoide* (Demain, 2000). Insect repellent ‘Naphthalene’ was obtained from the fungus, *Muscodorviti genus* (Daisy, 2002). Thus, the bioactive compounds from these endophytes could be a promising and safe alternative to synthetic insecticides, in future.

![Nodulisporic acid (Source-google.com)](image)
3.8 Antibiotic resistance

Antibiotic resistance in bacteria has been witnessed since 1950s for most of the major classes of antibiotics used to treat human diseases. Antibiotic resistances is the ability of micro-organisms such as bacteria, fungi, viruses and parasites to live and grow in the presence of a chemical (drug) that would normally kill them or limit the growth of microbes. Resistant microorganisms are not killed by antimicrobial drugs (e.g. antibiotics), therefore the treatment becomes ineffective and infections persist.

Fig.3.10 Chemical structures of Nodulisporic acid and Napthalene
Source-google.com

Fig.3.11 Drug resistance caused by micro-organisms
Examples of common multi-drug resistant bacteria:

- Methicillin-resistant *Staphylococcus aureus* (MRSA)
- Vancomycin-resistant enterococci (VRE)
  - Extended-spectrum beta-lactamase (ESBL)-producing Enterobacteriaceae (common *Enterobacteriaceae* are *Escherichia coli* and *Klebsiella pneumoniae*)
- Carbapenemase-producing *Enterobacteriaceae* (e.g. *Klebsiella pneumonia*)
- Multidrug-resistant *Pseudomonas aeruginosa*
- *Clostridium difficile*

### 3.8.1 Mode of antibiotic action

To understand the mechanism of antibiotic resistance, understanding of antibiotics mode of action is required. There are five major modes of antibiotic mechanisms of activity:

- Interference with cell wall synthesis
- Interference with nucleic acid synthesis
- Inhibition of protein synthesis
- Inhibition of a metabolic pathway
- Disorganizing of the cell membrane

### 3.8.2 Mechanism of drug resistance

Drug resistance has become a serious issue over the past several years. Drug resistance among bacterial species is acquired by few mechanisms. The fundamental mechanisms of antimicrobial resistance are:

1. **Inactivation of antibiotics**- Direct inactivation of the active antibiotic molecule (Wright, 2005).
2. **Target modification**- Modification of the antibiotic target site so that the antibiotic is unable to bind properly (Lambart, 2005).
3. **Efflux pumps and outer membrane (OM) permeability changes**- Changes in membrane permeability to antibiotics (Kumar, 2005).
4. **Target bypass** – Few bacterial species become refractory to specific antibiotics by bypassing the inactivation of a given enzyme (Mobashery *et al*., 1999; Happi *et al*., 2005).
3.9 Nanotechnology

Nanotechnology is a promising multidisciplinary science involving various aspects of technology and research (Uskokovic, 2008). The term “nanotechnology” was first time introduced in 1974 by Norio Taniguchi (Taniguchi, 1974). According to
Mansoori (2005) nanotechnology offers importance in production, characterization and application of nanomaterial structures, devices or systems with controlled shape and size at nano scale. Nanoparticles are ultimate building blocks of nanotechnology (Mansoori, 2007), comprising various organic and inorganic metal nanoparticles in the size of 1-100nm. Metal nanoparticles of gold, silver, platinum, copper, zinc have been extensively studied.

Feynman (1991) reported that, the nanoparticles have a unique physico-chemical, biological and optical properties, possible to manipulate for desired applications. As biological processes occur even at nano scale and due to the amenability of nanoparticles to biological functionalization, the nanoparticles are having significant applications in the medical field (Parak et al., 2003).

Global outbreaks of infectious diseases due to various pathogens and the development of drug resistance among the bacterial pathogens, led into the emergence of nanoparticles as novel antimicrobial agents (Morens et al., 2005; Kim et al., 2007). Bacteria, fungi and plants extracts are the primary biological systems involved in nanoparticles production.

![Biological systems for nanoparticle synthesis](image-url)
<table>
<thead>
<tr>
<th>Source</th>
<th>Types and size range of NPs (nm)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacteria</strong></td>
<td></td>
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</tr>
<tr>
<td><em>Bacillus thuringiensis</em></td>
<td>Ag 10-20</td>
<td>Jain <em>et al.</em> (2010)</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>Ag 30-50</td>
<td>Gurunathan <em>et al.</em> (2009)</td>
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<tr>
<td><em>Lactobacillus strains</em></td>
<td>Ag, Au 15-40</td>
<td>Sintubin <em>et al.</em> (2009)</td>
</tr>
<tr>
<td><em>Pseudomonas stutzeri</em></td>
<td>Ag&gt;200</td>
<td>Klaus <em>et al.</em> (1999)</td>
</tr>
<tr>
<td><em>Corynebacterium</em></td>
<td>Ag 5-15</td>
<td>Zhang <em>et al.</em> (2005)</td>
</tr>
<tr>
<td><em>Bacillus cereus</em></td>
<td>Ag 5</td>
<td>Ganesh Babu and Gunasekaran (2009)</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>Ag 150-180</td>
<td>Nanda and Saravanan (2009)</td>
</tr>
<tr>
<td><em>Ureibacillus thermosphaericus</em></td>
<td>Ag 1-100</td>
<td>Juibari <em>et al.</em> (2011)</td>
</tr>
<tr>
<td><strong>Fungi</strong></td>
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<td></td>
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<td>Ag 1-5</td>
<td>Duran <em>et al.</em> (2007)</td>
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<td><em>Aspergillus oryzae</em></td>
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<td>Binupriya <em>et al.</em> (2010)</td>
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<td>Thakkar <em>et al.</em> (2010)</td>
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<td><em>Klebsiella pneumonia</em></td>
<td>Se 100-400</td>
<td>Fesharakhi <em>et al.</em> (2010)</td>
</tr>
<tr>
<td><strong>Plant</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Jatropha curcas</em></td>
<td>Ag &gt;20</td>
<td>Pala <em>et al.</em> (2010)</td>
</tr>
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<td><em>Aloe vera</em></td>
<td>Au 50-350</td>
<td>Chandran <em>et al.</em> (2006)</td>
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<td>Ag 50</td>
<td>Huang <em>et al.</em> (2007)</td>
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<td>Shankar <em>et al.</em> (2004)</td>
</tr>
<tr>
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<td>Shankar <em>et al.</em> (2004)</td>
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<td><strong>Yeast</strong></td>
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<td>Dameron <em>et al.</em> (1989)</td>
</tr>
<tr>
<td><em>Schizosaccharomyces pombe</em></td>
<td>CdS 50-150</td>
<td>Dameron <em>et al.</em> (1989)</td>
</tr>
</tbody>
</table>

Table 3.4 Altered sources of nanoparticles synthesis (Ingale *et al.*, 2013)
3.9.1 Myco nanotechnology

Myco nanotechnology is the hybrid study which includes the study of “mycology and nanotechnology”. Due to its vast diversity of fungi species it has gained potential importance. Rai et al. (2009) and Gupta et al. (2012) revealed that nanoparticles of good dimensions and mono dispersity could be synthesized using fungi.

In nature, fungi are of vital importance as they are the larger secretors of proteins which are capable of hydrolysing the metal ions (Mandal et al., 2006). They can accumulate metal ions by various physico-chemical as well as biological mechanisms including polymers binding and extracellular binding by metabolites (Mukharjee et al., 2001). Hence, fungi are the ideal candidate in the mass production of nanoparticles. Another benefit of using fungi is their ease of cultivation and isolation (Mandal et al., 2006).

The morphology and particle size of nanoparticles depends on various physical as well as chemical parameters such as pH, composition of the culture medium, incubation time and growth in the light or dark (Joerger et al. 2000).

Mukharjee et al. (2001) studied synthesis of gold nanoparticles with well-defined dimensions using Verticillium sp. These results showed that the trapping of AuCl4 – ions on fungal cell surface occurs by electrostatic interaction with positively charged groups (such as lysine residues) in enzymes, present mycelial cell wall.

Nitrate dependent reductase and shuttle quinines from F. oxysporum are found to perform the synthesis of silver nanoparticles (Dura’n et al., 2005). Castro-Longoria et al (2012) were able to synthesize platinum nanoparticles of size 4-35 nm intracellularly by using Neurospora crassa. Bhainsa et al. (2006) demonstrated a rapid synthesis of monodispersed silver nanoparticles by using filamentous fungus Aspergillus fumigatus within 10 minutes. Trichoderma asperellum and Trichoderma reesei are able to produce silver nanoparticles while exposed to silver salts (Mukharjee et al., 2008; Nevalainen et al., 1994).

Hence the fungi have received a great attention as a novel method for the production of nanoparticles and have emerged as “Bio-nano-factories” in recent years.

3.9.2 Mechanism of nanoparticle synthesis in fungi

Two approaches for the synthesis of nanoparticles
1. Top down approach
This approach involves formation of nano materials from bulk substrate. The process includes cutting, etching, grinding of bulk materials by various mechanical, chemical or electrochemical methods (Singh et al., 2011).

2. Bottom up approach
This is opposite to top down approach. In this method, nano structures are formed by self-assembly into tubes or crystal followed by synthesis of nanoparticles (Moghaddam, 2010).

![Fig.3.14 Top down approach and Bottom Up approach of nanoparticle synthesis](image-url)
Fungi have an ability to synthesize intracellular as well as extracellular nanoparticles. Probable mechanism involved in intracellular synthesis of nanoparticles involves binding of heavy metal to fungal cell wall by enzymes present on it. Furthermore, the reduction of metal ions occurs with the help of cell wall enzymes. This results in aggregation and formation of metal nanoparticles (Kashyap et al., 2013).

Srivastava et al. (2013) confirmed the role of intracellular nitrate reductase, where silver nanoparticles were synthesized from *Halococcus salifodiane*. Extracellular synthesis of nanoparticles mainly depends on interaction of metal ions and enzyme released, mainly reductase. This leads to the formation of nanoparticles in solution (Kshyap et al., 2013).

Kumar et al. (2007) demonstrated the role of enzyme alpha NADPH dependent nitrate reductase in silver nanoparticles synthesis (Kumar et al., 2007). Nitrate reductase causes the reduction of Ag+ ions leading to the formation of silver nanoparticles. The nanoparticles formed were characterized by UV-visible spectroscopy, XRD and TEM analysis.

![Fig.3.15 Mechanism of nanoparticle synthesis](image-url)
Manivasagan et al., (2013) reported the role of nitrate reductase in the synthesis silver nanoparticles. They found extracellular secretion of nitrate reductase when silver ions were reduced in culture supernatant of *Nocardiopsis* sp. The production of nitrate reductase was confirmed by FTIR analysis.

Hamedi et al. (2014) reported the presence of nitrate reductase enzyme in cell free extract of *Neuraspora intermedia*, during nanoparticle synthesis. Recently, silver nanoparticles have been synthesized in presence of extracellular purified enzyme nitrate reductase. The enzyme was isolated from *Fusarium oxysporum* using a selective medium and purified by ultrafiltration and ion exchange chromatography. The synthesis of silver nanoparticles mediated by nitrate reductase was NADPH dependent, where gelatin was used as a capping agent (Gholami et al., 2014).

### 3.9.3 Applications of nanoparticles

![Applications of Nanotechnology](image)

Fig 3.16 Applications of nanoparticles
Nanotechnology has received a great attention of biologist because of their extensive applications in different fields. Few applications of nanoparticles produced by microorganisms are:

**Regulation of biological processes**

By using suitable nanoparticles, different biological processes can be regulated where nanoparticles can be used to enhance the gene expression in the cell in which hybrid polymer protein conjugate nanoparticles are useful which increases the delivery of the DNA to the cell nuclei. Silver nanoparticles are useful in the treatment of cancer, where a complex of antibody and a nanoparticle is used which targets cancer cell to create free radical which would then kill the affected cells (Dos et al., 2014).

**Antimicrobial agents**

Gold and silver nanoparticles acts as an antimicrobial agent, as those retain antibacterial effect against many pathogenic microorganisms. These nanoparticles have the ability to produce active oxygen species causing damage to the bacteria and also strongly binds to DNA or RNA interfering with the microbial replication (Dos et al., 2014). Copper nanoparticles are used as an antimicrobial agent, as they exhibit activity against many disease causing micro-organisms (zain et al., 2014).

**Intercalating agents**

Silver nanoparticles have a large number of applications such as intercalation materials in electrical batteries. It is also used as an optical receptors, which is useful in many chemical reactions where it acts as a catalyst and also in many healthcare devices as it poses an antibacterial properties (Dura’n et al., 2005).

**Catalytic agent**

Platinum nanoparticles are also used as a catalyst in many chemical reactions because of its superior catalytic activity. It is also used in the fuel cell technology, where it is used as a cathode as well as anode, as it shows an effective redox reaction (Chen et al., 2009).
Other applications

Magnetite (Fe2O4) and siliceous material produced by different bacteria and diatoms are used in optical coating for solar energy applications (Joerger et al., 1999).

Nanoparticles are also useful in the bioremediation of radioactive wastes generated by nuclear power plants. Uranium contaminated waste waters are clean-up by certain strains of bacteria (Dura’n et al., 2007).