DISCUSSION

Although there are several classes of antifungal drugs available for treatment of both superficial and invasive fungal infections, it remains a significant clinical problem till date. One major problem is that yeasts are eukaryotes and generally present more difficult therapeutic problems than bacteria as there are relatively few antifungal agents that can identify unique targets not shared with human hosts. The currently available drugs are effective but eventually lead to drug resistance. Moreover, the treatment costs, unpleasant side effects, associated host cytotoxicity and the fact that most available antifungal drugs are fungistatic in nature validate the search for new strategies. Natural products are a very good source of chemical scaffolds with diverse biological activities. They have a profound impact on antimicrobial drug discovery. Our findings suggest options for expanding the utility of plant active principles as antifungal agents.

*Mentha piperita* or peppermint is a perennial flowering member of the mint family. The herb has medicinal properties and has been used since antiquity as a digestive aid, for management of gallbladder and respiratory diseases (Ronald, 2003; Reichling *et al.*, 2009; Kligler and Chaudhary, 2007; Lei *et al.*, 2011). Extracts of peppermint are used in many cosmetic products and in over the counter medicines (Derwich *et al.*, 2011; Marta Cristina Teixeira Duarte *et al.*, 2005). The antifungal potential of plant essential oils and their components has been described in several studies (Knoblock *et al.*, 1989; Arras and Usai, 2001; Vishnu *et al.*, 2008; Edris and Farrag, 2003). *Ocimum sanctum* and its lead molecules have been shown to be good antifungal agents having multiple target sites in *Candida* (Khan *et al.*, 2010a). Although the inhibitory activity of Mint EO and its major constituents against various pathogenic yeasts has been
demonstrated earlier (Sardi et al., 2013; Carretto et al., 2010; Saharkhiz et al., 2012; Li et al., 2011; Pramila et al., 2012), their effect on certain crucial virulence factors has not been verified. In the present study, we have demonstrated the fungicidal activity of Mint EO and its three lead biomolecules and studied their effect on the virulence of *C. albicans* (ATCC 10261, ATCC 44829, and ATCC 90028), *C. tropicalis* (ATCC 750) and *C. glabrata* (ATCC 90030). Mint EO was used only after analysing its constituents by GC-MS. Since the oil was characterized by high amounts of Menthol, Carvone and Menthone, studies were also done with these natural compounds. Although the quantity of Menthol was higher in this particular chemotype of Mint EO, Carvone showed greater antifungal properties than Menthol and Menthone.

Our results show that both sensitive and resistant *Candida* strains were affected by Mint EO and its constituents. MIC was defined as the concentration required to inhibit 90% cell growth. MIC of Mint EO was 225µg/ml against all the *Candida* strains used in the present study. Comparative analysis between MIC and MFC showed that MFC of the compounds Carvone, Menthol and Menthone was twofold higher than MIC in all the *Candida* strains while in case of Mint EO, MFC was the same as MIC. MFC may be a better predictor of therapeutic response than MIC, especially in immunosuppressed patients (Carolina, 2009; Chakrabarti et al., 2009). Menthone was the weaker antifungal while Mint EO and Carvone proved to be the most effective with low MIC and MFC values. No systematic differences were observed between fluconazole sensitive and resistant strains. *Candida* isolates having an MIC≥ 64 mg L⁻¹ for fluconazole were considered resistant. The zones of inhibition formed on solid agar media by exposing the *Candida* strains with MIC and sub-MIC values of Mint EO and its constituents further demonstrated the fungicidal activity of these compounds while fluconazole showed fungistatic activity. The zones produced around the discs in diffusion assay were completely clear, an
indication of potential fungicidal activity, whereas in contrast fluconazole showed a turbid halo, an indication of its fungistatic nature.

Virulence characteristics of *Candida* species include adherence to host tissues, morphological changes, and secretion of hydrolytic enzymes like phospholipases and proteinases (Haynes, 2001). Yeast to hyphal transition is one of the initial stages of the infection process in *Candida*. The present work demonstrates that at sub inhibitory concentrations, Mint EO and its lead molecules, significantly inhibit cell transition from yeast to hyphae which is considered to be the more pathogenic form, thus preventing the initial stage of the infection process. Germ tube induction is essential for the invasion of host tissues in the *Candida* infection process and our results show efficacy of these natural oil components against the virulence attributes of *Candida*. In control cells, the germ-tube induction proceeds with the increase in incubation time period. Abundant Chlamydospores were seen around the hyphae after long incubation time periods. Pseudohyphae were not visible in the untreated cells while these structures could be seen in treated cells indicating that *Candida* cells were stressed in that environment.

The host target cell membrane is made up of proteins and lipids. *C. albicans* is capable of producing proteinases (Hube, 1996; De Bernardis *et al.*, 2001) and phospholipases (Ibrahim *et al.*, 1995) that hydrolyse peptide bonds and phospholipids, respectively and cause tissue invasion and damage. The secretion of proteinases by *C. albicans* depends not only on the type of strains and infection, but also on the type of phenotypic switching. Environmental conditions and the stage of infection also play a role in this process (De Bernardis *et al.*, 2001). Mint EO and its lead molecules have a significant inhibitory effect on proteinases and phospholipases which suggests that these natural compounds can prevent the hydrolysis of host tissue when it is colonized with *C. albicans*. These compounds not only prevent the transition of the yeast to a
more pathogenic hyphal form but also check the invasion of fungal cells into the host tissues. As phospholipases and aspartyl proteinases of *C. albicans* are considered the important virulence factors (Basu *et al.*, 2003), the lowered secretion of these enzymes may indicate the less virulent nature of *Candida* strains, in the presence of Mint EO and its lead molecules. In the absence of test compounds, secretion of hydrolytic enzymes was greater. There are several reports that throw light on the correlation between secretion of extracellular enzymes and virulence in *Candida* species (Kiliç *et al.*, 1996; Kustimur *et al.*, 1999; Mattews, 1994).

*Candida albicans* and other *Candida* species cause both superficial and systemic infection. An effective antifungal therapeutic agent should have fungicidal properties and should be non toxic. Mint EO and its lead molecules used in the present study have the ability to kill *C. albicans* at their respective MIC values. Since the oral and vaginal cavities are constantly exposed to secretions, the maintenance of correct concentrations of therapeutic agent is difficult. In addition the therapeutic agent should be insoluble or at least sparingly soluble in these secretions. All the four compounds used here are only moderately soluble in aqueous environment. Our results showed that all the test entities have the ability to reduce the transition of *C. albicans* from yeast to hyphal form and also decrease the secretion of proteinase and phospholipase at subinhibitory concentrations. Hence these essential oil components have the potential to act as topical agents or rinsing agents when applied to a susceptible infection site at high concentrations which are high and can be maintained. The toxicity of these essential oil components was studied by *in vitro* haemolytic assay. These compounds are significantly less toxic and showed less than 10% hemolysis while Fluconazole at the same concentration showed 100% hemolysis.

Natural products provide an unparalleled source of chemical scaffolds with diverse biological activities and have profoundly impacted antimicrobial drug discovery (Khan *et al.*, 2010a; Khan
et al., 2010b). Our findings suggest options for expanding the utility of plant active principles as antifungal agents.

As reported earlier (Burt, 2004; Palmeira-de-Oliveira et al., 2009), majority of the essential oils act by disturbing microbial cytoplasmic membranes. In the current study we exhibited membrane damage as a mode of action of Mint EO and its major constituents. The antimicrobial activity of essential oils and then components has been reported earlier (Xu et al., 2008; Cristani et al., 2007; Lucchini et al., 1990), but not much is known about this mechanism of action. This work is an attempt to assess the antifungal role of Mint essential oil, Carvone, Menthol and Menthone by studying their effect on structural and functional aspects of membrane integrity in terms of ergosterol content and PM-H⁺ ATPase activity.

Our studies suggest the presence of cellular target(s) that is available to the compounds externally. In fungal cell physiology PM-H⁺ ATPase plays vital role. It maintains electrochemical proton gradient across the cell membrane necessary for the nutrient uptake. It regulates pHₐ, changes in which are widely regarded as of crucial importance to physiology and morphogenesis (Kaur et al., 1988; Manzoor et al., 2002). We explored the effect of Mint essential oil and its major constituents on glucose induced H⁺ efflux which is an estimate of PM H⁺ ATPase activity. Inhibition of enzyme activity correlates well with the cessation of cell growth. This observation is in accordance with the previous studies as well. Azoles even at concentrations higher then MIC do not affect the acidification of external medium in yeast (Manavathu et al., 1999; Ben Josef et al., 2000) while Mint EO, Carvone, Menthol and Menthone could do so at MIC values. It is thus probable to speculate that the test compounds may be interacting directly with the enzyme, serving as the primary reason for their antifungal activity. Phytochemicals have been extensively shown to target different types of ATPases.
Thus, it would be useful to further investigate the interaction of the test compounds with purified PM-ATPase. Existing classes of antifungal have low efficacy, high toxicity and frequently lead to drug resistance. There is thus a critical need to develop more effective therapies to deal with such infections and natural essential oils like Mint EO and its active components offer a safer alternative. Our results suggest that PM H\(^+\)-ATPase can be explored as a potential surface active antifungal target for these and other potential drugs.

Even though natural antifungal compounds act by disrupting cytoplasmic membranes of microbes, specific mechanisms involved in their antimicrobial action remain poorly characterized. It was interesting to observe that cell leakage induced by Mint EO and its main constituents was far greater than that induced by FLC which is an established membrane targeting antifungal drug. Ergosterol is a unique membrane sterol of fungi and the selective cytotoxic behaviour of these drugs hint towards their affinity for this particular target and hence inhibits its biosynthesis pathway. Analysis of ergosterols obtained from FLC-sensitive and -resistant *Candida* strains showed no major difference in either the sterol contents or the sterol patterns in comparison to the untreated controls. Growth of *Candida* in the presence of sub-inhibitory concentrations of the test compounds altered the sterol patterns of the FLC-sensitive and resistant strains in a similar manner. These plant active principles completely blocked ergosterol synthesis at MIC values. Studying the effect of these compounds at various sub-inhibitory concentrations on the sterols of the FLC resistant *Candida* strains showed that they act in a dose-dependent fashion in decreasing the ergosterol content (Fig 4.12-4.15). In the present research, quite considerable inhibition associated with ergosterol biosynthesis seemed to be observed possibly in the sub-MIC attentiveness connected with Mint EO and its particular main constituents. The effect on membrane strength, so, appears too originated from ergosterol
biosynthesis inhibition in a very approach comparable to which connected with FLC. The antifungal effects were attributed to their ability to inhibit ergosterol biosynthesis and to make the cytoplasmic membrane porous in both drug sensitive as well as drug resistant strains. Scanning Electron Microscopy investigation performed in the present study on a representative Candida strain ATCC 90028 cells treated with compounds for 14 hours clearly demonstrates damaging effect of Mint EO and its active compound Carvone on membrane and cell wall structure.

Candida, contains approximately 200 yeast species (Eggimann et al., 2003), and it is an opportunistic commensal of the human oral, gastrointestinal, vaginal, cutaneous and mucosal surfaces. Other rising Candida species which include C.glabrata, C.krusei, C. tropicalis, C.parapsilosis, are currently posing severe nosocomical threats to patient populations (Koehler et al.,1999; Chakrabarti et al., 2009), but till now the most causative organism is Candida albicans (Filler and Sheppard, 2006). Since last few decades the occurrence of candidiasis and other fungal infections have been increased. And currently available drugs are proving to be insufficient as they prove unsuccessful against new or re-emerging fungi which develop resistance very quickly. Also the currently available drugs have undesirable side effects.

Azoles have been a predominant therapy drug for Candida infections for a long time now. Fluconazole (FLC) is the most widely used azole for systemic candidiasis due to its high solubility, low toxicity and wide tissue distribution (Brammer et al., 1990). It targets lanosterol 14α-demethylase in the ergosterol biosynthetic pathway (Vanden et al., 1987). However, treatment failures are increasing, especially in AIDS patients, as prolonged use of this drug has led to resistance in Candida spp. probably due to its fungistatic rather than fungicidal nature of
antifungal action (Sanglard et al., 2003; Uppuluri et al., 2008). New therapeutic strategies are required to reduce the toxicity of these drugs.

The perspective on antifungal drug resistance and other problems suggest several possible ways of overcoming, or preventing, drug resistance. The efficacy of FLC and other antifungal agents can be improved by using combination therapy (Pinto et al., 2009; Ghannoum et al., 1995; Mukherjee et al., 2005; Tariq et al., 1995). Combinational therapy can be used to attack more than one target simultaneously with a low probability that resistance to both drugs will arise, or to attack a drug target and its resistance mechanism. While combinational therapy sounds attractive and it has been reported that antimicrobial combinations can actually select against the development of resistance results is variable and there are increased efficacy testing requirements.

Additional in vitro synergy tests are required to be performed on new fungal strains and other pathogens to evaluate antimicrobial potentials of these and other essential oil components. With this synergistic combinatorial approach many antifungal agents may find even broader therapeutic applications.

In the combination experiments, Mint EO and its lead molecules carvone, menthol and menthone were used at sub a MIC value which at there was minimal hemolysis. Hence our studies indicate that Mint EO and its lead molecules are able to induce synergistic effect with azoles below their cytotoxic concentrations against all tested Candida strains. Although Mint EO showed lower sensitivity than FLC, it showed greater activity against FLC-resistant Candida strains.

In medical microbiology, synergism of natural products and antibiotics against pathogenic microorganisms is a thrust area that leads to the development of novel potential phytopharmaceuticals. Quite a few studies have proved synergistic action of essential oil
fractions of diverse plants with synthetic drugs as antifungal agents (Rosato et al., 2008). The present study however explores azoles (major class of antifungals in clinical practice) against a wide variety of Candida strains. The study is significant as it shows chemosensitization of azoles by Mint EO and its lead molecules carvone, menthol and menthone against FLC-susceptible Candida isolates as well as the increased sensitivity of resistant strains for Mint EO and its lead molecules. The study is significant as there has been a rise in cases of antifungal resistance, especially FLC resistance in patients. Our findings suggest options for expanding the utility of essential oil components as antifungal agents. It is worth speculating that the FLC resistance has been overcome by Mint EO and its lead molecules. Resistance to FLC and other drugs is due to over expression of efflux transporter genes (MFS, ABC superfamily) leading to increased drug efflux from the cell. Mint EO might be inhibiting either expression or the activity of these transporters and therefore the cells are showing increased susceptibility to FLC which otherwise is not observed (Table 4.8). All the Candida strains tested showed a synergistic interaction between Mint EO and its lead molecules carvone, menthol and menthone & FLC. None of the isolates showed antagonistic effects with either of the combinations.

Human erythrocytes are useful for studying toxicity of compounds, as they are readily available, their membrane properties are well characterized, and their lysis can be easily monitored by measuring the release of hemoglobin. Hence, the in vitro hemolytic assay is a feasible screening tool for investigating in vivo toxicity to host cells (Christie et al., 2007). Mint EO and its lead molecules Carvone, Menthol and Menthone were studied for hemolytic activity and the results indicate that these test compounds were significantly less cytotoxic than the conventional antifungal drug fluconazole.
In conclusion, Mint essential oil components, Carvone, Menthol and Menthone are structurally related fungicidal bioactive compounds having low MIC values and negligible cytotoxicity. Mint essential oil and its three lead compounds (Carvone, Menthol and Menthone) not only reduce the transition of *C. albicans* from yeast to the invasive and more pathogenic hyphal form at sub-inhibitory concentrations but has also a significant effect on the production of the hydrolytic enzymes secreted by the fungal cell during infection. Mint EO and its major constituents are fungicidal bioactive compounds that have a profound effect on PM-ATPase of *Candida* species making this membrane protein a very good potential antifungal target. The excellence of these compounds demand more insight studies into all possible mechanisms of these compounds. These compounds have a great therapeutic potential and further studies will lead to the development of new antifungal drugs. To determine the usefulness of the essential oil *in vivo* supplementary studies using animal models should be done.