CHAPTER: 5 Discussion, Conclusion, Limitations and Future research

Discussion

Fungal infections have been a neglected and forgotten topic for almost a decade, but now the burden of fungal diseases has become a growing threat to global health. Annually, fungal infections affect over a billion people with a kill rate of more than 1.5 million people (Bongomin *et al.,* 2017). This statistical figure is similar to the number of deaths caused due to tuberculosis per year and even 3-fold higher than malaria (Bongomin *et al.,* 2017). Among the fungal infections, candidiasis is the 5th most common nosocomial infection that accounts for ~700,000 deaths per year worldwide and is also associated with prolonged hospitalization and increased healthcare costs (Sievert *et al.,* 2013; Bongomin *et al.,* 2017). Candidiasis refers to a wide spectrum of diseases which vary from a superficial skin infection to a life-threatening systemic infection. At present, more than 160 species have been differentiated and accepted within the *Candida* genus (Turner and Butler, 2014). However, only around 30 *Candida* species have been found to cause infections in humans, and rank 2nd as the most common causative agents of fungal infections globally (Mancera *et al.,* 2019; Brown *et al.,* 2012). Furthermore, among these pathogenic species, *C. albicans* remains the most predominant and most isolated species that accounts for about 62% of all the isolates followed by *C. glabrata* (17 %), *C. parapsilosis* (9%), *C. tropicalis* (4%), *C. krusei* (3%) and other *Candida* species (St-Germain *et al.,* 2008; Pfaller *et al.,* 2007). *C. albicans* is an opportunistic pathogen, which is normally present inside the body in various locations such as mouth, throat, gut, vagina and on the skin as commensals in about 70% of healthy population (Kabir *et al.,* 2012). However, it may become pathogenic if allowed to grow abnormally, which is mostly due to a disturbance in the host environment or if the host becomes immunocompromised.

Since the 1970s, the incidences of candidiasis have increased constantly (Chin *et al.,* 2016). This significant rise in *Candida* infections is related to various factors and the most important include; increasing number of immunocompromised population as a consequence of other diseases such as AIDS, tuberculosis, cancer, organ transplantation, corticosteroid therapy and other modern-day surgeries, and the second factor which is addressed in this study is the limitations associated with the currently used antifungal drugs that have a narrow antifungal spectrum, are limited in number, are ineffective, and are characterised by adverse side effects such as high toxicity and emerging drug resistance. Further research in antifungal drug development could resolve these issues by discovering new antifungal agents with minimum or no toxicity and broad antifungal spectrum. In the field of drug development, natural products have been widely exploited due to the fact that nature is a vital source of drugs (Dias *et al.,* 2012). Numerous studies have reported antifungal property of natural products, while as on the other hand most of these products showed adverse physicochemical characteristics that become an obstacle for these natural compounds to reach clinics. In view of these challenges medicinal chemists have modified the natural products using advanced techniques resulting in the formation of secondary metabolites with significantly increased potency and safety. Several secondary metabolites, referred to as semi-synthetic drugs or derivatives/analogues, have already been reported effective against various infectious diseases including candidiasis (Stratton *et al.,* 2015; Ahmad *et al.,* 2015). The promising results have encouraged research in this area for the development of new antifungal molecules.

Against this background, in this study we focussed on the development of novel antifungal agents which can be used as an alternative to current antifungal drugs. For this, we synthesized seven novel eugenol tosylate congeners (ETCs) with different substituents on the pendent sulfonyl group from a parent compound eugenol (4-allyl-2-methoxyphenol), which has already been known for its antimicrobial and other pharmacological properties. The derivatives (ETC-1 to ETC-7) were obtained and structurally characterized by different

physical and spectroscopic techniques. All the derivatives (ETC-1 to ETC-7) and their parent compound eugenol were tested *in silico* and *in vitro* using different methods. *In silico* results confirmed that both eugenol and its derivatives obeyed the 'Lipinski Rule of Five' and thereby confirm the desirable drug likeness properties of these molecules. The *in vitro* antifungal susceptibility testing (MIC and MFC) of these compounds against various fluconazole susceptible and resistant *C. albicans* isolates revealed that tosylation of eugenol resulted in molecules with potent antifungal activity. In comparison to their parent compound eugenol, all the derivatives showed extreme decline in MIC and MFC values up to 4000 and 2000-folds respectively. These results are supported by our previous findings where tosylation of eugenol resulted in derivatives with superior antifungal potential (Ahmad *et al.,* 2015). The remarkable antifungal activity of these ETCs encouraged us to check their target site and mechanism of action. Therefore, for these tosylates we selected the sterol 14α-demethylase (CYP51) enzyme as a target in *C. albicans,* because it plays an essential role in sterol biosynthesis and is a principal drug target for a number of antifungals such as azoles. Molecular docking has been done to predict the binding-conformation of ETCs to CYP51 protein. The docking score reveals the binding affinity of the drug candidate to the enzyme active site. Our results demonstrated that among the test compounds ETC-5 had the best docking score and is efficiently involved in its high affinity to the active site of CYP51 protein, followed by ETC-6 and ETC-7 respectively.

Thus, based on antifungal susceptibility and molecular docking results we selected the three most active compounds (ETC-5, ETC-6 and ETC-7) for further studies. In addition to the *in silico* study, we further checked the *in vitro* effect of these selected ETCs on ergosterol biosynthetic pathway in *C. albicans* by quantifying the total intracellular sterol content and their effect on the *ERG11*, gene encoding sterol 14 α –demethylase. Our findings indicated a significant decrease in ergosterol biosynthesis in a concentration dependent manner, as well as down regulation in expression of the *ERG11* gene. There are several studies which

corroborate our results where eugenol and other natural compounds have been reported to target the ergosterol biosynthesis pathway in *C. albicans* (Ahmad *et al.,* 2010; Shreaz *et al.,* 2011). These results are also in line with our previous findings, where it was reported that ETCs with different functional groups reduced ergosterol biosynthesis by targeting lanosterol 14-α demethylase enzyme and also downregulates the expression of its related gene *ERG11* (Ahmad *et al.,* 2015).

After MFC determination, the fungicidal effect of these ETCs was further confirmed by viability testing using the MUSE Cell Analyzer. All the *C. albicans* cells after overnight treatment with test compounds showed drastic decrease in cell viability at their respective MIC values and exhibited >90% cell death in both fluconazole susceptible and resistant isolates of *C. albicans*. In the literature, fungicidal activity of eugenol against *C. albicans* has been found to be concentration and time dependent (Pinto *et al.,* 2009; Latifah-Munirah *et al.,* 2015). Our results for these ETCs are in line with these findings as the effect on viability was both concentration and time dependent. Pinto and colleagues reported that under the same experimental conditions, eugenol (2.5 µl/ml) induced cell death in 99.2% of the cells, and a conventional antifungal drug amphotericin B (2 µg/ml), that is fungicidal in nature, induced cell death in less than 20% of *Candida* cells (Pinto *et al.,* 2009). Eugenol also demonstrated the ability to disintegrate the cell wall and further decrease its permeability, which subsequently causes cell death (Latifah-Munirah *et al.,* 2015). This further strongly supports our findings, where ETCs demonstrated improved fungicidal activity in a dose dependent manner.

The development of new antifungal drugs is restricted due to the limited number of known drug targets in fungi. Thereby over utilizing of these targets is also contributing to the development of multidrug resistance in these species. Therefore, discovering new drug targets is also an important strategy to develop novel antifungal drugs. One such strategy is to target

virulence factors, which could be a new paradigm for the development of new and effective antifungal drugs. In line with that, we selected ETC-5, ETC-6 and ETC-7 to investigate their effect on major virulence factors of *C. albicans* such as adherence, morphological switching, secretion of tissue damaging hydrolytic enzymes, biofilm formation and also on expression of genes associated with these virulence factors (*ALS1*, *ALS2*, *ALS3*, *ALS9*, *CPH1*, *HWP1*, *SAP1*, *SAP2*, *SAP3* and *PLB1*). Our results revealed that the ETCs significantly inhibited adherence, morphogenesis, biofilm formation and reduced extracellular secretion of hydrolytic enzymes (proteinases and phospholipases) of *C. albicans*. In addition, down regulation in expression of pathogenicity related genes was also observed. Thus, it was shown from the results that these test entities target virulence factors of *C. albicans* and prevent the commensal organism from becoming pathogenic. This is the first study, where derivatives of eugenol or other semisynthetic compounds have been tested against virulence factors of *C. albicans* and improved results have been obtained in comparison to eugenol, which has already been reported effectual in reducing pathogenicity of *C. albicans* by targeting its virulence factors (He *et al.*, 2007; Halbandage *et al.*, 2017; Raut *et al.*, 2013). Moreover, eugenol has been reported to downregulate the expression of hyphae related genes resulting in inhibition of hyphal formation in *C. albicans* (Haque *et al.*, 2016). All these previous findings where eugenol has targeted virulence factors of *C. albicans* and their genes further validated our results.

To further investigate in-depth mechanisms behind the antifungal activity of ETC-5, ETC-6 and ETC-7, the mode of cell death was determined by studying apoptosis. Induction of apoptosis in yeast cells is considered as an ideal model for the screening/development of novel antifungal agents that can target fungal-specific apoptotic regulators with minimum or no toxicity to human cells. In the literature, natural products and their derivatives such as lycopene, Coumarin, nerol, limonene, plagiochin E, *Ocimum sanctum* essential oil and its two major constituent's methyl chavicol and linalool have been shown to induce apoptosis in

yeast cells (Jia *et al.,* 2019; Khan *et al.,* 2013; Wu *et al.,* 2010; Khan *et al.,* 2014). Eugenol has also been reported to induce apoptosis after decreasing ergosterol biosynthesis in *Candida* cells (Khan *et al.,* 2013).

The results of this study revealed dose-dependent effects of ETCs on the major markers of yeast apoptosis which include phosphatidylserine externalization, DNA damage, mitochondrial depolarization and decrease in cytochrome c oxidase activity. At high concentrations, the evidence of necrosis from the results of FITC Annexin V/PI-staining signifies lethal, rapid and irreversible antifungal action of the tested compounds. This might originate from their damaging effects on the structural and functional integrity of membranes, as discussed above. The results from TUNEL assay, where dose-dependent DNA damage has been observed again confirmed the results of FITC Annexin V/PI-staining. Moreover, ETCs significantly decreased mitochondrial membrane potential and cytochrome c oxidase activity in *C. albicans* cells, thus suggesting induction of apoptosis through intrinsic pathway which was further supported by the lipophilic nature (cLogP= 3.87-5.24) of these compounds. The intrinsic pathway of apoptosis is mitochondrial mediated and in yeast apoptosis the consequence of mitochondrial involvement is well studied (Guaragnella *et al.,* 2012). Cytochrome c is present as loosely and tightly bound pools attached to the inner membrane by its association with cardiolipin. Peroxidation of cardiolipin disrupts this interaction and generates a soluble pool of this protein. Furthermore, outer mitochondrial membrane permeabilization by Bax (from the Bcl-2 gene family) is needed to allow the release of cytochrome c into the cytosol. After its release from the mitochondria into the cytosol, it leads to the activation of yeast metacaspase Yca1p (the only caspase which plays an essential role in yeast apoptosis and is an ortholog of mammalian caspases), which in turn results in the activation of a caspase cascade inducing apoptosis. This is observed most commonly with such forms of cell death in yeast. The release of cytochrome c from the mitochondria into the cytosol directly correlates with a decrease in cytochrome c oxidase activity. The involvement

of cytochrome c in metacaspase activation has also been suggested (Silva *et al.,* 2005). Several studies have reported that release of cytochrome c is associated with yeast apoptosis (Ludovico *et al.,* 2002; Yang *et al.,* 2008).

Our results demonstrated potent apoptotic effect of these test entities in *C. albicans* cells, even at low concentrations. The significant mitochondrial depolarization, DNA fragmentation and decrease in cytochrome c oxidase activity suggested that these compounds possibly involve the mitochondrial routes for their mechanism of action to induce apoptosis, most probably by the release of cytochrome c which then activates metacaspase-dependent apoptotic pathway.

For the development of a successful pharmaceutical preparation, minimum or no toxicity is primary requirement for new molecules to further proceed. In this regard, we checked the cytotoxicity of the test compounds (ETC-5, ETC-6, and ETC-7) by *in vitro* haemolytic assay using horse RBCs. From the results, it was detected that test compounds at their different concentrations exhibited cell haemolysis in the range of 1.98% to 15.18%, which confirmed their low toxicity, and suggesting that they are potentially safe to use for further animal studies.

Conclusion

The findings of this study confirmed that tosylation of eugenol resulted in derivatives with superior antifungal potency and had multi-target mechanisms of action. The overall effect of these test compounds at both biochemical and molecular level was dose-dependent. Throughout the study ETC-5 was the most active derivative followed by ETC-6 and ETC-7 against both fluconazole susceptible and resistant isolates of *C. albicans*. These ETCs diminished virulence attributes of *C. albicans* by targeting its major virulence factors at inhibitory and sub-inhibitory concentrations. The antifungal activity of these compounds is related to their ability of inhibiting ergosterol biosynthesis and subsequent induction of apoptosis at lower concentrations and necrosis at higher concentrations. For the induction of

apoptosis in *Candida* cells, these congeners actuate metacaspase-dependent apoptotic pathway. Furthermore, low cytotoxicity effect associated with these ETCs advocate their further use for *in vivo* studies. All these findings suggested that these newly synthesized ETCs have the potential to be developed as new antifungal agents with multi-target mechanisms of action for the treatment of *Candida* and other fungal infections to overcome drug resistance.

Limitations

This study was an *in vitro* study, and therefore *in vivo* efficacy of these ETCs remained a limitation. The compounds were tested for their antifungal activity only against *C. albicans* however, other non-albicans *Candida* species should have been included to evaluate the broad-spectrum of their antifungal activity. Further, the inadequate number of *C. albicans* isolates used remains a limitation in this study.

Due to the limited availability of funds in the project, only a few assays associated with yeast apoptosis were performed. Other related assays may further reveal the in-depth mechanisms of apoptosis targeted by these ETCs. In addition, horse red blood cells were used to check the cytotoxicity of these test compounds due to its low cost and easy availability, however human cell lines are more reliable for *in vitro* cytotoxicity studies.

Future Research

Future testing using animal models is required to understand the in-depth antifungal mechanisms of these tosylates, which may take these compounds to the next step of drug development. Increasing the number of test isolates, including other non-albicans *Candida* species and large-scale genetic analyses for genes which encode different virulence factors of *C. albicans* may provide more information and better understanding about the antifungal effect of these tosylates. The compounds induced apoptosis in *C. albicans* cells, which could be one of the important factors behind their antifungal activity, however further in-depth

study related to apoptosis by targeting other possible apoptotic pathways will be an important study.

Furthermore, drug combination studies are widely used for the treatment of various diseases, thus combination studies of these ETCs with known conventional antifungal drugs will be an interesting study. To further explore the safety of these ETCs, use of human cell lines and different methods for cytotoxicity studies will be helpful.