India has been known to be rich repository of medicinal plants that can provide biologically active molecules with enhanced activity and can provide lead compounds for various drug targets. Our indigenous tribes and ancient civilizations used medicinal plants to treat ailments that they used even fungi grown on roasted green corn to treat intestinal ailments. Medicinal plants are known to harbour endophytic fungi that are believed to be associated with the production of pharmaceutical products (Zhang et al., 2006). Being rich in biodiversity they still remain unexploited and hence reviews stress upon the importance to explore endophytic mycoflora in the medicinal plants.

The chosen medicinal plants *Asparagus racemosus* and *Hemidesmus indicus* are well known medicinal plants growing among the tropics being used in traditional systems of medicine such as ayurveda and siddha. The roots of shatawari (*A. racemosus*) have been used for the treatment of diarrhea, rheumatism, diabetes and brain complaints etc. (Chadha 2003) while the roots and leaves of nannari (*H. indicus*) is used for treating chronic rheumatism, veneral diseases and urinary disorders.

Tropical areas have high medicinal value due to their large plant diversity and therefore possess high endophyte diversity. The endophytic counterparts in our chosen medicinal plants growing well among tropics had the most time to evolve and create complex and specific relationships with plants in creating more active secondary metabolites of biological importance. Most mycologists suggest that fungal diversity peaks in tropical areas where woody angiosperm diversity is also at its highest (Arnold et al., 2000). Studies show that endophytes are not host specific. They envisage different parts of the same host and can also be isolated from different plants belonging to the different families and classes and grow under different ecological and geographical conditions (Cohen, 2006).

This forms the rationale of the study regarding fungal endophytes occurring in different plant hosts occurring wide in tropics. The chosen plants *A. racemosus* and *H. indicus* were belong to different families and clade that the endophytes isolated from these plants constitute a diverse range of Hyphomycete and Coelomycete members with recurrence in different seasons along with sterile morphotypes. Sterile forms have often been isolated as endophytes from many
plants (Rajagopal and Suryanarayan, 2000). Lacap et al., (2003) also reported that sterile mycelium prevails in most of the endophytic research studies. In the present study nearly 12 sterile mycelia occurred with Morphotype sp.10 being dominant recurring in most seasons in both A. racemosus and H. indicus possessing 40.6% and 37.6% of sterile isolates, respectively. Studies carried out by Amrita et al., (2012) in medicinal plants reveals that overall relative percentage occurrence of sterile forms (48%) were maximum when compared to Hyphomycetes (25%), Coelomycetes (14%) and Xylariales (13%).

Host endophyte relationship is variable from host to host and the endophyte (Jalgaonwala et al., 2011). Similarly the endophyte assemblages occurring in A. racemosus were differing from H. indicus that the occurrence of certain Hyphomycetes like Syncephalastrum sp., Nigrospora sp., Curvularia sp., Chaetomium sp., and Gloeosporium sp., were isolated from H. indicus but not from A. racemosus. Although the endophytic diversity was higher in H. indicus than A. racemosus their colonization and isolation rates from the foliar parts were comparatively lesser than the latter. However the overall colonization and isolation rates as well as the relative colonization densities were higher in wet periods of Sep-Nov for both medicinal plants. The overall colonization and isolation rates of endophytic fungi were similarly higher in 29 Chinese medicinal plants growing in tropics constituting 31 taxonomic groups (Huang et al., 2008). Further their studies yielded Colletotrichum sp. and Phomopsis sp. to be the dominant genera following mycelia sterilia. The endophytic diversity of A. racemosus also possessed Colletotrichum sp., and Phomopsis sp., as dominant genera followed by Acremonium strictum. Yet in H. Indicus, A. strictum was the dominant genera occurring likewise to the Egyptian medicinal plants studied by Selim et al., (2010).

Endophytic fungi have been reported from plants under various environment conditions including tropic (Mohali et al., 2005), temperate (Ganley et al., 2004), xerophytic (Suryanarayanan et al., 2005) and aquatic (Krizic et al., 2006). Moricca and Ragazzi (2008) showed that the type of interaction between an endophyte and a plant is controlled by the genes of both organisms and modulated by the environment. Therefore a concurrent study performed on the seasonal recurrence of these endophytes suggests how the environmental conditions impact upon the isolation and colonization of endophytes during the wet and dry periods of a year. Studies likewise on foliar endophytes of Plumeria rubra carried out by Suryanarayan and Thennarasan (2004) helped them to assess whether there are temporal changes in endophyte
communities. In both *A. racemosus* and *H. indicus* the endophyte diversity, their isolation and colonization rates peaked during the wet periods of Sep-Nov rather than other periods of a year. This may be due to precipitation, one of the major factors that influence infection by foliar endophytes. Several reviews suggest that a strong correlation exist between endophyte infection levels and cumulative precipitation (Wilson, 2000). Studies carried out by Rodrigues (1994), Suryanarayan *et al.* (1998), Kathiravan *et al.* (2010), Shobana *et al.* (2011) found the same apparent trend where leaves sampled during wet season harbors more endophytes than in dry seasons where leaves become fully matured with very little precipitation. Previously Krishnamurthy *et al.* (2008) reported higher colonization frequency of endophytes occurring in *H. indicus* during wet seasons rather than dry seasons.

Climate change may alter the degree of mutualism between plant and fungi that even changes the efficacy of transmission of the endophyte from mother to daughter plant. Additionally the effect of climatic conditions like relative humidity, temperature, rainfall, moisture etc. influence upon the stomatal conductance and mesophyll conductance of leaves controlling transpiration rates and availability of CO$_2$ which in turn impact on the colonization of foliar fungal endophytes (Brosi *et al*., 2010). Also during wet seasons, higher rainfall promotes the dispersion of fungal spores and the moderate temperature helps in greater viability of these fungal propagules for successive colonization in plant tissues. These fungal spores are detached from the host by raindrops and dispersed in splash droplets. The mucilage surrounding splash borne spores protects them from desiccation and loss of viability under unfavourable conditions (Fitt *et al*., 1989).

Isolation of endophytes also depends on the protocols employed that different surface sterilization methods are effective in isolating diverse and more endophytes. However these methods have to be optimized depending on the leaf tissue characteristics as few can destroy epiphytes while certain sterilizing agents can destroy even the endophytes depending on their concentrations used and the time of exposure (Romero *et al*., 2001). The study has shown an optimal procedure of using 0.2% HgCl$_2$ for 30 s, and 75% EtOH for 1 min for effective isolation of foliar endophytes from *A. racemosus* and *H. indicus*.

Spectrophotometric assay methods based on turbidimetric measurements have been used in a small number of mycological growth studies where fungal biomass is correlated with optical density for monitoring fungal growth over time (Schnurer 1993; Rodrigues *et al*., 2009). Initially
there is a delay before the optical density increased due to fungal growth referred to as the lag phase which was shown to be due to germination of the spores and initial hyphal formation (Meletiadis et al., 2001). The lag phases are species dependent with small inter strain variation and highly reproducible. Considering the growth of A. strictum it reached maximum on 18th day with an O.D of 3.26 during log phase. The growth of filamentous fungi is usually characterized by smoother curves and long transition periods that are being evident for A. strictum too. However the growth of filamentous fungi is also dependent on the medium and environmental conditions employed. An optimal nutrient medium should provide not simply adequate growth but the best possible growth in order to allow molds to grow without restriction and express all phenotypes. Different types of media, pH and temperature conditions were studied that revealed changes in turbidometric measurements and the growth curves. Use of basic nutrients amended with minimal essential elements like salt chelators etc. in PDB-YE media might have resulted to obtain better growth curve with maximum O.D of 3.35 yielding higher biomass for A. strictum. The media components and the culture conditions affects the germination and elongation rates of spores and hence the growth rates. Similar studies conducted reveals Yeast Nitrogen Base medium and Sabouraud broth were observed to be highly nutritious media providing the highest growth for R. microsporus and A. fumigatus with growth rates three to four times higher than those achieved in RPMI media (Meletiadis et al., 2001). Comparison of media types and culture conditions and checking for corresponding growth rates also aid us to optimize the media for standardization for yielding higher biomass concentrations.

The fungal exo-metabolome (Thrane et al., 2007) consisting of secondary metabolites and certain parts of the endo-metabolome consisting of primary metabolites are produced as a reaction to the biotic and abiotic environment. These secondary metabolites are extracted by organic solvents and separated whose profiling has been used quite extensively for taxonomic purposes (Frisvad et al., 2007). The secondary metabolites extracted from mycelial mat and culture filtrate of A. strictum using different organic solvents yielded varied quality of chemical compounds like alkaloids, flavonoids etc. explains their qualitative presence as well as the polarity of the organic solvent used to obtain metabolites of hydrophilic and hydrophobic nature (Belofsky et al., 1998; Holler, 1999 and Lin et al., 2000). Similarly secondary metabolites have been extracted from various endophytic fungi like Alternaria sp., Chaetomium sp., Curvularia sp., etc. using various solvents like methanol, hexane, dichloromethane, ethyl acetate and butanol
tested for potency via antimicrobial, antileishmanial, anticancer and antioxidant studies. Extracts from hexane, dichloromethane, methanol were more effective indicating their potency in efficient extraction of biologically active metabolites present in those endophytes. Similarly in our results dichloromethane and acetone were better solvents in extraction of various metabolites. Given the chemical nature of such small organic molecules they can be detected by different spectroscopic tools such as IR, UV, FLD, MS etc. (Nielsen et al., 2004). These organic extracts following UV and IR spectral analysis suggest the presence of various compounds evident through their absorption spectra and chemical bonds. IR spectra infers the physical properties of the active compounds that the absorption peaks indicate C-H stretching for alkanes, C=O stretching for ketones, asymmetric nitro groups, etc. that in particular the existence of absorption peaks 1683, 1684, 1688 indicates C=C stretching vibration reveals the presence of β Unsaturated amines bonds which can be related to the presence of unsaturated fatty acids in the organic extract.

Filamentous fungi can also be characterized by quantitative profiles of fatty acids (Blomquist et al., 1998), their pattern of utilization of C- and N-sources their temperature, water activity, pH, atmosphere, redox relationships (Frisvad et al., 1998; Andersen and Frisvad, 2002) etc. Fatty acid production from the fungal mycelial mat is almost directly proportional to the biomass production. Etten and Gottlieb (1965) in *Penicillium atrovenetum* analyzed the changes in the lipid constituents during the growth and development that the total fatty acids increased to a maximum during the log phase and the major fatty acids were Palmitic, Stearic, Oleic and Linoleic. Younger mycelium contained a much lower percentage (on the basis of total fatty acids) of linoleic acid compared to the ungerminated spores. According to the results inferred in present study, optimization of the media conditions in standardizing for higher biomass yields have proven the fact that higher the biomass, higher was the percentage of free fatty acids and hence the acid value. Higher yield of nearly 0.87, 0.85 and 0.65 gm of biomass per 100 ml of culture media were obtained with the cultures grown in PDB-YE under conditions of pH 6 and room temperature, with 12 h cycle of light and dark conditions yielding 36.3, 27.6 and 15.2 percentage of free fatty acids with an acid number of 72.23, 54.9 and 30.24 compared to other cultures. Lowering growth temperature from 48 °C to 25 °C increased the synthesis of unsaturated fatty acids in the spores and the mycelium of the thermotolerant and thermophilic fungi of order mucorales as studied by Sumner and Morgan (1969). Optimization of the conditions favoring
production of the biologically active compounds (i.e. medium, incubation period, rpm, temperature and pH value) was studied in *Penicillium brevicompactum* and found that the medium containing Malt Extract with peptone was suitable substrates for metabolite production. Optimal conditions of 30°C, 200 rpm for 7 days yielded maximum dry biomass and GLA, respectively in oleaginous endophytic fungi of order mucorales (Ahmaed *et al.*, 2006). Similarly highest dry weights (11–12 g/l) and lipid contents (~24%, w/w) were observed when glucose or fructose was used as carbon source whereas the highest amount of γ-linolenic acid (~26%) was determined in starch-grown cells of *Mortierella* sp. (Hansson and Dostalek, 1988). These volatile fatty acid metabolites can be separated and detected by Gas Chromatography (GC) that fatty acid profiling finds various applications like estimation of fungal biomass via signature fatty acids, multivariate discriminant analysis among the species at intra specific level etc. The type of fatty acid present and its relative concentration are useful characteristics for separating taxa of varied filamentous fungi, including Oomycetes, Zygomyces, Basidiomycetes and even sterile mycelia that have emerged as recent developments in fungal taxonomy (Guarro *et al.*, 1999).

Fatty acid production can be enhanced by elicitor compounds like metallic salts acting as metal ions interfering with the growth of fungi and hence their metabolites. Minimal increase in metal ion concentration from 1 μg to 500 μg gave a significant increase of biomass yield in ASIS01 strain. Also in ASIS01 the production of fatty acids was increased at 12% that the overall percentage of total monounsaturated fat reveals 80% increase of 87.86 g/100 g and Polyunsaturated fat reveals 12% increase of 12.14 g/100 g, indicating the effect of metal ions on the lipid accumulation and fatty acid synthesis in the improved filamentous fungi. In ASIS01, percentage of Oleic acid was higher of about 22.99 g/100 g of fat sample indicating an 11 fold increase when compared to control strain as a result of metallic salt elicitors. Similarly in *A. terreus* and *Alternaria altrenata* two unsaturated fatty acids Linoleic acid (18:2) and Oleic acid (18:1) were markedly increased at high copper concentration (Abboud and Alawlaqi, 2011).

Similar studies carried out by Muhid *et al.* (2008) reveals that Mg²⁺, Fe²⁺ ions and Zn²⁺ affected GLA production in *Cunninghamella* sp. with Zn²⁺ resulting in increase of upto 74% of GLA yield. Further, Mg²⁺, Fe²⁺ ions and Zn²⁺ had profound effect on lipid accumulation resulting in 64%, 43% and 33% increase in lipid content followed by Cu²⁺ and Mn²⁺ in *Cunninghamella* sp. These metal ions affect the enzymes involved in lipogenesis by playing a role as cofactors. For example Mg²⁺ is involved in regulation of Malic enzyme and ATP citrate lyase which provides
NADPH for fatty acid synthase activity and Acetyl CoA precursor for lipogenesis. A similar report in *Penicillium spiculisporum* reveals the dependence of metal ions as cofactors for increased malic enzyme activity in turn for lipogenesis. (Totani *et al*., 2000 and Yang *et al*., 2000). The influence of Ca$^{2+}$, Mg$^{2+}$, Mn$^{2+}$, and Fe$^{2+}$ ions on lipid accumulation, fatty acid composition and Arachidonic acid (ARA) production was studied by Sajbidor *et al*. in *Mortierella* sp. S-17. Their results yielded beneficial effect of Mn$^{2+}$ in the concentration range of 2–500 mg/l on lipogenesis.

The increase in Oleic acid production due to salt elicitors implies the role of metal ions to act as cofactors for the enzymes involved in fatty acid synthesis. The production of oleic acid from palmitic or stearic acid is catalyzed by the enzyme complex δ-9-desaturases. This non-heme iron-containing Endoplasmic Reticulum (ER) membrane-bound enzyme is a part of a three-component enzyme system involving cytochrome b5, cytochrome b5 reductase, and the δ-9 fatty acid desaturase. This complex catalyzes the NADH and oxygen-dependent insertion of a cis-double bond between carbons 9 and 10 of the saturated fatty acyl substrates, palmitoyl (16:0)-CoA or Stearoyl (18:0)-CoA yielding the monoenoic products palmitoleic (16:1) or Oleic (18:1) acids respectively. They constitute three histidine residues that are reported to be catalytically essential and proposed to be the ligands for the iron atoms ([http://bioinf.umbc.edu/dmdm/generatelogo.php?accession=cd03505](http://bioinf.umbc.edu/dmdm/generatelogo.php?accession=cd03505)). Shanklin *et al*., (1994) also showed that the δ-9-desaturases from rat and yeast and the δ-6, δ-12 and δ-15-desaturases from higher plants and cyanobacteria have three corresponding primary sequences containing histidine residues. Further Stukey *et al*. (1990) proposed a model for yeast δ-9-desaturases in which each protein has two long hydrophobic domains each capable of spanning the membrane twice such that the three hydrophilic domains (containing the conserved histidine residues) reside on the cytoplasmic face of the ER membrane. For these reasons the δ-9-desaturase with three infrequent histidine-containing motifs is assumed to be associated with the ER and to catalyze desaturation at the cytoplasmic side. Hence the metal ions in particular Fe$^{2+}$ can bind with the ligand binding domains of these histidine residues in δ-9-desaturase and might influence the production of Oleic acid in ASIS01 which however needs an extensive study. However, higher the metallic salt concentrations it inhibited the growth as well as decreased the production of free fatty acids in *A. strictum*. The cell membrane structure is made up fatty acids that might be
affected with stress of elevated concentration of metals. This may result in decline in membrane integrity generally manifested by leakage of mobile cellular solutes and cell death.

Various methods have been developed to measure total antioxidant capacity such as the ORAC (Cao et al., 1993) or the 2,2- diphenyl-1-picrylhydrazyl (DPPH) assay, etc. The Radical scavenging activity of Oleic acid fraction was determined using the α,α-diphenyl-β-picrylhydrazyl (DPPH) assay. Free radical scavenging capacity of the Oleic acid was noted to be increased in a concentration dependent manner that at a concentration of 200 µg/ml. The scavenging activity of Oleic acid reached 51.2%, which was comparable to that of reference standard BHT used. Certain studies conducted by Cho et al, (2010) showed a functional oil containing Monoacylalcohol-Oleic Acid had the strongest radical scavenging and antioxidant activities against copper mediated low-density lipoprotein (LDL) oxidation and the strongest inhibitory activity against LDL-associated phospholipase A(2) and exhibited potent activation of paraoxonase activity which contributes to the maintenance of antioxidant activity. Fatty acid micelles scavenged superoxides in an unsaturation-dependent manner that is evident through studies of Richard et al. (2008) where supplementation of Human Aortic Endothelial Cells with unsaturated fatty acids resulted in lower formation of ROS.

A number of mono and polyunsaturated fatty acids have shown to inhibit the growth of malignant cells in vitro. Oleic acid is a monounsaturated fatty acid with a symmetrically placed double bond. Its IUPAC name is cis-9-octadecenoic acid, its lipid shorthand name is 18:1 cis-9 and the CAS registry number is 2027-47-6. The effect of Oleic acid on the viability of human breast cancer (MCF-7) and liver cancer (Hep–G2) cell lines evaluated through MTT assay induced a dose dependent inhibitory effect against the cell lines tested. The IC50 values of Oleic acid were found to be 1.25 mg/ml against MCF-7 while, 62.5 µg/ml against Hep–G2 cell lines, respectively. Previously the discovery of Oleic acid as the major component of olive oil in a healthy Mediterranean diet to protect against breast cancer was examined by David Tin Win (2005). Additionally Oleic acid and its two branched-chain derivatives were tested in vitro for their anticancer activities against two cancer cell lines MCF-7 (human breast) and HT-29 (human colon) exhibited highly significant antitumor activity (Dailey et al., 2011). The ability of certain fatty acids like oleic, linoleic, alpha-linolenic, gamma-linolenic, arachidonic, docosahexaenoic and eicosapentaenoic acid to inhibit significantly the growth of three human pancreatic cancer cell lines in vitro revealed EPA to be the most potent (ID_{50} 2.5-5 gm) comparatively.
Anticancer assays is often sustained with DNA fragmentation studies to check for cell apoptosis. The induction of toxicity can be investigated by changes in cell size, granularity, membrane integrity and DNA fragmentation using flow cytometry and electrophoretic studies. Present studies have proven to induce fragmentation that IC50 concentration of Oleic acid - 1.25 mg/ml and 62.5 µg/ml was optimum enough in MCF-7 and Hep-G2 cell lines to cause DNA smearing (slight DNA degradation) and the DNA ladders consisting of fragments were observed in agarose gel stained with ethidium bromide. Similar to MTT assay conducted for Oleic acid the cytotoxicity of palmitic, stearic, oleic, linoleic, arachidonic, docosahexaenoic and eicosapentaenoic acids on a macrophage cell line (J774) was investigated by De Lima et al. (2006) proven the same results. Several studies have also reported the induction of DNA fragmentation and loss of membrane integrity in different cell types after treatment with FAs (Otton, R. and Curi, 2005). Muralidhar et al. (2004) showed that OA, LA, AA and DHA promote apoptosis of human monocyte-derived macrophages. The mechanisms of fatty acids to induce cell death involved changes in mitochondrial transmembrane potential and intracellular neutral lipid accumulation. It involves Reactive Oxygen Species (ROS) mediated DNA fragmentation that is being enhanced by unsaturated fatty acids leading to necrosis. Mitochondrial dysfunction on decrease of Trans membrane potential, accumulation of ROS, membrane permeability transition and release of apoptotic factors during apoptosis or necrosis has been implicated (Higuchi, 2004). Further studies indicate that OA, the main monounsaturated fatty acid of olive oil, suppresses Her-2/neu overexpression, which in turn interacts synergistically with anti-Her-2/neu immunotherapy by promoting apoptotic cell death of breast cancer cells with Her-2/neu oncogene amplification (Menendez et al., 2005). X-ray diffraction of PE-FA systems demonstrated that Oleic acid (OA) produced important concentration-dependent alterations of the lipid membrane structure. The effects of free fatty acids (FFAs) on membrane structure are relevant for the particular reasons like some biological membranes contain very high levels of FFAs. Intake of diets rich in OA have been shown to exert protective effects against tumoral and hypertensive pathologies, FFA derivatives have been developed as antitumoral and antihypertensive drugs (Funari et al., 2003).

Increase in Oleic acid production in ASIS01 suggests an increased expression of δ-9 fatty acid desaturase gene responsible for Oleic acid production. The pathway for fatty acid desaturation and elongation from palmitic acid and stearic acid to MUFAs (Monounsaturated
fatty acids) and long-chain polyunsaturated fatty acids (LCPUFAs) has been elucidated both by biochemical means and by studying mutant strains that multiple fatty acid δ-9-desaturases encoded by distinct genes have been found so far in Fungi, Rat, Mouse, Carp, Drosophila, Caenorhabditis and Arabidopsis. Initially genomic DNA was extracted from the mother culture and ASIS01 followed by PCR amplification using degenerate primers to isolate δ-9- fatty acid desaturase gene. However it resulted in multiple bands indicative of binding of degenerative primers with the DNA apart from the specified locus. Yet the first set of primer (δ-9, FAS Primer 1) produced the amplified products of expected size of approx. 469 bp and therefore can further be employed in future for PCR optimization and gene sequencing studies. Similar attempts have resulted in isolation of the δ-9-desaturase cDNA and genomic DNA from Mortierella alpine 1S-4 using a degenerate PCR approach based on the amino acid sequence motifs conserved in other microsomal fatty acid desaturases. This genomic gene encoding δ-9-desaturase has only one intron, which consists of 153 bp (Sakuradani et al., 1999). Shanklin et al., showed that the δ-9-desaturases from rat and yeast and the δ-6, δ-12 and δ-15-desaturases from higher plants and cyanobacteria have three corresponding primary sequences containing histidine residues. A third gene (δ-9-3) encoding a fatty acid δ-9-desaturase was isolated from the oil-producing fungus M. alpina. The predicted protein of 512 amino acid shared 53% sequence identity with the two fatty acid δ-9-desaturases, ole1p and ole2p already reported from this organism and contained three histidine boxes, four putative transmembrane domains and a C-terminal cytochrome b5 fusion that are typical of most fungal membrane-bound fatty acid desaturases (MacKenzie et al., 2002).

Altogether the studies henceforth describe the importance of fatty acid enhancement using simple metallic salt elicitors from an endophytic counterpart of medicinal plants. The Oleic acid can be scaled up for production and absolute purification in terms of quality and quantity in turn can find applications in therapeutic, clinical, medical and industrial arenas. Also attempts performed for the isolation of δ-9 fatty acid desaturase gene can be optimized in future by improving primer designing and standardizing PCR conditions that gene expression profiling through qRT-PCR techniques can comparatively highlight the molecular background for elevated levels of Oleic acid in the improved strain ASIS01. The study thus proves the efficiency of monounsaturated fatty acid - Oleic acid as better anticancer and antioxidant agent and its extraction from novel source of endophyte associated with a medicinal plant improved using salt elicitors.