Figure 6.1: Graphical representation of the anticandida effect of Mint EO and lead molecules
Candida albicans an opportunistic human commensal, is the predominant cause of virtually all type of candidiasis, but other Candida species, including C.glabrata, C.krusei, C.tropicalis, and C.parapsilosis are now posing serious nosocomical (hospital acquired) threats to immunocompromised patients. Risk factors that increase incidence of Candida infections include compromised immunity, hormonal imbalances, prolonged use of broad spectrum antibiotics and oral contraceptives, pregnancy, metabolic and nutritional disorders. The currently available drugs have low efficacy, high toxicity and frequently lead to drug resistance. They have undesirable side-effects and are ineffective against re-emerging fungi. Treatment options for invasive infections are limited and almost always involve the use of nephrotoxic amphotericin B and azoles- which lead to resistance on prolong use probably due to their fungistatic rather than fungicidal nature. There is thus a critical need to develop more effective therapies to deal with such infections and natural products offer a safer alternative. Another useful strategy would be to improve the efficacy of the existing antifungal drugs using combinational therapy. Plant essential oils possess a broad spectrum of antimicrobial properties due to the presence of a wide variety of bioactive natural molecules. In the present work we studied the relative antifungal efficacy of Indian medicinal plants traditionally known for their antimicrobial potential- Mint (Mentha piperita) against C.albicans.

There are only a few antifungal drugs available largely due to the eukaryotic nature of fungal cells and hence the difficulty in identifying unique antifungal targets not shared with human hosts. Plasma membrane H$^+$ ATPase is unique and crucial to fungal cells and hence is a promising antifungal target. It will help in the development of new mechanism based drugs. The objective of this study was to further elucidate the antimicrobial mechanism of action of Mint
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EO and its lead compounds by determining their effect on the activity of H⁺ ATPase located in the membrane of pathogenic *Candida* species.

The effect of Mint EO and its lead molecules, namely Carvone, Menthol, and Menthone was investigated on the viability of various Candida species. The MIC₉₀ values for both fluconazole-susceptible and resistant Candida strains were determined *in vitro*. The present work is an attempt to explore the antifungal activity of Mint EO and its lead molecules on the growth and pathogenicity of 5 susceptible and resistant *Candida* isolates by turbidometric and susceptibility testing methods (microtitre assay, growth curve studies, resistotyping). The synergistic effect of these compounds with fluconazole was also studied (Checkerboard microdilution assays) to explore the combinational potential of these compounds as chemosensitizing agents for conventional drugs.

All the 5 *Candida* strains used in the present study were susceptible to the test compounds. At sub-MIC values of the compounds, all the strains showed less than 50% growth while at the MIC values, the inhibition was greater than 80%. An increase in the lag phase was also observed with increasing concentrations of the compounds. It was encouraging to observe that all the test compounds decreased cell growth tremendously even in fluconazole resistant strains. The decrease was concentration dependent and no growth was observed at the MIC values of the compounds. Disc diffusion assay was conducted to evaluate the efficacy of test compounds in solid media against various *Candida* strains. It was observed that the diameter of the inhibition zones (ZOI) and hence the inhibitory activity increased with increasing concentrations of the test compounds in the order Mint EO > Carvone > Menthol > Menthone. The zones formed around the disc were completely clear indicating towards their fungicidal nature while fluconazole showed a turbid zone, an indication of its fungistatic nature.
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The proteinase and phospholipases are responsible for adhesion, tissue damage and invasion of host tissues. The secretion of these enzymes has been implicated as potential virulence factors for some *Candida* species. Dimorphic transition (yeast to hyphae) is another important property of this fungus characterizing virulence. Hence we further investigated the effect of Mint EO and its lead compounds on the secretion of proteinases and phospholipases and morphological transition. At half MIC values, the compounds inhibited both proteinases and phospholipases secretion in the order Mint EO > Carvone > Menthol > Menthone. Mint EO was the most effective as it inhibited enzyme secretion by around 50% at ½ MIC. Yeast to hyphal transition was affected tremendously by the test compounds. The *Candida* cells grown in Lee’s simplified media showed more than 95% cells undergoing yeast to hyphal transition after 210 min. After the same time period the percentage of cells undergoing transition decreased to 8, 15, 30 and 35% in Mint EO, Carvone, Menthol and Menthone, respectively at ½ MIC values. Hyphal length was even more profoundly affected in the presence of test compounds. The present work demonstrates that Mint EO and its lead compounds interfere by inhibiting one of the initial stages of the infection process. As elongation of the hyphae is crucial for the pathogenicity of the fungus, its inhibition in the presence of Mint EO and its lead compounds is encouraging and will help in controlling the inception of the disease itself. These compounds also seem to check the damage caused by hydrolytic enzymes and hence invasion of fungal cells into the host tissues.

PM-ATPase plays a crucial role in fungal cell physiology and maintains an electrochemical proton gradient across the cell membrane necessary for the uptake of nutrients. Regulation of PM-ATPase activity is directly linked with the growth of the fungus, hence we explored the effect of Mint EO and its lead constituents on the glucose induced H⁺ efflux which is a consequence of H⁺ ATPase activity. Our results illustrated that Mint EO and its lead constituents
have a profound effect on PM-ATPase of Candida suggesting that this protein can be explored as a potential surface active antifungal target. Glucose induced H⁺ efflux was inhibited to the same extent in both the susceptible and resistant strains. At their respective MIC₉₀ values, the Mint EO, Carvone, Menthol and Menthone show about 47%, 42%, 35% and 29%, inhibition in rate of H⁺-efflux by PM-ATPase of Standard Candida strains respectively. In case of FLC-resistant strains, the decrease in efflux was by 52%, 48%, 32% and 30%, respectively; a trend similar to the susceptible cases. Vanadate (5mM), a specific inhibitor of the proton pump showed 100% inhibition as expected while fluconazole, a commonly used antifungal drugs showed only 24% inhibition in standard Candida strains suggesting that the natural compounds may be having a binding site(s) on the PM-ATPase. These results throw some light on their mode of action.

To get a clearer picture of the drug induced disruption of cell membrane, we estimated the total ergosterol content by the Sterol Quantification Method (SQM) after treating the Candida cells with the test compounds. A dose dependent decrease in ergosterol biosynthesis was observed when the yeast cells were grown in SD broth in the presence of MIC and sub-MIC values of the test compounds for 16h. Our results showed that Mint EO and its lead constituents impair ergosterol biosynthesis in all the Candida strains including the resistant ones. At their respective MIC values, the test compounds showed 100% inhibition in ergosterol content. Cells grown in FLC (8µg ml⁻¹) also showed 100% decrease in susceptible cells which inhibit ergosterol synthesis by interacting with lanosterol 14α-demethylase. As expected in case of FLC-resistant strains, the inhibition observed was only 16% when cells were exposed to 64µg ml⁻¹ FLC (MIC of resistant strains). This was expected and correlated well with our disc diffusion data. Significant inhibition was observed even at sub-MIC values. The disruption of membrane integrity thus, appears to be due to inhibition in the ergosterol biosynthesis in a manner similar to
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that observed with fluconazole. The toxicity of these compounds was studied by in vitro hemolytic assay using human RBCs. All the four test compounds were found to be negligibly hemolytic (< 5%) in comparison to the conventional drug (fluconazole) even at very high concentrations.

Microscopic analysis of the treated Candida cells was performed to get a clear picture of the drug induced disruption of the cell wall and cell membrane. Scanning electron microscopy (SEM) micrographs of treated cells obtained in this study showed extensive damage and breakage in the cell wall and cell membrane.

Combinational studies were performed to investigate the use of chemo-sensitization by Mint EO and its lead compounds to augment the efficacy of conventional antifungal drugs, especially azoles. The test compounds exhibited strong synergism with fluconazole even in fluconazole resistant species. No antagonistic interaction was observed. Our findings are encouraging in view of the growing treatment failure and antibiotic resistance in managing Candida infections and suggest a way of treating resistant infections through a drug combination approach. Present study encourages the use of Mint EO and its lead compounds as an alternative fluconazole therapy for the treatment and prevention of candidiasis and for topical application in the management of superficial infections as all the four test compounds has negligible cytotoxicity and in combination with fluconazole could be used in combating other type of Candida infections. Further studies using animal models are necessary to see the in vivo efficacy of the compounds.