5. DISCUSSION

The epidemiology of dermatophytosis has changed significantly. All races are usually affected and the clinical varieties and prevalence appear to reflect changes in socioeconomic conditions, lifestyles and migration mainly on environmental factors. There are evidence that predominance of species of dermatophytes not only differs from region to region but may change with the passage of time (Naseri et al., 2013). Due to its contagious nature, an early diagnosis is needed to control transmission of these infection. Tamil Nadu being as a tropical state, the climatic conditions of three cities of the Salem zone vary from moderately dry to dry consisting primarily of farmers and a large proportion of the workers or laborers particularly working in granites, handlooms and poultry. Besides the climatic conditions favorable for the sustained growth of dermatophytes, other factors such as the migration of laborers and workers, overcrowding, unhygienic life style of the community might contribute to the development of dermatophytosis in this region of the state.

5.1 DETERMINATION OF THE PREVALENCE OF DIFFERENT CLINICAL PATTERN, AETIOLOGICAL AGENTS AND PREDISPOSING (RISK) FACTOR OF DERMATOPHYTOSIS

The present study comprised a total of 168 patients (114 male and 54 female) attending the outpatient Departments of three dermatologic clinics in and around Salem District, Tamil Nadu, India over a period of one year from January 2013 to December 2013. All clinically diagnosed cases of dermatophytosis in all age groups and of both sexes were taken for the study and patients who were already on treatment for dermatophytosis were excluded from the study. Out of the 168 cases, 153 skin scrapings were collected along with 8 hair and 7 nail clipping samples.

The males were most predominantly affected as the dermatophytosis was observed in 114 cases (68%) when compared to females in 54 cases (32%) and the male to female ratio was found to 2.1:1 which was similar to the studies of Sahai and Mishra, 2011; Balakumar et al., 2012; Jha and Murthy., 2013; Bhatia and Sharma, 2014; Hitendra et al., 2012; and Agarwal et al., 2014 The supremacy of these infections in male was extravagant due to the fact of high exposure to the
outdoor environment such as higher physical activity leading to excess of perspiration in a hot humid climate, more prone to trauma, warmth and occlusive footwear and maceration effect of hyperhidrosis in comparison to females. Some hormonal factors may also predispose to infection; the female hormone progesterone was an effective inhibitor of fungal growth while the male dihydrotestosterone is an effective inhibitor of progesterone binding site. The lower incidence in females may be also due to the non-reporting of the female patients to the hospitals due to the prevailing social stigma in the rural population in India.

The highest incidence was seen in the second and third decade of age group (15-30) with 67 cases (40%) with a mean of 23 years followed by 30-45 in 49 cases. This result coincided with the studies of Sarika et al., 2014; Surendran et al., 2014 and Sonth et al., 2013. The probable reason for higher prevalence in this group could be that the individuals in this group are often most active because of their involvement in the outdoor activities such as studies, jobs etc. Post pubertal changes in hormones resulting in acidic sebaceous gland secretions are responsible for decrease in the incidence with age.

The occupational predisposition showed students as the predominant group affected by dermatophytosis comprising 39 cases (23%) followed by 38 cases of housewives (23%) may be due to increased wet work and heavy physical work which predisposes 26 cases of agricultural workers (15%) to excess perspiration in a humid environment. The higher incidence of student community was due to certain facts such as low level of parental education because parents are liable to minimize or completely exterminate the high occurrence of dermatophytes from the student population as it was their obligation to train their wards on the fundamentals of hygienic and healthy living (Metintas et al., 2004). The student hostile environment and living in dormitory contributive for facile spread of dermatophytes because of the rooms often flooded beyond their normal bearing capacity, thereby fostering firm body contact. Despite the contact, the hygienic requirements of the student hostels are inferior as so many of them are made to share inadequate toilet facilities and of course the habit of sharing clothing and items such as combs, hair pins, towels, shoes and beddings, predisposing them to fungal infections.

The co-morbidity of these infections was noted in 7% of clinically diagnosed cases. The commonest associated condition being diabetes mellitus in 5 cases(3%)
followed by rheumatoid arthritis and hypertension which was contrary to the study of Ghosh et al., 2014 who reported atopy as the primary co-morbid condition. Besides, to colonize a keratin-covered skin, fungal agents need to cross a natural barrier which was immunocompromised and deeper epidermal layers are not able to activate the immune response against infections, as both mechanisms are impaired in the skin of Diabetes Mellitus patients (Foss et al., 2005). Diabetic patients may present with complications involving all systems of the body. Fungal nail infection can contribute to the severity of diabetic foot and may be a major cause of morbidity in feet of diabetic patients, and the presence of mycotic nail may result in infection to the adjacent nail or skin injury and provide a reservoir of pathogen fungi (Avila et al., 2011). Although the primeval role of the drug was anti-inflammatory it also plays a crucial part in immunosuppression which depresses the immunity and increases the frequency of the fungal infections like tinea versicolor, tinea cruris developed earlier in patients treated with methotrexate than paronychia after commencement of treatment in rheumatoid arthritis patients (Douri, 2007).

Tinea corporis (32%) was the most predominant clinical type in 54 cases followed by tinea cruris (n = 39, 23%). The result of the present study were similar to the studies of Pandey and Pandey et al., 2013; Bhatia and Sharma, 2014; Singh et al., 2015; Bhagra et al., 2014; Agarwal et al., 2014; Sonth et al., 2013. However, an exception was found in the studies of Shama et al., 2015 and Ghosh et al., 2014 who reported T. cruris and T. unguium as the predominant clinical types. The main reasons behind the high prevalence of T. corporis were severe itching, cosmetic appearance and due to fear of early leukoderma which induces the patient to seek medical advice. Tinea conditions were acquired as the consequence of exhaustive physical work and prolonged exposure to sun leading to excessive sweating. T. capitis rarely occur in normal healthy adults. This may be due to increased fungistatic triglyceride content of sebum in adults. Some authors associated low incidence of Tinea capitis in India with use of hair oil.

The annular type of clinical presentation was found prevalently in 58 cases (34%). In the case onychomycosis, distal lateral subungual onychomycosis accounted for 6 (3%) cases followed by total dystrophic onychomycosis in 2 (1%) cases. Among the T. capitis kerion infection was found in 3 (2%) cases while grey-patch in 2 cases (1%) which was controversial to the study of Mane et al., 2013 who reported grey-patch T. capitis in higher incidence.
Distribution of the dermatophytes varies with the geographical area and during the course of time leading to a change in the spectrum of dermatophytic isolates. Out of the 168 samples, 105 (62%) samples were culture positive and 24 (14%) samples were culture negative. Among the culture positive cases, dermatophytes accounted for 81 (48%) cases, which included 70 (86%) Trichophyton, the predominant species, followed 8 (10%) Microsporum and 3 (4%) Epidermophyton species. Amidst the Trichophyton species, Trichophyton rubrum was the predominantly isolated in 19 cases (23%) followed by Trichophyton mentagrophytes var interdigitale (16%). Several reports from various parts of South India have also shown Trichophyton as the commonest genus and T. rubrum as the commonest species (Hanumanthappa et al., 2012; Balakumar et al., 2012; Madhavi et al., 2011; Kumar et al., 2012; Surendran et al., 2014; Sonth et al., 2013; Sumathi et al., 2013; Lakshmanan et al., 2015; Doddamani et al., 2013 and Sarada and Kumari, 2015. The reason for overall high isolation of T. rubrum from most of clinical variants is. T. rubrum has an affinity for inhospitable and tough keratin, like that of palms, soles and nails as no age group is spared by this organism. This species is highly communicable with remarkable adaptability. Among the non-dermatophytic molds (NDM) isolated in this study Aspergillus spp. (23%) was the predominant species. These molds reported in this study not only causes superficial infections but if untreated can develop in to serious and sometimes causes fatal mycosis (Warnok, 2006). Hence, these nondermatophyte moulds should always be kept in mind while investigating and treating a case of dermatophytosis and the common practice of discarding them as contaminant should be avoided.

5.2 COMPARISON OF THE EFFICACY OF POTASSIUM HYDROXIDE (KOH) MOUNTS IN RELATION TO THE CULTURE FOR RAPID DIAGNOSIS OF DERMATOPHYTOSIS

Direct microscopic examination of KOH-prepared material was a simple and cheap method used in the diagnosis of mycotic infections, possibly which requires minimum technical aids. When compared to mycological culture, KOH mount had the ability to detect more fungal agents from clinical specimens. KOH smear and fungal culture to be complementary laboratory exams, with higher sensitivity in the former test and higher specificity in the latter. KOH smear serves as a good screening test for determining presence of disease, both before and at the end of therapy. The fungal culture, which can take up to three weeks to become positive,
can serve as a more specific confirmatory test. The costs of empiric treatment without either laboratory test include the risk of missing an alternate diagnosis and the cost of medication used to treat a non-existent tinea infection which suggests that this routine test has high sensitivity compared with culture.

In this study, the KOH mount had a sensitivity of 81% which was higher than the study of Sarika et al., 2014 (73%) and specificity of 79% with a positive predictive value of 93% and a negative predictive value of 56%, a false positive rate of 44% and a false negative rate of 0.13 %, when used as a diagnostic aid for dermatophytosis. In a similar study, Dass et al., 2015 reported the positive predictive value of KOH was found to be 60%, negative predictive value of KOH was found to be 88%. Out of 168 cases, direct microscopy was positive in 113 (67%) cases and culture was positive in 129 (77%) of cases which was contrary to the study of Shrihari et al., 2012 who insisted 80-85% of fungal infections have been diagnosed based on the findings of microscopy alone and there was agreement ranging from 70-80% between direct microscopy and culture. 105 (62%) cases were both KOH and culture positive. 8 (5%) cases were KOH positive but culture negative whereas 24 (14%) cases were KOH negative but culture positive. 31 (18%) cases were both KOH and culture negative. The results obtained by microscopic examination by KOH were in agreement with those of culture.

An assessment of the merits of KOH as a diagnostic tool for dermatophytosis revealed that the KOH mount remains as the standard method for rapid diagnosis of dermatophytosis. This method does not require any sophisticated equipment and it is less cost. This microscopic method is simple and reliable. The culture negative results may reflect the administration of an antifungal treatment initiated before sampling as some patients may have not given incorrect information on their receipt of antifungal treatment. High false negative results were reported in various studies and this may vary depending on the experience on the mycology laboratories and their isolation methods. High false negative results in mycological culture may be due to various other factors such as non-viability of certain fungal agents, inappropriate or insufficient sample collection (Nail clippings collected distal to the fungal disease) and insufficient crushing of clinical material before subjecting for test.
5.3 PRELIMINARY SCREENING OF THE DERMATOPHYTES FOR PROTEASE ENZYMES ON VARIOUS MEDIA CONTAINING DIFFERENT PROTEIN SUBSTRATE

Proteinases are produced by dermatophyte in vitro and play an important role in the pathogenesis of fungal infections *in vivo* thereby enabling them to parasitize tissues such as the stratum corneum, nails and hair (Singh, 2014). Regarding the role of keratinases in the hydrolysis of proteins, two closely linked mechanisms appear to be operative: (1) mechanical penetration of mycelium into keratin substrates and (2) enzymatic degradation by the keratinases in the cell walls (Singh, 1997). The keratin, introduced as a source of carbon and nitrogen to the mineral agar medium, allows a quick selection of active strains, providing additional information about the character and localization of the enzyme in the cell. Native keratin contained in hairs or feathers did not constitute such a universal source of carbon and nitrogen for dermatophytes as the preparation of keratin substrate employed a suitable method for acting as the substrate for enzyme production. In this study along with the keratin substrate, casein and gelatin were used as protein sources.

In qualitative screening, out of 81 dermatophytic isolates, 32 isolates showed a zone of clearance around the colony in keratin media while 26 showed positive results for the preliminary screening of protease on gelatin-peptone media and 23 in the casein agar plates. *T. rubrum* was the highest protease producer (30%) on three of the media especially on keratin media (25%) followed by *T. mentagrophytes var interdigitale* (20%), *T. tonsurans* and some of the Trichophyton species (17%). The keratin followed by gelatin-peptone and casein were the best nitrogen sources for protease production by *T. rubrum* which was controversy to the report of El-Said in 2002, who insisted the utilization of peptone as a best source for protease production. Abdel-Hafez *et al.*, 1995 also reported casein followed by gelatin and ammonium nitrate were the best nitrogen sources for protease production by *T. soudanense*.

In quantitative analysis using mineral medium containing raw chicken feathers as substrate, out of 26 isolates screened only one strain K-42 *T. rubrum* showed the highest mean proteolytic activity 44.17 KU/ml and the enzyme activity reached at its peak on the 10th day of incubation while Gokulshankar *et al.*, 2010 reported enzymatic activity among different anthrophilic dermatophytes in which *T.*
rubrum showed high enzymatic activity in between 16- 20 days of incubation. Venkatesan et al., 2010 reported that the T. rubrum recorded the 25.75 KU keratinase activity among the tested organisms on the 20th day. Mahmood et al., 2008 reported T. mentagrophytes had ability to produce extracellular protease which was indicated by proteolysis activity 111.9 U/ml against casein substrate. Whereas the proteolytic activity of T. rubrum NKL69 used in this present study was found to be 121.4 KU/ml which utilized keratin as a substrate. The presence of protease was evidenced by the hydrolysis of the chicken feather keratin employed in the medium. This is mainly due to the classical nature which facilitates obligate parasitism and complete anthropization of T. rubrum. (Venkatesan et al., 2010). Although there are studies on proteolytic activity using various substrates use of chicken feathers and report of characterisation of proteases of T. rubrum in this region is the first report.

The present experiments indicate the possibility of applying modified keratin of chicken feathers or keratin substrate as a very useful model for a preliminary estimation of keratinolytic activity of dermatophytes. This keratin, introduced as a source of carbon and nitrogen to the mineral agar medium, allows a quick selection of active strains, also providing additional information about the character and localization of the enzyme in the cell.

5.4 GENOTYPIC IDENTIFICATION AND PHYLOGENETIC ANALYSIS OF THE STRAIN SHOWING THE HIGHEST PROTEOLYTIC ACTIVITY

Molecular techniques can be used as an epidemiological tool for the detection of dermatophytes. Therefore, an accurate identification of dermatophytes at the species or the strain levels can be done best by using molecular methods for epidemiological surveys. The dermatophyte specific primer based PCR targets the 18S rDNA is useful in the direct identification of dermatophytosis from clinical specimens and it can be applied in the routine diagnostics wherever the laboratory facilities are adequate (Elavarashi et al., 2013). The sequencing result of 18S Ribosomal DNA of Trichophyton rubrum showed that 640 nucleotide base pairs which included ITS1, 5.8S ribosomal RNA coding gene and ITS2. The BLAST result showed that the obtained sequence showed 100% similarity with T. rubrum 18S ribosomal DNA and hence the strain K-42 was confirmed as T. rubrum UM1. The phylogenetic evaluation with other Trichophyton spp. showed that the Trichophyton rubrum UM1 showed 98% bootstrap with T. rubrum which was similar to the study
of Nascimento and Martinez-Rossi, 2001) who partially sequenced 18S rDNA comprised 604 bp, of T. rubrum.

5.5 DETERMINATION OF METALLOPROTEINASE (MEP1-5) AND SUBTILISIN (SUB1-7) GENES AND SEQUENCE ANALYSIS OF Trichophyton rubrum STRAIN UM1

Multiplex PCR amplified product showed approximately 1400bps in size. The sequence result showed that the amplified product comprised of 1452 nucleotides. The Blast result showed that the obtained sequence showed similarity to Trichophyton rubrum Subtilisin gene type 3. The SUB3 was placed in the phylogenetic clade of dermatophytes.

To the best of our cognizance, this study is the first one to reveal the subtilisin gene SUB3, a serine protease in an anthropilic dermatophyte, T. rubrum strain UM1 in Tamil Nadu. The same gene was also reported by Burmester et al., 2011 in the secretome analysis of Arthroderma benhamiae in which three of the subtilisin-like serine proteases (SUB3, SUB4, and SUB7), three fungalysin-type metalloproteases (MEP1, MEP3, and MEP4), the leucine aminopeptidases Lap1 and Lap2, as well as the dipeptidyl-peptidases DppIV and DppV were detected in the secretome. Likewise, Leng and Wei, 2011, identified and quantified more than 90 proteins in secretome of T. rubrum which suited to proteases such as subtilase and metalloprotease, peptidases such as leucine aminopeptidase, and cell wall remodeling related enzymes such as endochitinase and beta-glucosidase. Similarly, the genes, which coded subtilisin-like proteases was identified and the homology study was determined by Moallaei et al., 2009 as the obtained nucleotide as well as amino acid sequences indicated different rates of homology with other subtilisin-like proteases genes in other pathogenic dermatophytes. Lemsaddek et al., 2010 studied a high diversity of virulence gene profiles in 86 dermatophytes and the presence of at least one gene was revealed in 91% of the dermatophytes, with 44% and 27% being positive for the five MEP and seven SUB genes, respectively.

The data shown by Tarabees et al., 2013 showed that, the incidence of MEP1-4 was only 10% of the screened samples, while it was 20% for MEP5. A total of seven genes encoding putative serine proteases of the subtilisin family (SUB) were isolated in T. rubrum. Based on sequence data and intron-exon structure, a phylogenetic analysis of subtilisins from T. rubrum and other fungi revealed a
presumed ancestral lineage comprising *T. rubrum* SUB2 and *Aspergillus* SUBs. All other SUBs (SUB1, SUB3–7) were dermatophyte-specific and have apparently emerged more recently, through successive gene duplication events. Chen *et al.*, 2010 investigated the different expression patterns of *T. rubrum* secreted endoproteases genes and the results indicated that SUB7 and MEP2 were the dominant endoproteases. Bagut *et al.*, 2012 first revealed the expression of the keratinolytic protease SUB3 in *M. canis* arthroconidia produced *in vitro*. These data supported previous hypotheses concerning the major role of SUB in the earliest events of the infectious process, i.e. adherence to corneocytes.

5.6 PREDICTION OF PROTEIN STRUCTURE AND FUNCTION OF THE SUBTILISIN3 (SUB3) GENE THROUGH INVESTIGATION OF SIGNAL PEPTIDES, CATALYTIC TRIADS AND PROTEIN STRUCTURES BY 2D AND 3D PREDICTION TOOLS

Advancements in decoding the DNA transformations through transcript profiling and computational analysis had single-handedly catapulted the field of fungal pathogenesis into the molecular era. Comprehending the molecules mediating interactions at the host-fungal interface is crucial to building new approaches for treatment and management of fungal infections. Consistent with this hypothesis, during in vitro cultivation with keratin as the sole source of carbon and nitrogen, dermatophytes were proven to secrete multiple proteases, the potential virulence determinants. The implementation of bioinformatics tools, will furnish us with a working knowledge of host-pathogen interactions and the immune evasion strategies adopted by pathogenic organisms, which will in turn guide the development of therapeutics or vaccines.

The sequence alignment with various SUB3 genes result showed that the Open reading frame with 1392 nucleotide base pairs including 4 exons and 3 introns coding for SUB3 proteins. The protein translation result of this study gave one open reading frame comprising of 389 amino acids from exon regions and the signal peptide sequence consist of 20 amino acids coded by 60 nucleotides and a pI of 10.21. which was contrary to the study of Cruz *et al.*, 2010 identified a similar sub3 keratinase transcript during growth of *T. rubrum* in keratin cultures whose *TrSUB3* gene coded to a protein with 397 amino acid residues (41 kDa) and a pI of 8.51. In an another study reported by Moallaei *et al.*, 2009 sequenced DNAs contained 3 open reading frame of approximately 1095bp in *T. vanbreuseghemii*
and 1082 in *M. gypseum* encoding a 358 amino acids protein in *T. vanbreuseghemii*
and a 333 amino acids protein in *M. gypseum*. Thus, from this point, it was
confirmed that protein sequence of each protease varies with ecology and
epidemiology. Jousson *et al.*, 2004 also reported a SUB3 of *T. rubrum* was 1415
gene length with 3 coding regions, comprising 281 amino acids with 19 amino acids
in its signal peptide and a molecular mass of 28 kDa.

The aminoacid sequence alignment with other SUB3 genes result showed
that the catalytic triads Aspartic acid-Histidine-Serine is present in the 149, 221, 430
of the nucleotide positions respectively. The alignment of deduced translation
products showed that all *T. rubrum* Sub sequences display the catalytic triad (for
instance Asp158/His190/Ser345in Sub1) and the motifs around these residues
characteristic of proteinases of the subtilisin family. Among *T. rubrum* Subs, the aa
sequence identity ranged from 29.4% (between Sub2 and Sub6) to 67.4% (between
Sub3 and Sub4). The previously characterized N-terminus of mature Sub3 from *M.
canis* (Mignon *et al.*, 1998) and the analysis of the sequence alignment suggested
that dermatophyte Subs would be synthesized as preproproteins with a propeptide
of 96 to 103 amino acid residues. The mature domain generated after cleavage of
the prosequences predicted a molecular mass ranging from 28.2 to 39.7 kDa.

The molecular formula for Sub3 was found to C_{1969}H_{3074}N_{572}O_{515}S_{18}. The 2D
and 3D structures revealed that the protein contains 7 numbers of alpha Helix and
17 numbers of beta pleats. The catalytic triads aspartic acid is located in 2nd alpha
helix, histidine is located at 2nd beta pleats and the serine is located at 5th beta
pleat. Monod *et al.*, 2005 described the putative catalytic triad of the serine
proteases as Ser613, Asp690, His725 (DppIV) and Ser558, Asp641 and His673
(DppV).

The protein subtilisin protease contained 5 predicted pockets whose
multiplicity represents the frequency with which the selected pockets found in a set
of ligand-binding protein structures. The MG ligand of pocket 1 in domain 2 showed
the highest pocket multiplicity (76) with binding site residues at S35, I37, F78, N87,
R110, C111 followed by CA ligand in pocket 1 of domain 1 whose multiplicity was
69 with binding site residues at P311, P313 and R336 positions. The least
multiplicity (20) was found in 3rd pocket of DU ligand with V39, K41, D42, N81, G82,
W83, R84 and R106. The ProDom analysis results showed that the enzyme Sub3
was closest to the domain SUB3_TRIRU 345-393 Protease serine hydrolase with 100% identity confirming the Subtilisin protease family. The signal peptide was present between the 27th and 28th position of the aminoacid sequence. The phosphorylation sites were found at 10 cleavage sites of serine along with 9 threonine cleavage sites. There was no phosphorylation sites presented at tyrosine cleavage sites. Pannkuk et al., 2015 in a similar study reported the specific order of Asp160-His192-Ser345 catalytic triad (for family S8 serine endopeptidases) and two predicted calcium-binding sites in PdSP1 (C1: K294, A296, D319; and C2: G132, S135, H136) in a subtilisin-like serine protease secreted by the bat pathogen Pseudogymnoascus destructans. Cruz et al., 2010 also predicted the novel biochemical functions of the Sub3 keratinase from T. rubrum The CombFunc results of Sub3 protein was predicted as this enzyme exhibited serine-type endopeptidase activity in the proteolysis mechanism thus involving in pathogenesis and binds to host IgE thus prohibiting the active evasion host immune response via regulation of host complement system.

5.7 MOLECULAR MODELLING OF SUB3 PROTEIN OF T. rubrum UM1

The best method for predicting the structure of a protein depends on whether it has sequence homology to a protein of known structure. If there was such a similarity, relatively accurate models can be built using the known structure as a template. In the absence of such similarity, models can be built using de novo prediction methods, which do not rely on a template structure. Similar approach was carried out in the present study due to the low similarity with the PDB database structure, a stumpy query covered modeled structure was obtained and hence the structure models were generated using Robetta server the structure prediction was made by using the de novo Rosetta fragment insertion method. The geometrical and structural consistencies of the modeled Sub3 protein was carried out by PROCHECK. This analysis revealed that only two residues (0.7%) in the Ramachandran plot of subtilisin protein falls under the disallowed region while 86.0% of the residues were in the most favored region, 12.7 % of the residues were in additionally allowed region and 0.7% of the residues were in generously allowed region. Overall, the modeled structure was reliable since 86% of the residues falls in the most favored region which was controversial to the study of Wadood et al., 2014 whose Ramachandran plot revealed that 81.1% of residues are in favored region, 13.3% are in additionally allowed region and 5.6% are in outlier region.
Elengoe et al., 2014 also evaluated the backbone psi and Phi dihedral angles of the NBD which revealed that 81.7%, 15.4%, 2.1% and 0.9% of residues were falling within the most favored regions, additionally allowed regions, generously allowed regions and disallowed regions (ASN33, ASP44 and ASP97) respectively. In general, a score close to 100% implies good stereo-chemical quality of the model and therefore, these PROCHECK results suggest that the predicted model was of good quality.

5.8 VIRTUAL SCREENING OF PHARMACOPHORE MODELS FOR TARGET SUB3 PROTEIN

The history of drugs intended to conceal protease activity dates back to the 1950s. Many new protease inhibitors were currently in development, with at least 50 different proteases being considered as potential targets. Many of these targets were used to screen for inhibitors, whereas others were rationally developed using structure-based drug design (Turk, 2006).

Docking runs were performed with Glide in Extra Precision (XP) mode against Maybridge, ChemBridge and Allium databases (Vitas ML) both in presence and in absence of constraints involving (i) an hydrophobic region corresponding to the side chain of residues (ii) an aromatic site placed on the side chain and (iii) a hydrogen bonding group to mimic the hydrogen bonding properties. The top ranked molecules (hits) yielded a list of four small molecules in Vitas ML Allium database, five lead molecules from Chembridge and one lead molecule in Maybridge database with glide score ranging from -9.76 to -7.29. The lead compound 12799 from the Maybridge database posed the highest glide score value -9.76. with the glide energy of -51.91 possessing side chain and backbone hydrogen bonds interactions. From the screened compounds, top five compounds was chosen from each databases totally 15 compounds on the basis of their Glide XP score, mmGBSA score and interactions. The highest the negative value of mmgbsa was directly proportional to the free binding energy. Frequencies of the vibrational modes were computed at 300 K for these minimized structures including all snapshot atoms and using a harmonic approximation of the energies. The energy contributions to the free energy of ligand binding of the novel lead compounds. The five final docked conformations obtained for the different inhibitors were evaluated based on the number of hydrogen bonds formed and bond distance between atomic co-ordinates of the active site and inhibitors. All the compounds formed
hydrogen bonding and pi stacking (non-covalent interactions between aromatic rings) which were the important factors in rational drug design. The active lead compounds showed hydrogen bond and hydrophobic interactions with active site residues of Sub3 such as Trp217, Ala343, Asn62, Asn60, Met277 and Ala244. These top ten lead compounds mainly preserved the Trp217 and Ala343 interaction and these interactions has been exploited in the rational design of novel Sub3 inhibitors except for Chembridge_86971799 which preserved Asn62 and Chembridge_ 10582039 Asn60 respectively along with Trp217. Thus, these interactions may play an important role in SUB-3 inhibition. Hydrogen bonds formed between the compound and the protein usually contribute to the stability of the protein-ligand complexes; a large number of hydrogen bonds form more stable complexes. Similarly, in the study of Elengoe et al., 2014 the active residues of the T204V mutant (Thr13, Thr14 and Arg72) were also involved in the formation of hydrogen bonds, suggesting the protein (T204V) forms a more stable complex than the NBD and K71L. Susithra et al., 2014 obtained glide score ranging from -7.85 to -2.51 which showed good hydrogen bond interactions with PL II against lichen metabolites atranorin, lecanoric acid, salazinic acid and dibenzofuran (usnic acid) derivatives showing effective H-bond interactions and hydrophobic contact with the active residues viz. Thr217, Gly36, Ser79, Asp214, Ser218, Tyr192, Gly216, and Tyr77 present in the PL II protein. Analogous to these studies, Gupta et al., 2015 reported the interacted residues Leu26, Tyr29, Asn31, Asp33, Pro315 and Thr316 residues were involved in hydrogen bonding interactions with selected ligands and can be used as prominent active binding sites and which was common to the predicted active site.

Parallely, the analyzed results proved that the docking score was high for Protease with Dolichin A (-6.6899) and Dolichin B (-6.6944) among reverse transcriptase and integrase (Auxilia et al., 2013). The docking simulation of the most active chromone derivatives 9 toward poly [ADP-ribose] polymerase (PDB ID 3SE2) showed that the most enzyme–inhibitor complex was stabilized by hydrophobic interactions occurring between the aromatic moieties of the ligand and lipophilic residues of the binding site. In particular the compound 3 group was oriented towards the hydrophobic region lined by Ile857, Arg726, His523, His518, His513 and Ser510.(Shanthi et al., 2013).
Hierarchical clustering algorithm method was performed to select structurally dissimilar compounds in order to get more potent inhibitors. Fig 5 showed the process of hierarchial clustering framework. The hierarchical clustering started with the dataset of all sampled ligands for a complex 1 and uses fuzzy c-means (FCM) to divide the set into three subsets. Each ligand belongs to each subset with different probability degrees depending on its distance from a randomly chosen center (centroid ligand) of that cluster. Since there were three subsets, three centroids were selected that were not strongly biased to one subset or the other were removed from the three main partitions.

Most of drug candidates fail in clinical trials due to poor ADME properties. Thus, an important aspect of drug discovery is to avoid compounds not having drug likeliness and good ADME property. So to streamline the virtual screening, drug likeliness and ADME properties of all the thirty compounds were predicted using QikProp, version 3.4 of Schrodinger 2013. Lipinski filter and reactive filter were applied before virtual screening to avoid false positive lead molecule using Qikprop script of Schrodinger (Meraj et al., 2013). Lipinski filter rejected ligands not following Lipinski rule of five and reactive filter rejected ligands with reactive functional groups and in case non-violation of rule shoed the value of zero. Drug likeliness, log P, log S, molecular weight and toxicity risks may be used to judge the compound’s overall potential to qualify a ligand as potential drug candidate. In order to streamline the virtual screening, drug likeliness and ADME properties of all the ten lead compounds were predicted using QikProp, version 3.4 of Schrodinger 2013. Lipinski filter and reactive filter were applied before virtual screening to avoid false positive lead molecule using Qik prop script of Schrodinger. All the structures showed significant values for the properties analyzed i.e. in the acceptable range and showed drug-like characteristics based on Lipinski’s rule of 5. Molecular weight of most of the ligand falls within the range of 252-478 Daltons.

The properties were based on Lipinski rule of five, molecular weight (mol_MW) less than 650, partition coefficient between octanol and coefficient between octanol and water (QlogPo/w) between -2 and 6.5 and solubility (QPlogS) between -6.5 to 0.5, PHOA greater than 80%, brain/blood partition coefficient (QPlogBB) parameter between (–0.2 to -1.6 ), predicted skin permeability (QPlogKp) between -0.4 to -3.5 indicated the mandatory for the unique properties of a novel drug. The efficiency to cross blood brain barrier and high percentage of
human oral absorption provided the lead compounds more impetus over other drugs. Each of the lead compound obeyed Lipinski rule of five and three violations

Docking studies in *T. rubrum* have not been conducted till date, to the best of our knowledge; hence, molecular docking was performed for the Sub3 of the organism. As these top ten lead compounds mainly preserved the Trp217 and Ala343 interactions, making it a highly lucrative antifungal target in Sub3 of *T. rubrum*. Moreover, existing literature on the subtilisin protease enzyme revealed that the enzyme was one of the vital importance to fungal virulence and pathogenicity.