Opportunistic fungal infections are the major cause of morbidity in immuno-compromised individuals such as cancer and HIV patients. The mortality rate of invasive fungal infections is high despite of antifungal chemotherapy with amphotericin B, azoles and caspofungin (Fridkin 2005). The unprecedented increase in such patients has challenged the management of fungal infections. Furthermore, problem of host toxicity associated with antifungal drugs and emergence of drug-resistance in primary and opportunistic fungal pathogens has impeded the success of antifungal therapy. Therefore, new and effective antifungal agents and approaches for treating these infections are warranted. To combat above problems various strategies have been made under investigation which include combinational therapy, development of new antifungal agents with improved efficacy, novel approaches of drug delivery and search of novel compounds which can interfere with fungal virulence and biofilm formation both at classical and molecular levels of drug targets.

Natural products derived from medicinal plants have been well known for their contribution in developing modern drugs as well as herbal formulation in traditional medicine system (Cowan 1999; Braun and Cohen 2010). It is believed and recommended by leading scientists that with improved strategy and intelligent design of test assay novel bioactivity of natural products may be discovered against various ailments. Essential oils from medicinal plants are known for their antifungal activity and uses but these oils and their major active compounds have not yet been screened for novel anti-virulence potential and its synergy with currently used antifungal drugs against drug-resistant strains of fungi. Considering the need of alternative antifungal therapy, a systematic investigation on certain plant essential oils known for their ethnomedicinal uses in Indian traditional system against fungal infections was made to exploit their antifungal and anti-virulence activities against drug-resistant strains of yeasts and filamentous fungi. The findings of this investigation are discussed below.

6.1. Occurrence of virulence factors in Candida spp and filamentous fungi

In the present study, 26 clinical and 11 reference strains used were isolated/collected, identified and confirmed on the basis of standard biochemical, cultural and morphological characteristics as Candida albicans, Candida glabrata, Candida krusei, Candida tropicalis, Cryptococcus neoformans, Aspergillus flavus, Aspergillus fumigatus,
Aspergillus niger, Alternaria solani, Fusarium oxysporum, Mucor rouxii and Trichophyton rubrum. Identification of strain \( C. \) albicans 04 based on 18S rRNA gene sequencing confirmed our biochemical basis of identification. Further, virulence attributes namely germ tube formation, cell surface hydrophobicity, proteinase and haemolysin were studied for their expression among \( Candida \) spp at the phenotypic level both qualitatively and quantitatively. In addition, ability to form biofilm by these strains was also determined. Filamentous fungi were tested for production of extracellular enzymes viz. lipase, phospholipase, gelatinase, elastase and keratinase.

In this study, varying level of phenotypic expression of these virulence factors was observed among test strains of \( Candida \) spp and other filamentous fungi. Four strains of \( C. \) albicans produced all the tested virulence factors i.e. germ tubes, proteinase, haemolysin and CSH whereas two strains of non-albicans \( Candida \) produced proteinase, haemolysin and CSH and others produced either one or two virulence factors. Production of these virulence factors in the strains of \( Candida \) spp isolated from different sites of infection have been reported by several other workers in solid or liquid media (Luo et al. 2001; Kantarcioğlu and Yucel 2002; Kuriyama et al. 2003; Dagdeveren et al. 2005; Kumar et al. 2006; Gokce et al. 2007; Furlaneto-Maia et al. 2008). Among the filamentous fungi, \( A. \) niger IOA-3 and \( T. \) rubrum IOA-9 exhibited production of several virulence factors such as phospholipase, proteinase, gelatinase and keratinase. These findings are in agreement with the reports from other workers who have shown production of these virulence factors by clinical strains of \( Aspergillus \) spp and \( Trichophyton \) spp (Muhsin et al. 1997; Alp and Arikan 2008).

Biofilm forming ability was demonstrated by 77% of \( C. \) albicans strains from different clinical sites as revealed by both visual and spectroscopic methods. This may reflect similarities in the ability of strains of \( C. \) albicans from different sites of infection to form biofilms and aid to the pathogenesis of \( Candida \) spp. Formation of biofilms by albicans and non-albicans \( Candida \) strains isolated from different clinical specimens and their relevance to pathogenicity has been reported by several other workers (Shin et al. 2002; Gokce et al. 2007; Furlaneto-Maia et al. 2008; Lal et al. 2008; Ferreira et al. 2009).

Incidence of one or other virulence factors in 50% or more test strains in our study highlights the relevance of virulence factors in these isolates to be more pathogenic in
nature as reported by various researchers that increased expression of virulence factors is attributed to the increased pathogenicity of drug-resistant strains leading to severity of disease. Several *in vitro* studies have reported incidence of these virulence factors in clinical isolates of *Candida* spp including their drug-resistant variants (Sweet *et al.* 1995; Koga-Ito *et al.* 2006; Antony *et al.* 2007; Ramesh *et al.* 2011; Tay *et al.* 2011).

Expression and quantity of virulence factors produced by microorganisms is attributed to the specificity of isolates, its association with different clinical conditions, and or physiological condition and genetic make up of the strain. For example, blood isolates generally produce much higher levels of virulence factors than do isolates from wounds and urine (Price *et al.* 1982; Dagdeveren *et al.* 2005). Since strains under study were isolated from different clinical conditions such as patients of known vaginitis, candidemia and urinary tract infections, so variation in amount and expression of virulence factors was obvious.

### 6.2. Susceptibility of test fungi to antifungal drugs

The sensitivity behavior of test strains of *Candida* spp and *C. neoformans* has revealed a cross resistance to azoles namely, fluconazole, itraconazole and ketoconazole showing wide range of resistance level (2-512 µg/ml). Our findings are similar with the reports of Cross *et al.* (2000) who have reported cross-resistance between miconazole, clotrimazole, and itraconazole and fluconazole in the clinical isolates of *C. glabrata* and *C. albicans*. The increased cross resistance to azoles is probably due to the frequent use of these drugs in medical practice. Several other workers also have reported resistance to azoles in *Candida* spp. and *C. neoformans* (Krcmery *et al.* 2002; De Logu *et al.* 2005; Swinne *et al.* 2005; Hamza *et al.* 2008; Arechavala *et al.* 2009). Although some of the strains of this study were sensitive to amphotericin B but co-resistance was also clearly displayed. Other workers have also reported the development of resistance in *C. albicans* to amphotericin B (Tseng *et al.* 2005). Multi drug resistance is a serious issue during treatment of opportunistic fungal infections of immunocompromised individuals such as transplant recipients and cancer patients undergoing cytotoxic chemotherapy (Thakur *et al.* 2008). Furthermore, similar to yeasts multi-drug resistant human pathogenic fungi from different genera of *Aspergillus, Trichophyton, Mucor* and *Fusarium* were evident from our study. Increasing resistance to azole-drugs amongst various filamentous fungi
has become a common problem and has been reported by several other workers (Howard et al. 2006; Santos and Hamdan 2007; Snelders et al. 2008; Mortensen et al. 2010; Arendrup et al. 2008).

However, the exact incidence of antifungal resistant strain required more extensive sample size and such data are commonly available with clinical observations. The purpose of this study was to obtain a set of drug-resistant and virulent strains of human pathogenic fungi to be tested for various dimensions of assays. Fluconazole is considered to be one of the safest drugs in treating fungal infections but emergence of resistance against it has diminished its efficacy in clinical setting. On the other hand newly developed echinocandins have shown efficacy against Candida spp but lesser activity to Aspergillus spp and poorly active against C. neoformans, in addition, these drugs are costlier in use (De Logu et al. 2005; Onyewu and Heitman 2007).

6.3. Antifungal activity of essential oils against drug-resistant fungi

Increasing incidence of mycoses in spite of available antifungal drugs triggers interest in the development of safe and effective antifungal drugs or alternative antifungal therapy from various approaches including medicinal and aromatic plants. Plant essential oils have long been used safely and effectively in ethnomedicine in treatment of various ailments including fungal infections (Cavaleiro et al. 2006; Park et al. 2007; Bajpai et al. 2009) and are expected to be useful in combating current fungal infections (Aqil et al. 2010). Therefore, considering the problem of drug-resistance in pathogenic fungi, certain essential oils and some of their major active compounds were screened for their in vitro efficacy against C. albicans and other pathogenic fungi.

The present study has revealed broad spectrum inhibitory activity from oils of C. copticum, C. citratus, C. martini, C. verum, S. aromaticum and T. vulgaris, and major active ingredients like cinnamaldehyde, eugenol, geraniol and citral, against multi-drug resistant human pathogenic fungi from different genera of Candida, Aspergillus, Trichophyton, Mucor and Fusarium both in solid and liquid media. Cinnamaldehyde, citral and oil of C. martini exhibited promising anti-candidal activity against multi-drug resistant strains of C. albicans with the MIC range of 45 to 200 µg/ml.

In general, active compounds namely cinnamaldehyde, citral, eugenol and geraniol, were more active than essential oils in their antifungal activities. GC-MS analysis further
supported that such active constituents are present in the test essential oils which collectively or synergistically interact to impart a more rapid mode of action. In this regard, the above-mentioned essential oils and active compounds may act as potential antifungal agents against drug-resistant fungi. The antifungal activity of these essential oils was reported by several other workers (Park et al. 2007; Bajpai et al. 2009; Tyagi and Malik 2010a). However, this is probably first attempt to assess the inhibitory activity against drug-resistant strains of this group of fungi including *C. albicans*, Non-albicans *Candida*, *Cryptococcus* spp, *Aspergillus* spp, *Trichophyton* spp and other filamentous fungi. The variation in susceptibility to these oils might be due to the variation in the structural, physiological and genetic makeup of the different strains of fungi tested as well as composition and nature of active compounds present in essential oils tested as revealed by GC/GC-MS analysis.

Further, these oils exerted a concentration dependent effect on growth of filamentous fungi in terms of biomass production and mycelial radial growth even at lower concentrations (18 to 40 µg/ml). Cinnamaldehyde was found to be most inhibitory against *T. rubrum* and *A. fumigatus*. Antifungal activities of these oils and compounds are also reported by several other workers (Saikia et al. 2001; Wang et al. 2005; Cheng et al. 2008). In our study, light microscopy revealed the concentration and time dependent toxic effects of oils of *C. verum* and *S. aromaticum* on mycelial and reproductive structures of test fungi. Formation of chlamydospores is considered to be the indicator of stress conditions produced in the presence of oils and in response hyphal shrinkage and autolysis of cytoplasm is achieved. It was also observed that cinnamaldehyde, eugenol, *C. verum* and *S. aromaticum* inhibited significantly both ungerminated and germinated conidia of *A. fumigatus* MTCC2550 and *T. rubrum* IOA-9 at a concentration range from 5 to 80 µg/ml. Cinnamaldehyde exhibited edge over tested oils in activity against both ungerminated and germinated conidia of these fungi at sub-inhibitory concentrations. Our findings are comparable with antifungal drugs such as amphotericin B, itraconazole and voriconazole inhibiting both ungerminated and germinated conidia in clinical isolates of *A. fumigatus* as reported by Manavathu et al. (1999).

Furthermore, to determine that whether the antifungal activities from these oils are cidal or static in nature, time kill assays was performed. The data obtained reflected the
efficacy of test oils higher than fluconazole in killing *C. albicans* strains exhibiting resistance to fluconazole and amphotericin B or susceptible to amphotericin B. The potency of cinnamaldehyde in killing the fungi as revealed by time kill assays against *A. fumigatus* MTCC2550 and *T. rubrum* IOA-9 was found to be higher than fluconazole and the other essential oils or active compounds. These findings have indicated the fungicidal activity of essential oils and active compounds over the fungistatic nature of fluconazole. Fungistatic nature of fluconazole may be attributed to its lower killing potential but these oils even appeared to be more cidal than amphotericin B.

In spite of well known antifungal properties of these essential oils, less information is available on their mechanism of action against the fungi included in this study. Therefore, to investigate mechanism of action of these essential oils or active compounds against yeasts and filamentous human pathogenic fungi, the strains of *C. albicans* 04, *A. fumigatus* MTCC2550 and *T. rubrum* IOA-9 were selected as representative of each group and subjected to microscopic observations and other assays. To ascertain the surface and ultrastructural changes produced by oils against yeasts and filamentous fungi, the cinnamaldehyde and eugenol were selected as test agents as were more inhibitory to fungal growth at lower concentrations in our study. The morphological changes produced were confirmed by SEM of *C. albicans* 04, *A. fumigatus* MTCC2550 treated with sub-MICs of cinnamaldehyde or eugenol. In the SEM micrograph, the differences between treated and untreated *Candida* cells were clearly visualized. The cell envelope of yeast is appeared to be damaged by the oils as evident by shrinkage of cell surfaces, presence of non-polar bud scars, and receding of cytoplasm leading to lysis of cells. Increase in buds and bud scars implies that drug affect normal division process of yeasts resulting in a single cell with multiple attempt to divide but not resulting in increase in viable number of cells. The deposition of vesicles on the cell surface is an indicative of broken cells releasing cytoplasmic materials upon damage of cell wall and therefore, suggests fungicidal nature of oils towards yeast cells. The surface alterations are most probably due to the change in cell permeability showing that the first changes are localized at the plasmamlema and cell wall before any alteration can be detected in the cell interior (De Nollin and Borgers 1974). The indentations of the walls in collapsed cells are indicative of permeability changes that provoke osmotic imbalance. The folds in the cell wall giving
bulging appearance may be due to the deposition of membranous material between plasmalemma and cell wall or in the cell wall itself (De Nollin and Borgers 1975). Almost similar effects were also observed for thymol and eugenol against Saccharomyces cerevisiae as reported by Bennis et al. (2004). Tyagi and Malik (2010a) have shown membrane damaging effect of oil of C. citratus against Candida albicans.

In case of filamentous fungi A. fumigarus MTCC2550, severely collapsed and squashed hyphae due to the lack of cytoplasm were evident. Apical growth at the hyphal tip is correlated to the highest metabolic activity (Heath 1990) and it has been reported that 1,3-β-D-glucan synthase, an fungal cell wall synthesizing enzyme, is located in hyphal tip (Beauvais et al. 2001). Therefore, disruption of these sites in filamentous fungi may suggest inhibitory activity of oils against cell wall-synthesis similar to micafungin (Nishiyama et al. 2005). The disruption of hyphal and lateral branches tips may induce extracellular leakage of cellular material leading to collapsed hyphae or autolytic destruction of whole hyphae as evident from our study. Similar changes in hyphal morphology may be related to the disruption of cell wall integrity and, therefore, it could be postulated that essential oils or active compounds are interfering with cell wall synthesis.

Further, to ascertain whether site of action of essential oils against yeasts and filamentous fungi is at cell wall, cell membrane or other intracellular structures, strains of C. albicans 04, A. fumigatus MTCC2550 and T. rubrum IOA-9 were subjected to TEM at sub-MICs of cinnamaldehyde or eugenol. TEM observations of treated Candida cells showed shrinkage of the protoplast, disruption of the cytoplasmic membrane, decomposition of inner organelles and even undulant cell wall was observed. Our findings find support from the studies of Tyagi and Malik (2010b) who have reported similar effects on cell wall and membranous structure of Candida albicans when exposed to oil of C. citratus. Similarly ultrastructural analysis of treated cells of filamentous fungi (Aspergillus fumigatus and T. rubrum) highlighted multiple sites of action of oils in fungal cell including damages to the cell wall, cell membrane, nucleus, nuclear membrane, cytoplasmic contents, mitochondria and other organelles. These observations indicate that
mechanisms of action of oils are disruption of overall intracellular endomembranous system. An increase in number and expansion of endoplasmic reticula serves to detoxify the drugs or pesticides inside the cell (Park et al. 2007) and indicates a stimulation response of the cell to oils as evidenced from our findings. Further, accumulation of polysaccharide granules is responsible for the rupture of plasmalemma structure (Ghahfarokhi et al. 2004).

Therefore, it appears that cell wall and cell membrane integrity, along with other membranous structures are the target sites for essential oils or active compounds. This effect may be attributed to the lipophilic properties of essential oils or active compounds and indicates their ability to penetrate the plasma membrane (Knobloch et al. 1989). Also, alterations in the cytoplasm and cell organelles may be responsible for the change of cell architecture causing deterioration of the cell envelope. These findings of ultrastructural changes are in agreement with the effects reported in T. rubrum by onion extracts (Ghahfarokhi et al. 2004) and in A. niger by oil of Thymus sp (Rasooli et al. 2006). Increase in cell wall thickness of yeast or filamentous fungi due to comparable deposits in the form of electron dense vesicles and or irregular shaped membranous materials were observed in our study. This effect is similar to the fungal cells treated with inhibitors of ergosterol synthesis such as azoles and allylamines which are known to damage membrane because ergosterol is an essential lipid component of the cell membrane (Nishiyama et al. 2005).

Essential oil components have the ability to alter the cell permeability by entering between the fatty acyl chains of membrane lipid bilayers and therefore, disrupt the lipid packing (Hammer et al. 2004). Due to this the cell membrane properties like fluidity/permeability and other functions may get changed (Sikkema et al. 1995). This may also affect the regulation and function of the membrane bound enzymes that alter the synthesis of many cell wall polysaccharide components (i.e. β-glucan, chitin and mannan) and therefore the cell growth and morphogenesis is disturbed (Hammer et al. 2004).

The findings of electron microscopic studies have highlighted the multiple sites of action of oils in fungal cells including damages to the cell walls, cell membranes, other membranous structures and cytoplasmic contents; leading to cell death. Further additional level of evidence on mechanism of action was assessed by sorbitol assay for detection of
cell wall as target sites and K\(^+\) leakage for detection of cell membrane damage. Assays for UV absorbing material that are released after cell death were also performed.

A distinctive feature of compounds acting on the fungal cell wall is that the antifungal effect can be reversed in a medium containing an osmotic stabilizer such as sorbitol. Therefore, MIC of cinnamaldehyde, eugenol, *C. verum* and *S. aromaticum* and fluconazole (negative control) and congo red (positive control) were evaluated against *C. albicans* 04 and *A. fumigatus* MTCC2550 in the presence of sorbitol. There was no increase in MIC of test agents against *C. albicans* 04 and *A. fumigatus* MTCC2550 except eugenol and cinnamaldehyde exhibiting increase in MIC up to two and four-folds, respectively. This marginal increase in MIC is not enough to conclude the cell wall as target site by these compounds. Therefore, it may be suggested that primary target of these compounds would not be the cell wall synthesis or assembly. However, the morphological changes observed in the treated fungi lead to suggest that fungal membrane could be the target for them. Therefore, further experiments such as absorbance of cytoplasmic content leakage, effect on ergosterol biosynthesis or activity were performed to ensure the mode of action of these compounds.

Effect of these oils on to cell permeability was clearly evident from data obtained in 260 nm absorbing materials and K\(^+\) leakage assays. The data obtained for test oils were at par with positive control drug amphotericin B, a membrane disruptive-agent known to affect cell permeability and lethal action against fungal cell. The result showed that concentration and time dependent increase in release of cytoplasmic content (increase in absorbance values at 260 nm compared to untreated control) is accompanied with change in K\(^+\) concentration in cell membrane (evident from increased extracellular concentration in oil/drug treated supernatant) and leads to cell death at higher concentrations of these test oils. Similar mechanism of action by palmarosa and tea tree oils has been reported on yeasts (Prashar *et al.* 2003; Hammer *et al.* 2004) and filamentous fungi (Wang *et al.* 2010). In our study delayed (30 min after exposure to agent) release of 260 nm absorbing material from fungal cell was observed that may be due to the time required to induce the membrane bound cell wall autolytic enzymes leading to lysis (Gilbert 1984). While the activation of autolytic enzymes may have been responsible for this effect, the lysis may also have been due to the weakening of the cell wall and the subsequent rupture of the
cytoplasmic membrane due to osmotic pressure or a specific action on the cytoplasmic membrane. However, in our study substantial leakage of $K^+$ from treated cell has indicated that cytoplasmic membrane is targeted by test oils and its fluidity and permeability is changed leading to leakage of cytoplasmic material. Loss of significant amount of 260 nm absorbing material suggests that nucleic acids were lost through damaged cytoplasmic membrane. Marked leakage of cytoplasmic material is considered as indicative of gross and irreversible damage to the cytoplasmic membrane (Hugo and Longworth 1964).

Therefore, to further confirm membrane damage or lesion the flow cytometry of treated fungal cells were performed using PI as fluorescent marker. In a dose dependent manner eugenol at 4X MIC resulted in 50.90% dead cells, with an edge over cinnamaldehyde (40.21%) and amphotericin B (30.42%), an ergosterol-binding fungicidal antifungal agent. Our findings are in accordance with the reports on membrane damaging action by oils of *Thymus vulgaris*, *Syzygium aromaticum*, eugenol, thymol and carvacrol against *Candida albicans*, *Aspergillus* spp and dermatophytes (Pinto *et al.* 2006; Pinto *et al.* 2009; Ahmad *et al.* 2011). Permeation to PI, particularly following short incubation periods as was shown in our study, indicates that the mechanism of action of the eugenol and cinnamaldehyde against *Candida* cells involves a primary lesion of the cell membrane with substantial morphological changes leading to cell death. Since the membrane damaging effects of antifungal agent might be resulted from different mechanisms of activity involving direct binding to ergosterol (amphotericin B) or indirectly inhibiting the ergosterol biosynthesis (fluconazole). Therefore, additional experiments for differentiating these kinds of mechanism, ergosterol quantitation and ergosterol binding assays were performed for *Candida* cells in the presence of test oils. Our studies revealed a dose dependent decrease in the synthesis of ergosterol content at sub-MICs of *C. verum*, *S. aromaticum*, cinnamaldehyde and eugenol, and the effect was far greater than fluconazole. These oils at MICs blocked the ergosterol synthesis almost completely in the order of eugenol >cinnamaldehyde >*S. aromaticum* > *C. verum*. The similar dose dependent effect on ergosterol biosynthesis in *Candida albicans* by oils of *Coriaria nepalensis*, *Thymus vulgaris*, *Syzygium aromaticum*, eugenol, thymol and carvacrol has been reported by other workers (Pinto *et al.* 2006; Pinto *et al.* 2009, Ahmad
The effect on membrane integrity, thus, appears to originate from the inhibition of ergosterol biosynthesis in a manner similar to fluconazole or other ergosterol synthesis inhibitors. However, when these oils were tested for their ability to bind ergosterol resulted in increase in MIC against *Candida albicans* cells in the presence of increasing concentration of ergosterol in a manner similar to amphotericin B. These observations together led to make tentative suggestion that these oils viz. *C. verum*, *S. aromaticum*, cinnamaldehyde and eugenol primarily exert membrane damaging effect that may be attributed to their ability to inhibit ergosterol biosynthesis and simultaneously interacting with membrane by binding to ergosterol. This is to be mentioned here that a whole line of current antifungals such as amphotericin B, azoles, allylamines and other target the ergosterol biosynthesis pathway or its end product, which is unique to fungi. Ergosterol is an important target because it maintains membrane fluidity, asymmetry and integrity and therefore various cell functions (Rodriguez et al. 1985). In this regard, these oils with potential antifungal activity are supposed to be effective antifungals to be exploited in drug development. Ergosterol also contributes to proper functioning of membrane bound enzymes (Lupetti et al. 2002). That may involve interference with the cell wall synthesizing enzymes and this may pertain to partial or secondary action of these oils affecting cell walls as observed under electron microscopic studies.

In light of the present findings, it could be proposed that antifungal action of oils of *C. verum*, *S. aromaticum*, cinnamaldehyde and eugenol against yeasts in particular *C. albicans* and filamentous fungi in particular *A. fumigatus*, takes place via two step process. The first step involves the passive entry of oils and its components being amphiphillic in nature into plasma membrane in order to initiate membrane disruption by altering fluidity and integrity. The second stage is the accumulation of oils/components in the plasma membrane resulting in the inhibition of cell growth due to increased bilayer disorder, leakage of ions and cytoplasmic materials resulting in disorganized cytoplasm. These effects disturb the osmotic balance of cell leading to membrane associated proteins inefficient to perform cellular functions and eventually leading to cell death. Therefore, the excellence of these oils/compounds exhibiting multiple sites of actions demands more insight studies into all of the possible mechanisms of these compounds.
6.4. Antifungal activity of essential oils in combination with antifungal drugs against drug-resistant fungi

Development of new and ideal antifungal agents is remained a challenge for researchers and has encouraged the search for alternative strategies to combat Candida infections including combinational approach of two or more antifungal agents. Considering this view, the essential oils viz. C. copticum, C. citratus, C. martini, C. verum, S. aromaticum, T. vulgaris, cinnamaldehyde, citral, eugenol and geraniol exhibiting promising antifungal activity alone against Candida spp, Aspergillus spp and Trichophyton rubrum were tested in combination with antifungal drugs viz. amphotericin B and fluconazole to explore more effective approach. Combination of antifungal agents has advantages over monotherapy because it can exhibit improved efficacy due to the increased rate of killing, a broader spectrum of action covering multiple infections by different pathogens, a reduced duration of therapy resulting in the decreased likelihood of developing resistance and minimization of the dose-related toxicity of antifungal drugs (Vazquez 2003; Vitale et al. 2005). Combination therapy with available antifungal drugs has been recommended and used in treatment for various mycoses (Cuenca-Estrella 2004; Baddley and Pappas 2007; Segal and Steinbach 2007; Wirk and Wingard 2008); however, combination therapy with plant products is less explored. In spite of their diverse activity, essential oils or active compounds in combination with antifungal drugs have been less frequently investigated. Since natural products are cheaper and considered safer, they could be better explored for their synergistic interaction with drugs of choice against Candida and other mold infections potentially resulting in more cost effective and safer formulations.

Some in vitro studies have revealed synergistic interactions for antifungal activity including latex from Euphorbia characias with ketoconazole (Giordani et al. 2001), oils of Alium and Pelargonium graveolens with ketoconazole against Trichophyton spp (Shin and Lim 2004; Shin and Pyun 2006), Agastache rugosa oil with ketoconazole against B. capitatus (Shin and Kang 2003), and santolina oil with clotrimazole, allicin with azoles, oils of Thymus vulgaris and Myrtus communis with amphotericin B against Candida albicans (Suresh et al. 1997; Giordani et al. 2004; Khodavandi et al. 2010; Mahboubi and Bidgoli 2010); but little information is available against drug-resistant fungi. In this perspective, the current findings highlight the synergistic interaction of certain essential
oils or active compounds with amphotericin B/fluconazole against the drug-resistant strains of *C. albicans, A. fumigatus* and *T. rubrum*. Promising results are coming in the form of potentiation of these drugs by their partner drugs in combination, preferably, by combining agents with different antifungal mechanisms.

In the present study, eugenol, citral and *T. vulgaris* being promising anti-candidal agent alone, also exhibited synergistic interaction with both fluconazole and amphotericin B. Although, oil of *C. martini* was an effective anti-candidal agent alone, but in combination was indifferent with both fluconazole and amphotericin B. Geraniol exhibited moderate anti-candidal activity alone, and also showed a significant level of synergy with both fluconazole and amphotericin B. Also, a relatively higher limit of tolerance (greater MIC) to amphotericin B and fluconazole as observed in test strains was reduced substantially by 16- to 32-fold and, thereby indicated effectiveness of these combinational approaches. All test combinations showed synergistic interactions against the test strains of filamentous fungi except the oils of *C. martini* and geraniol against *A. fumigatus*. Differences occurring with the same combination may be because the geraniol and geraniol acetate shared more than 50% of the *C. martini* oil. It is possible that the behavior of *C. martini* oil is greatly affected by geraniol and that *C. martini* oil and its major ingredients share the same mechanistic differences in the interaction results. Cinnamaldehyde was most effective in the combination showing strongest synergy with fluconazole against both *A. fumigatus* and *T. rubrum*. Cinnamaldehyde also reduced the MIC of fluconazole up to eight-fold and its MIC decreased up to 16- and 32-fold.

Treatment of fungal infections caused by the drug-resistant fungi may require higher treatment doses. If fluconazole is used in higher doses in monotherapy it can lead to adverse side effects such as hepatotoxicity (Groll et al. 1998). With this in consideration, our data suggests that dose-related toxicity associated with amphotericin B and drug resistance against fluconazole can be overcome in combinations with essential oils or active compounds. As revealed in this study, the interactive responses of oils and active compounds did not appear to be affected by the susceptibility behavior of the strains to amphotericin B or fluconazole rather variation in a combinational effect may pertain to the nature of oils, their constitutional compounds and their concentrations. However, this
study is based on *in vitro* observations and thus required careful investigation under *in vivo* system of diseased model to develop effective combination.

The synergistic interactions of essential oils or active compounds with fluconazole/amphotericin B may be related to the simultaneous inhibition of different target sites by test agents and drugs. Fluconazole inhibits fungal cytochrome P450 dependent enzyme lanosterol 14-α-demethylase and thereby blocks ergosterol biosynthesis, and also inhibits P450 dependent enzymes involved in fungal respiration. Amphotericin B binds to ergosterol in the cell membrane leading to perforation and leakage of cytosol and hence cell death (Vazquez 2003; Onyewu and Heitman 2007). The synergistic combination of these drugs with oils may be explained mechanistically that oils exerting effects in ways other than available antifungal drugs, mainly on cell wall, plasma membrane and other membrane structures of *Candida* and other filamentous fungal cells as evidenced from our study; aids these drugs in their mode of action. Also, it could be possible that fungicidal action of essential oils or active compounds lower the burden for these drugs to act upon. Since fluconazole is a hydrophilicazole, it is less effectively absorbed compared to lipophilic azoles like ketoconazole, clotrimazole and itraconazole by cytosolic components of fungal cells (Scheven and Schwegler 1995). The oils damaging the cell membrane may facilitate its improved entry to the cell leading to more effect on the ergosterol biosynthesis inhibition and adding to the effect of cell membrane destruction. Regarding amphotericin B, its binding to ergosterol in the cell membrane may be aided by alteration in membrane permeability or fluidity by test oils and may lead to enhanced effect of cell membrane damage. The exact mechanism for disruption of cell membrane or cell wall synthesis by oils is not understood but multiple target sites by such kind of antifungal agents are added advantages in the combination therapy. Furthermore, efficient fungicidal activity of oils against azole- and amphotericin B-resistant strains suggests that these oils are effective against strategy or adaptive mechanisms of resistance exhibited by test fungi.

6.5. Inhibition of virulence factors production/activity in drug-resistant fungi by essential oils

Another approach to deal with drug resistance in fungi is the development of an agent that can target essential virulence factors whose absence or inhibition, although not lethal
for fungi may result in loss of virulence of the organism. Therefore, compounds influencing the release of virulence factors may be useful in combating fungal diseases caused by drug-resistant strains. In the present study, oils *C. copticum*, *C. verum*, *C. citratus*, *C. martini*, *S. aromaticum*, *T. vulgaris*, eugenol, cinnamaldehyde, citral and geraniol exhibiting significant antifungal activity were assessed for their ability at sub-MICs (0.5X and 0.25X MIC) to reduce the production of virulence factors namely germ tube formation, cell surface hydrophobicity, proteinase and haemolysin in drug resistant strains of *C. albicans*, and keratinase and elastase production/activity in the drug-resistant strains of *Aspergillus* spp and *Trichophyton* spp.

Test oils especially *C. verum*, *C. copticum*, *S. aromaticum*, *T. vulgaris*, eugenol and cinnamaldehyde significantly inhibited the ability of GTF. This is probably the first report on the anti-GTF activity by these oils in pathogenic strains of *C. albicans*. However, oils of Tea tree and certain other plants have been reported to inhibit GTF by some other workers (Hammer *et al.* 2000; Pozzati *et al.* 2010). Anti-GTF activity by oils is a promising anti-virulent property because germ tubes have been shown to contribute in increased adherence and invasiveness of *Candida* cells in models of skin, mucosal and disseminated candidiasis (Wellmer and Bernhardt 1997).

It is considered that hydrophobic interactions play an important role in the adherence of opportunistic pathogen yeasts to eukaryotic cells and is an important event in the initiation of the pathogenic process (Hazen 1989). Considering this the above mentioned oils were tested for their effect on hydrophobicity of *Candida* spp. Except *C. martini*, all other test oils significantly (*P*≤0.05) reduced the CSH at both 0.5X and 0.25X MIC in one or other strains of *Candida* spp tested. Similar to our findings, concentration dependent inhibition of CSH in *C. albicans* by tannins from *Stryphnodendron adstringens* has been reported by Ishida *et al.* (2006). Hydrophobic cells are more adherent and resistant to phagocytosis and, therefore more virulent than hydrophilic cells. In this regard, lowering of CSH of *Candida* cells by test oils suggests their importance in arresting the candidal colonization during pathogenesis.

Production of proteinase is a highly regulated process and transcriptionally co-regulated with other virulence attributes in *C. albicans*. Our findings have revealed a strong inhibition of proteinase production in *Candida* spp by at least six essential oils (*C. verum*, *C. copticum*, *S. aromaticum*, *T. vulgaris*, eugenol, cinnamaldehyde).
*C. copticum, S. aromaticum, T. vulgaris,* cinnamaldehyde and eugenol) at sub-MICs. Similar findings have also been reported by Hofling *et al.* (2011), who have reported inhibition of proteinases in *C. albicans* by six other plant extracts. Proteinase activity helps *in vivo* in obtaining nutrients, adherence, invasiveness, dimorphism, phenotypic switching, biofilm formation and degradation of immune components such as antibodies, complements and cytokines (De Bernardis and Sullivan 2001; Kuriyama *et al.* 2003; Khan *et al.* 2010). Therefore, test oils exhibiting strong anti-proteinase activity could be considered as important anti-pathogenic agents. Moreover, these test oils also exhibited inhibition of haemolysin production at varying level. To the best of our knowledge, this is the first report on inhibition of haemolysin production in *Candida* spp by test essential oils particularly *C. verum, S. aromaticum,* cinnamaldehyde, eugenol and geraniol. The role of haemolysin is to obtain iron as a nutrient from haemoglobin during disseminated growth of candidal infections; adherence is also shown to be reduced in iron limiting environment (Almeida *et al.* 2009). Therefore, exhibition of anti-haemolysin activity in test oils further may aid to their anti-pathogenic efficacy against *Candida* strains. These drug targets have been attempted by many researchers to obtain effective anti-pathogenic drugs against fungal infections as reviewed by Gauwerky *et al.* (2009). In general, test oils or compounds inhibited different virulence factors in one or other strains in both *C. albicans* and non-albicans *Candida* in the order of efficacy of proteinase >CSH >GT >haemolysin. Oils *C. copticum, C. verum, S. aromaticum, T. vulgaris,* eugenol and cinnamaldehyde were highly effective in reducing all the tested virulence factors at 0.5X and 0.25X MICs. Other oils could effectively inhibit one or two virulence factors at 0.5X MIC. Therefore, oils exhibiting broad spectrum anti-virulent activity may be potential candidates as anti-pathogenic drugs. It is to be considered that not all the virulence factors are expressed in infections or necessarily in a particular stage of infection (Naglik *et al.* 2003), the inhibition of even single virulence factor by test agents is of practical value. Eugenol constitutes 74.32% of oil of *S. aromaticum* and cinnamaldehyde shares 79.10% of *C. verum* as major active ingredients as evident from our study. Activity of these ingredients being at par with their respective oils is an indicative of that mechanistic
role of these oils is attributed to their major active ingredients and other minor compounds are of less importance.

Essential oils interrupt cell walls, cell membranes, nuclear and mitochondrial membranes and respiration in fungal cells as evident from our study together with reports from other workers (Inouye et al. 1998; Ishida et al. 2006). It has been suggested that where membrane integrity is adversely affected, membrane associated functions may also be compromised (Sikkema et al. 1995). Membrane associated enzymes such as chitin, mannan, 1,3-β-D-glucan synthase and adenosine triphosphatase (ATPase) are involved in GTF (Odds 1998). Therefore, loss of these enzymes together with lack of energy production may prevent the morphological transition from blastoconidia to hyphae, a form that is more adherent and invasive. In addition, mannoprotein acting as principle adhesion protein is associated with cell wall (Hazen 1989). The haemolytic factors produced by *C. albicans* are also believed to be mannoproteins (Watanabe et al. 1999). Interference with the production of this protein upon damage of cell wall or cell membrane may result in reduced cell adherence and low haemolytic activity. Essential oils being hydrophobic in nature will aggregate more hydrophobic cells and severe action of oils onto cell walls and membranes of *Candida* cells might be achieved. This could result in the damage of hydrophobic interaction bonds and, therefore lowering of cell wall associated CSH. These oils except *C. martini* at sub-MICs effectively reduced the production of virulence factors in *C. albicans* SC5314 a well known strain to study virulence.

To explore broad spectrum anti-virulence activity against filamentous fungi these oils were assessed for their inhibition of elastase and keratinase production or activities in *A. fumigatus* and *T. rubrum*. Oils of *C. copticum*, *C. verum*, *T. vulgaris* and cinnamaldehyde were significantly effective against production of elastase at 0.5X MIC. All the test oils except *C. citratus* and citral significantly reduced the elastase activity whereas only *C. martini* and geraniol were effective against keratinase production and activity. Production of elastases and keratinases are reported to aid in the pathogenesis of *Aspergillus* sp. and *Trichophyton* sp. (Okumura et al. 2007; Vermout et al. 2008). Since proteinases contribute to fungal virulence by destroying host tissues and digesting immunologically important proteins such as antibodies and complement factors (Santos et al. 2007), the
inhibition of these enzyme production or activities may reduce the pathogenesis of filamentous fungi. Therefore, ability to inhibit proteinase production/activity in filamentous fungi is added to the effectiveness of these oils as an anti-pathogenic candidate against wide range of fungal infections.

At higher concentrations these oils are cidal for candidal growth but at lower sub-lethal concentrations these lead to attenuation of virulence in *Candida* cells and may augment its susceptibility to immune cells, antibodies and natural killer cells. Moreover, at the tested sub-MICs of oils viability of *Candida* cells was non-significantly affected compared to untreated cells and, indicates that reduction in the production of virulence factors by oils is not due to the inhibition of growth rather interference with their synthesis. Therefore, mode of action of these oils towards fungal cells highlights their ability to arrest both the fungal growth at higher concentrations and inhibition of virulence factors at lower concentrations. Further, investigations are needed to know the exact mechanism of inhibition of virulence factors production. Inhibition of one or more virulence factors in fungal cells by oils at lower concentrations would result in no survival pressure of killing in cells as exerted by conventional antifungal drugs. Since most of the virulence traits are not essential for fungal growth, the chances of developing resistance are minimized. This approach might be more effective in immunosuppressed patients, where drug-resistant strains lead to relapses of infection due to their virulence potential and result in high mortality rate accounting for nosocomial fangaemia (Perfect and Cox 1999; Krcmery and Barnesz 2002).

6.6. Anti-biofilm activity of essential oils against drug-resistant strains of *C. albicans*

Extending the view of virulence in fungi, the formation of biofilms is considered as important aspect of microbial pathogenesis. The majority of manifestations of candidiasis are associated in one way or another with the formation of *Candida* biofilms on the surfaces of inert or biological materials (Crump and Collignon 2000). Sessile (biofilm) cells display unique phenotypic traits in comparison with planktonic cells. The most notable of these is that sessile cells are notoriously resistant to antimicrobial agents and withstand host immune defenses (Douglas 2003). This is the main reason why biofilm-associated infections are frequently refractory to conventional antibiotic therapy. Therefore, essential oils/active compounds namely *C. copticum, C. citratus, C. martini,*
C. verum, S. aromaticum, T. vulgaris, cinnamaldehyde, citral, eugenol and geraniol exhibiting potential inhibition of growth and virulence in test fungi, were also tested for their anti-biofilm activity against C. albicans strains.

In our study, antifungal susceptibility studies of the test strains have demonstrated a rise from the planktonic MICs to corresponding sessile MICs, a characteristic of C. albicans biofilms first described by Hawser and Douglas (1995). The drug susceptibility of these strains under biofilm conditions exhibited several folds increase in MICs. Sessile MIC (SMIC) of amphotericin B was raised up to 16- and 512- folds in C. albicans 04 and C. albicans SC5314, respectively. Similarly, SMIC of fluconazole was increased to 8- and 1024- folds, respectively. Increased resistance to fluconazole and amphotericin B Candida biofilms has been reported by other workers as well and antifungal drugs have been reported to exhibit 30 to 2000 times higher MIC against sessile cells compared to planktonic cells (Hawser and Douglas 1995; Seneviratne et al. 2008). Increases in MIC of test oils/compounds against sessile cells compared to their planktonic counterparts were only 2- folds higher for C. copticum, C. verum, S. aromaticum, cinnamaldehyde and geraniol and 4- folds for C. martini and citral. Remarkably, C. citratus, T. vulgaris, and eugenol showed no increase in SMIC compared to planktonic MIC against test strains. Furthermore, efficacy of test compounds was evaluated in terms of the time-dependent killing of established C. albicans biofilms. Data obtained have showed highest cidal activity from C. citratus, geraniol and eugenol compared to other oils and antifungal drugs tested.

It was interesting to observe the ability of test oils to destroy preformed biofilms of C. albicans. This prompted us to evaluate the effect of these compounds at sub-MICs to inhibit the formation of biofilms when the oils/drugs were added to the medium at the same time as the cells. Biofilm formation is considered to be an important virulence attributes for establishing and maintaining candidiasis. If an agent is added at the beginning of the experiment, the agent might act before the biofilm formed and inhibit development of biofilm; it could be of greater interest in combating recalcitrant infections of Candida biofilms. Our data revealed varying level of attenuation of biofilm formation by planktonic Candida cells in the presence of oils and drugs in a dose dependent manner. Among the tested agents, T. vulgaris showed most inhibitory effect on biofilm
formation at 0.5 X and 0.25 X MIC followed by *C. citratus*, eugenol, *C. copticum*, cinnamaldehyde. Other test oils and antifungal drugs were less effective in preventing the formation of biofilms.

These findings of concentration dependent inhibition of formation of biofilms were visually confirmed by light microscopy and scanning electron microscopy of *C. albicans* 04. Under light microscopy, untreated cells resulted in intact biofilm formation in 48 h whereas treated cells exhibited disorganization of biofilm stages with the increasing concentration of oils at sub-MICs. Ability to form hyphal forms plays a pivotal role in the development of the spatially organized architecture of mature *C. albicans* biofilms (Ramage *et al.* 2005). Inhibition of filamentation results in atypical biofilm architecture consisting of densely packed yeast cells that are easily detached from the substratum as shown in our observations. Therefore, it is speculated that anti-pathogenic (especially inhibition of dimorphic transition) activity of oils/phytocompounds of plant origin against planktonic cells might prevent the initiation and development of different stages of biofilms. Our findings have strengthen the exploitation of anti-biofilm properties from plant products including essential oils of *Cymbopogon citratus*, and active compounds like thymol, eugenol and terpenoids against *Candida* spp as recently reported by several other workers (He *et al.* 2007; Agarwal *et al.* 2008; Braga *et al.* 2008; Dalleau *et al.* 2008; Sangeetha *et al.* 2009; Sandasi *et al.* 2011; Taweechaisupapong *et al.* 2011).

In our study, SEM observations have clearly indicated the interference of eugenol and cinnamaldehyde with cell membrane integrity in *Candida* cells as evidenced by shrinkage of cell surface in planktonic cells. Other workers have also shown that compounds from essential oils affect cell membrane integrity of the yeast cells (Bennis *et al.* 2004; Tyagi and Malik 2010a). The similar mode of action was also observed against sessile cells of *C. albicans* as evidenced from our study. These observations lead to suggest that these compounds target cell membranes in both the planktonic (higher sterol content) and sessile (lower sterol content) cells of *C. albicans* and their mode of action remain unaffected with the phenotypic variation in terms of ergosterol content exhibited by planktonic and sessile cells.

Mature biofilms are notoriously difficult to eradicate and represent a source of infection that is recalcitrant to antifungals. This is puzzling because planktonic populations of the
same strain can be susceptible to a wider range of antifungals. Sessile cells exhibit drastic phenotypic changes compared to their planktonic counterparts resulting from a decreased growth rate or nutrient limitation, therefore, modes of action of conventional antifungal drugs have been restricted or ineffective in biofilms.

The most commonly used antifungals such as amphotericin B and fluconazole directly or indirectly target the ergosterol in planktonic *Candida* cells. Azoles act by inhibiting ergosterol biosynthesis, and amphotericin B binds to ergosterol. Decreased ergosterol content ([Mukherjee et al. 2003a](#)) and a diminished level of ergosterol biosynthetic gene expression ([Garcia-Sanchez et al. 2004](#)) have been reported in mature *Candida* biofilms. Since sterol metabolism is the primary cellular process affected by the most widely employed antifungal drugs, the diminished levels of ergosterol present in sessile *C. albicans* may reflect a physiological state more conducive to resistance in these cells. These all mechanisms of drug resistance are supposed to be overcome by phytocompounds exhibiting mode of action other than that of conventional antifungal drugs. This mode of action of these classes of compounds towards sessile cells could be compared to that of chlorhexidine, a membrane-active antiseptic that is very effective against bacteria that lack sterols ([LaFleur et al. 2006](#)).

Furthermore, the hydrophobic nature of such compounds may result in increased uptake of oils through charged polysaccharides of extracellular matrix making more cells in contact to compounds and exerting greater membrane permeation into *Candida* cells. Moreover, as per literature reports, one of the predominant reasons for drug-resistance of the *C. albicans* biofilms appears to be the restricted penetration of drugs inside the exopolymeric matrix, which can bind or restrict the diffusion of antifungals ([Douglas 2003; Ramage et al. 2005](#)). But the volatile nature of essential oils or its constituents can overcome this barrier by exerting more penetration inside the exo-polysaccharide matrix and therefore, may act as better antifungal against preformed biofilms. This may result in an increased spectrum of action of these compounds on sessile cells including persister cells leading to a retarded or disorganized development of biofilms. However, further work is needed to explore the exact mechanism of oils for overcoming the drug-resistant phenotype of biofilm cells.
It is to be considered that combination therapy overcomes the problem of drug-resistance and toxicity associated with the conventional antifungal drugs (fluconazole and amphotericin B) when used alone. Therefore, an attempt was made to determine whether synergism of fluconazole or amphotericin B and phytocompounds from essential oils in combinations could be extended to \textit{C. albicans} biofilms. The compounds exhibiting strong anti-biofilm activity viz. eugenol and cinnamaldehyde were tested for their interaction with amphotericin B or fluconazole to examine possible synergy with drugs against established biofilms of \textit{C. albicans}. Eugenol being a potential anti-biofilm agent against preformed biofilms and formation of biofilms alone, also exhibited synergistic interaction with fluconazole against biofilms formed by test strains. However, combination with amphotericin B was indifferent. Similar interaction result was also obtained for cinnamaldehyde. SMIC of fluconazole was reduced substantially by 32-fold and, thereby indicated effectiveness of these combinational approaches. This could be explained that when phytocompounds exhibiting cidal activity against biofilm cells are combined with fluconazole, the fungistatic nature of this drug is converted to fungicidal. In overall, combination approach may result in cell death and better activity in combination is achieved against \textit{Candida} biofilms.

\textbf{6.7. Conclusion}

I. The findings of our \textit{in vitro} study have highlighted the occurrence of multi-drug resistance among the strains of \textit{Candida} spp, \textit{Aspergillus} spp and \textit{Trichophyton} spp from different diseased conditions.

II. In addition, these strains exhibited production of various virulence factors namely germ tube formation, cell surface hydrophobicity, proteinases, phospholipases, haemolysins and biofilm formation in \textit{Candida} spp and production of lipases, gelatinases, keratinases and elastases in filamentous fungi. This has indicated a possible role of various virulence factors in the pathogenicity of fungi to cause fungal infections.

III. A fair number of common essential oils used in this study especially \textit{C. copticum}, \textit{C. citratus}, \textit{C. martini}, \textit{C. verum}, \textit{S. aromaticum}, \textit{T. vulgaris} and some of their major active compounds such as cinnamaldehyde, citral, eugenol and geraniol
demonstrated strong antifungal activity against drug-resistant strains of *Candida* spp, *Aspergillus* spp, *T. rubrum* and other filamentous fungi.

IV. The mechanism of action of some of these oils primarily appeared to be the damage of cytoplasmic membranes by inhibiting ergosterol biosynthesis and or binding to ergosterol in the membrane and thereby altering the membrane fluidity and integrity. In a consequence they may damage cell wall as a secondary effect as observed under electron microscopy studies.

V. An alternative combinational approach to antifungal agents, these oils exhibited synergistic interaction in a varying capacity when tested in combination with amphotericin B/fluconazole against drug-resistant strains of *Candida* spp, *A. fumigatus* and *T. rubrum*. This has indicated their possible effective exploitation in combination approach to combat mycoses caused by these drug-resistant fungi.

VI. These oils at sub-MICs also showed varying level of anti-virulence potential by inhibiting production of germ tubes, cell surface hydrophobicity, proteinase and haemolysin in *Candida* spp and inhibition of elastase and keratinase productions/activity in the strains of *Aspergillus* sp and *Trichophyton* sp. This is an indicative of their possible role in reducing the virulence of pathogenic fungi.

VII. Varying level of anti-biofilm activity of these oils against preformed biofilms or formation of biofilms alone and or in combination with fluconazole in *C. albicans* further indicated their potential role in inhibition of biofilm on medical implants in the host tissue. This also justify their effective use in combating the problems of drug-resistance associated with the biofilm mode of growth.

6.8. Future direction
The broad spectrum antifungal and anti-pathogenic activities of these test oils/compounds need to be evaluated *in vivo* alone and in combination with antifungal drugs to uncover their therapeutic potential against fungal infections caused by strains of *Candida* spp, *Aspergillus* spp and *Trichophyton* spp. Biochemical and molecular mechanism of anti-virulence activity of these oils need to be explored and also to check loss of virulence in animal model.