3. MATERIALS AND METHODS

3.1 GENERAL

The samples of skin, hair, and nail clippings were collected from patients suspected with dermatophytosis for this clinical condition attending the outpatient Departments of dermatologic clinics in and around Salem District, Tamil Nadu, India, over a period of January 2013 to December 2013. Patients presenting in this study had not previously undergone any treatment for their condition. This study was approved by the institutional ethics committee for the enrolment of patients (Ref. No: IEC/PU/HR/2012/002). A total of 168 samples which included skin scraping, hair clipping and nail clipping were subjected to microbiological evaluation.

3.2 HISTORY - TAKING AND CLINICAL EXAMINATION

The informed consent form was obtained from these patients prior to enrolling them for the study. The patient’s history and the clinical details were collected and recorded on a separate proforma meant for this study. A detailed clinical history of the enrolled patients was taken in relation to name, age, sex, address, occupation, duration of illness and involvement of more than one site. The clinical examination of patients was made in bright light which included site of lesion, number of lesions, types, presence of inflammatory margin, etc. The clinical details were also recorded on the proforma. The patients were selected based on the following inclusion criteria for this study.

Inclusion criteria

Patients presenting in this study should not have been on any prior treatment.

Exclusion criteria

Patients having superficial infection other than dermatophytosis and pregnant women.

3.3 MYCOLOGICAL INVESTIGATIONS

3.3.1 Collection of Specimen

The collection of clinical specimen (skin, hair and nails) for this study was done by following standard methods of Santosh et al., 2015.
a. Isolation of Fungi from Skin Scrapings

After cleansing the affected area with 70% ethanol, the skin specimen was collected by using a sterile No. 15 scalpel blade. The epidermal scales was scraped from the periphery of the lesions and was placed directly on the slide for direct microscopic examination. A sterile petridish or sterile black paper envelop for transport was used for transport of the specimen to the laboratory (plate 3.1).

b. Isolation of Fungi from Hair clippings

The infected hairs was removed by plucking with the roots intact using epilating forceps along with the base of the hair shaft around the follicle. Brush samples was used for culture of scalp infections. Scrapping was also performed from the scalp, to collect the specimen.

c. Isolation of Fungi from Nails clippings

The affected area was cleaned with 70% ethyl alcohol and the material from the nails was obtained by clipping or scraping from the undersurface of the nail plate. Ample subungual debris was obtained for direct microscopic examination and culture.

3.3.2 Direct Microscopy

The clinical specimen (skin, hair and nail) collected was subjected to direct microscopic examination by potassium hydroxide (KOH) wet mount by following the method of Singh et al., 2015.

a. Potassium Hydroxide (KOH) Wet Mount Preparation

A small portion of the clinical material was placed on a dust free glass slide and 2-3 drops of 10% KOH was added. A cover slip was placed over the specimen and the slide was gently heated. The slide was allowed to cool and ‘ripen’ for few minutes before examination. The slides were examined under bright field microscope first with low power (10X) and then under high power objectives (45X). For nail specimen 20% KOH was used and the slide was allowed to be kept in an incubator at 37°C for 1 hour for proper viewing. Hair specimen was examined as soon as after mounting.
3.3.3 Identification of the molds from clinical specimens (Choudhary et al., 2015)

The dermatophytes grown were identified based on macroscopic and microscopic features. The macroscopic features included the color of the surface and the reverse of the colony, the texture of the surface (powdery, granular, wooly, cottony, velvety or glabrous), the topography (elevation, folding, margins) and the rate of growth. The microscopic features included arrangement of spores, size (micro or macro), septation of conidia on the conidigenous cell, type of conidiation, chlamydospores, and hyphal modification (septate, spiral, pectinate bodies, favic-chandliers, nodular structures).

a. Isolation of dermatophytes by culture on different media

The clinical specimen was inoculated onto three sets of plates, one containing Sabouraud’s dextrose agar (SDA) (containing dextrose 40 g/l, mycological peptone 10 g/l and agar 15 g/l, deionized water 1L (pH 5.6) purchased from Himedia, Mumbai), Sabouraud’s dextrose agar (SDA) with 0.05% chloramphenicol (Himedia, Mumbai), Sabouraud’s dextrose agar with 0.05% chloramphenicol and 0.5% cycloheximide (Himedia, Mumbai) and dermatophyte test medium (DTM) (papaic digest of soyabean meal 10 g/l, glucose 10 g/l, phenol red 0.2 g/l and agar 20 g/l, deionized water 1L (pH 5.5±0.2) purchased from Himedia, Mumbai. The specimens was inoculated onto the petriplates containing these media and incubated at 28°C for upto four weeks, and was observed periodically for growth. If no growth was found after four weeks, it was taken as negative for the growth of dermatophytes. Dermatophyte test medium was incubated at 28°C for upto ten days and was observed for color change (Refai et al., 2013).

The culture was deemed significant if both the microscopy and the culture were positive. The assessment of non-dermatophytic molds was confirmed by repeated sampling and culture from the same site of infection in the patients.

Control

The sterility of the media was checked by inoculating unopened plates of each medium 28 - 30°C for up to two weeks. To rule out contamination of the media while inoculating the specimens, plates of solid media were periodically opened for
15 to 20 seconds and then incubated at the same temperature and for the same duration as the inoculated media.

b. Lactophenol Cotton blue Staining (LPCB) (Ellis et al., 2007)

The dermatophytic and non-dermatophytic cultures were examined microscopically by removing a portion of aerial mycelium and placing on the glass slide. To it a drop of lactophenol cotton blue was added and cover-slip was placed on it. The mount was observed under low (10X) and high power (45X) objective of microscope, for the presence of hyphae, macroconidia, microconidia and other accessory structures of the vegetative hyphae and the characters of each was noted.

c. Hair Perforation Test

This test was performed according Robert and Piheret, 2008 to distinguish between atypical isolates of *T. mentagrophytes* and *T. rubrum*, may also be used to differentiate *M. equinum* from *M. canis*. *T. mentagrophytes* and *M. canis* perforate hair whereas *T. rubrum* and *M. equinum* were negative for the test. *T. mentagrophytes* produce hair perforating organs that penetrate hair radially and cause wedge shaped perforation. A filter paper disk (approximately 90 mm in diameter) was placed onto the bottom of the sterile petridish and 10-15 ml of sterile distilled water was added to it. Infants’ hair was sterilized and placed on the filter paper. One percent yeast extract was added to it and the test fungus was inoculated on the hair strands. The petridish was incubated at room temperature and hair strands were examined weekly for a period of four weeks. The hair strands were examined under high power of microscope for wedge shaped perforations.

d. Slide culture

The non-sporulating dermatophytic mould which did not sporulate were subjected to slide culture by following the method of Chander et al., 2009. A microscopic slide was placed on a ‘V’ or ‘U’ shaped bent glass rod at the bottom of a petridish along with 1-2 coverslips and a filter paper. Petridish was closed with the lid, wrapped with craft paper and sterilized using hot-air oven. Blocks of (1 x 2 cms) Sabouraud’s dextrose agar with chloramphenicol and cycloheximide were cut to a depth of 4 mm using sterile scalpel blade. The block was transferred to the surface of the glass slide. The corners of agar block were inoculated with the fungal strains
to be identified. A cover-slip was placed on surface of agar block using sterile forceps. A few drops of sterile distilled water was added to the petridish and the lid was closed and incubated at room temperature. When the sporulation has occurred, the cover-slip was carefully removed from the agar block and placed on to the drop of Lactophenol Cotton Blue stain taken on a separate glass slide. Agar block from the original slide was carefully removed and decanted into 5% phenol solution. One or two drops of Lactophenol Cotton Blue stain placed on the slide with the fungal growth and cover-slip was placed on it. Both the slides were examined under the high power (40X) objectives microscope.

e. Biochemical Tests

Urease Test

This test was performed to differentiate between *T. mentagrophytes* and *T. rubrum*. Christensen’s urea agar slant (peptic digest of animal tissue 1 g/l dextrose 1g/l, sodium chloride 5 g/l, disodium phosphate 1.2 g/l, monopotassium phosphte 0.80 g/l, phenol red 0.012 g/l, agar 15 g/l, pH 6.8 ± 0.2 distilled water 1000 ml purchased from Himedia, Mumbai) was inoculated with the dermatophytic isolate. The change in color of media from yellow is indicative of positive (Robert and Pihet, 2008).

Bromocresol purple glucose milk solid glucose agar (BCP)

Bromocresol purple milk solids glucose agar (Solution A - skim milk powder 80 g/l, bromocresol purple (1.6% solution in alcohol) 2 ml, distilled water 1000 ml; Solution B - glucose 40 g, distilled water 200 ml; Solution C - agar 30 g, distilled water 800 mL pH 6.6) was a differential media useful in the characterization of dermatophyte species. Hydrolysis of the milk solids resulted in a zone of clearing around the colony (Kane *et al.* 1977).

3.4 COMPARISON OF THE EFFICACY OF POTASSIUM HYDROXIDE (KOH) MOUNTS IN RELATION TO THE CULTURE

The mounting medium was prepared as described by Singh *et al.*, 2015 containing 10-20% KOH solution. Nail specimens were slow to clear, the full thickness nail clippings was placed in a weak (5%) KOH solution in a watch glass for 24 hours to obtain adequate clearing. The rate of growth in culture was also observed and recorded. The sensitivity, specificity and predictive values of KOH mount as diagnostic aid for dermatophytosis were determined using isolation in
culture as the "gold standard" for the diagnosis of dermatophytosis (Levitt et al., 2010).

3.5 PRELIMINARY SCREENING OF THE DERMATOPHYTES FOR PROTEASE ENZYMES

3.5.1 Qualitative Screening of protease activity

The proteolytic activity of dermatophytes isolated during this study period was determined by using two different media’s namely casein agar and gelatin peptone agar respectively.

a. Casein Agar Media (Vijayaraghavan and vincent, 2013).

The casein agar plates (containing peptic digest of animal tissue 5 g/l, beef extract 1.5 g/l, yeast extract 1.5 g/l, sodium chloride 5 g/l, casein 10 g/l, agar 15 g/l, 0.0015% (w/v) bromophenol blue, deionized water 1000 ml, pH 6.8 ± 0.2) were prepared and sterilized by autoclaving. The dermatophytic molds were inoculated on casein agar plates in triplicates and incubated at 28°C for 7 days. After incubation, the colour change were observed. A known strain of T. rubrum MTCC 3272 with known proteolytic activity was used as a positive control.

b. Gelatin peptone agar (El-Said, 2002).

The dermatophytic molds was inoculated on to gelatin peptone agar (containing gelatin peptone 5 g/l, agar 15 g/l, deionized water 1000 ml with final pH 7.0 ± 0.1 purchased from Himedia, Mumbai) plates in triplicates and incubated at 28°C for 7 days. After incubation the plates were flooded with mercuric chloride solution and were examined for the appearance of uncolored zone, indicating the enzyme production. A known strain of T. rubrum MTCC 3272 with known proteolytic activity was used as a positive control.

c. Keratin Media

The keratinolytic activity of the dermatophytic isolates obtained during this study were determined by using solid and liquid keratin media respectively.

Preparation of soluble keratin

Soluble keratin protein was prepared from native chicken feather. About 10 g of chicken feather was dissolved with 500ml of DMSO by heating at 100°C for 2 hours. This solution was precipitated by 2 volume of cold acetone for 1 volume of
protein and the caseous precipitate of keratin protein (0.06%) and was suspended in 0.1 M phosphate buffer (Sharma et al., 2011).

**Proteolytic activity on solid keratin media**

The keratinolytic activity of the dermatophytic molds were examined on the solid mineral medium (containing MgSO₄·H₂O - 0.5 g/l; KH₂PO₄ - 0.1 g/l; FeSO₄·7H₂O - 0.01 g/l; ZnSO₄·7H₂O - 0.005 g/l, agar 1.5 g/l, deionized water - 1000 ml) supplemented with 0.06% keratin substrate as the sole source of carbon and nitrogen. The culture were inoculated on to solid media and incubated at 25°C for 8 days. The zone of clearance was indicated as keratinolytic activity (Wawrzkiewicz et al., 1991).

**3.5.2 Quantitative Screening of proteolytic activity on liquid keratin media**

The liquid mineral media was prepared as described earlier without adding agar in 100ml conical flask containing 1% keratin substrate using chicken feather. The native Chicken feathers was washed with ethanol dried and hammer milled individually prior to addition to the medium. The inoculated conical flasks was incubated on a rotary shaker (130 rpm) at room temperature. From 5th day of incubation each flask per strain was taken for keratinase enzyme assay by Bradford (Bradford, MM. 1976) method. The keratinase was assayed on every alternate day from 5th day of incubation till 25th day. The proteolytic activity was measured spectrophotometrically at 595 nm and was expressed in keratin units (KU), and 1KU is defined as an increase of 0.01 OD at 595 nm (Venkatesan et al., 2010).

**3.6 GENOTYPIC CONFIRMATION OF THE Trichophyton rubrum UM1 (Strain K42) WITH HIGHEST PROTEOLYTIC ACTIVITY**

The strain Trichophyton rubrum UM1 (K-42) showing the highest proteolytic activity was further subjected to genotypic confirmation by PCR amplification of 18S Ribosomal DNA sequencing using internal transcribed spacers (ITS1 and ITS4) targeting genes of ITS1, 5.8S and ITS2 of rDNA by following the method of Nascimento and Martinez-Rossi, 2001).

**3.6.1 DNA Extraction from Trichophyton rubrum UM1 (K-42)**

Extraction of DNA from the dermatophytic strain with highest proteolytic activity was prepared essentially as described by Elavarashi et al., 2013. DNA extraction, preparation of PCR mixture and post PCR analysis were carried out in
separate rooms using equipments designated for each to minimize the possibility of specimen contamination.

Around 100 mg of pure mycelium was taken in a sterile mortar and 500 µl of extraction buffer was added. The tissue was ground using clean pestle and the contents were transferred into a sterile Eppendorf tube. Equal volume of chloroform-isoamyl alcohol mixture was added and the tube was vigorously shaked for several minutes to get uniform emulsion. The tube was centrifuged at 13,000 rpm for 10 minutes and using clean Pasteur pipette, the top aqueous layer was transferred into a clean microfuge tube. The re-extraction was made using less volume of (250 µl) chloroform-isoamyl alcohol mixture. Equal volume of ice cold isopropyl alcohol was added to the separated aqueous solution and the tube was vortexed for adequate mixing. Then the tube was stored at –20°C for overnight for DNA precipitation. The precipitated DNA was collected by centrifugation at 13,000 rpm for five minutes and the upper alcohol layer was removed. The dried pellet was dissolved in TE buffer and was run in 0.8% agarose gel electrophoresis.

### 3.6.2 PCR amplification of 18S Ribosomal DNA

All PCR reaction was performed using a mastercycler gradient (Eppendorf, Germany).

**a. Primers**

The 18S rDNA universal primers used for the genotypic confirmation of *T. rubrum* UM1 strain K-42 were forward primer and reverse primers, ITS1 5′-TCCGTAGGTGAACCTGCGG-3′ and ITS4 5′-TCCTCCGCTTATTGATATG-3′ (Sigma-Aldrich, Bangalore) respectively.

**b. Amplification cycle**

The PCR amplification was carried out in the reaction mixture (sterile water - 38 µl, 10x assay buffer - 5 µl, dNTPs mix (10 mM each) - 3 µl, Template DNA (20-30 ng) - 1 µl, ITS1 (10 µM) - 1 µl, ITS4 (10 µM) - 1 µl, Taq polymerase (1 U) - 1 µl, the reaction mixture without any template DNA was kept as negative control. The cycling conditions consisted of initial denaturation at 94°C for 4 min, followed by 30 repeats of denaturation at 90°C for 45 s, annealing of primers at 50°C for 45 s, chain extension at 72°C for 90 s and final extension at 72°C for 5 min.
c. Detection of amplified DNA fragments by Agarose Gel Electrophoresis

To visualize the amplified PCR product, the amplified products were subjected to electrophoresis in Tris-borate EDTA (1X TBE) pH 8.0 on a 0.8% agarose gel incorporating 4μl of Ethidium Bromide (1μg/ml) was added to stain the amplicons. 8μl of the 1000bp DNA ladder (Genei, Bangalore) were run in parallel to the samples in each gel. The amplified PCR products were visualized under a gel documentation unit (GelDocMega, UK).

d. Purification of PCR products

The purification of amplified PCR product was carried out using QIAQuick (Qiagen) Spin Column Gel Extraction Kit as per the manufacture instruction. The purified DNA fragments of this isolate were taken for sequencing.

3.6.3 DNA sequencing

The direct gene sequencing was carried out by the method of Sanger et al., (1977) using DTCS quick start Dye terminator kit (Beckman Coulter) and Beckman Coulter CEQ 8000 auto analyzer. The removal of unbound dye and nucleotides from cycle sequenced product was carried out using DyeEx spin columns (Qiagen).

3.6.4 Phylogenetic analysis

The obtained sequence was subjected to BLAST (Basic Local Alignment Research tool) analysis, an online tool with the preexisting 18S rRNA coding genes sequences available in NCBI/Genbank to confirm the obtained 18S rRNA coding gene sequence. The obtained 18S rRNA coding sequence of the strain was aligned with various Trichophyton spp. 18S rRNA coding sequences obtained from GenBank, NCBI using Clustal-X Ver.2.0 aligning tool. The phylogenetic analysis was carried out using MEGA (Molecular Evolutionary Genetic Analysis) Ver.4.0. (Frealle et al., 2007).

3.7 EVALUATION OF MULTIPLEX PCR FOR DETERMINATION OF MEP(1-5) AND SUB(1-7) GENES

3.7.1 Extraction of DNA and PCR amplification

a. Primers

Extraction of DNA from the T. rubrum UM1 (K-42) with highest proteolytic activity was prepared by Elavarashi et al., 2013 as described earlier. All PCR reaction was performed using a mastercycler gradient (Eppendorf, Germany). The
forward primer and reverse primers (Table 3.1) specific for metalloproteinase (MEP 1-5) and subtilisin protease gene (SUB 1-7) purchased from Sigma-Aldrich, Bangalore, were used for this study.

Table 3.1 Primers used for PCR amplification of MEP(1-5) and SUB(1-7) genes and expected size of amplicons

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Expected product size</th>
</tr>
</thead>
</table>
| MEP1 | GCCACTGAGCTGGTTAAG  
CTTTGGATCGAATTTAGC | 1600 |
| MEP2 | AGAGTTCTGACTCGGAC  
ACTCGTGGATGACAATACC | 1400 |
| MEP3 | GCCATGTCTTCGTCAAG  
AGACCAGCTTACGCAAG | 2000 |
| MEP4 | ATCGTGATTCCTTACGACC  
TCGCCCATGTTATAGTCG | 2000 |
| MEP5 | CCAGCTACATGAGTCAATG  
ACAGGATGTGTAGACAAATGG | 1600 |
| SUB1 | ATCCCTGCTATGCCTCATG  
AATCGAAGTCTGACACAC | 1500 |
| SUB2 | ATATCTCAGCTACACTGAAG  
CCTGGATGCAATTGTACAC | 1400 |
| SUB3 | TTATCTCGTCTTCGTACG  
AGCAACGCTAAACACCTG | 1300 |
| SUB4 | AAGACTCAAGGCCAACAAG  
TTCCGATCATGAGCAACC | 1200 |
| SUB5 | GAAGTTGTGAGCCAAATGG  
CTCCAGGCCTAGCAGAAG | 1100 |
| SUB6 | CGATTCAGAAGTATTGATG  
GAGGTTCGGAGCCAGGG | 1650 |
| SUB7 | CTTGAGCTAGACACTGAAATG  
ATGAGGATTGCAACGA | 1384 |
b. PCR amplification

The PCR amplification was carried out in the reaction mixture (sterile water - 38 µl, 10x assay buffer - 5 µl, dNTPs mix (10 mM each) - 3 µl, Template DNA (20-30 ng) - 1 µl, forward primer (10 nM) - 1 µl (all primers), reverse primer (10 nM) - 1 µl (all primers), Taq polymerase (1 U) - 1 µl, the reaction mixture without any template DNA was kept as negative control.

c. Amplification cycle

The cycling conditions consisted of initial denaturation at 94°C for 4 min, followed by 30 repeats of denaturation at 90°C for 45 s, annealing of primers at 48°C for 45 s, chain extension at 72°C for 95 s and final extension at 72°C for 5 min.

d. Detection of amplified DNA fragments by Agarose Gel Electrophoresis

To visualize the amplified PCR product, the amplified products were subjected to electrophoresis in Tris-borate EDTA (1X TBE) pH 8.0 on a 0.8% agarose gel incorporating 4µl of Ethidium Bromide (1µg/ml) was added to stain the amplicons. 8µl of the 1500bp DNA ladder (Genei, Bangalore) were run in parallel to the samples in each gel. The amplified PCR products were visualized under a gel documentation unit (GelDocMega, UK).

e. Purification of amplified products

The purification of amplified PCR product was carried out using QIAQuick (Qiagen) Spin Column Gel Extraction Kit as per the manufacture instruction. The purified DNA fragments of these isolates were taken for sequencing

3.7.2 DNA sequencing

The direct gene sequencing was carried out by the method of Sanger et al., (1977) using DTCS quick start Dye terminator kit (Beckman Coulter) and Beckman Coulter CEQ 8000 auto analyzer. The removal of unbound dye and nucleotides from cycle sequenced product was carried out using DyeEx spin columns (Qiagen).

3.7.3 Sequence analysis

The nucleotide sequence was subjected to BLAST (Basic Local Alignment Search Tool) analysis in NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi.). The nucleotide BLAST was run using megablast (high similar sequence) mode with maximum target of 50 for confirmation of the sequence. The sequence was aligned
with similar sequence using Clustal-X ver.2.0 for the prediction of Exons and introns.

### 3.8 STRUCTURAL AND FUNCTIONAL PREDICTION OF PROTEIN FROM THE TRANSCRIBED SUB3 GENE OF *T. rubrum* UM1

#### 3.8.1 Translation of SUB3 sequence

The Open Reading Frame (ORF) sequence was predicted using tool called ORF finder (http://www.ncbi.nlm.nih.gov/projects/gorf/) open reading frame finding online tool. The sequences were subjected to aminoacid translation using Swissprot server.

#### 3.8.2 Prediction of aminoacid sequence

The translated peptide sequence was subjected to the investigation of aminoacid composition, signal peptide sequence and catalytic triad. The aminoacid composition was predicted by PredictProtein online tool (http://www.predictprotein.org) and the signal peptide was predicted by SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/). To find out the catalytic triad, the translated amino acid sequences of SUB gene of various *Trichophyton* spp. derived from GenBank were aligned with obtained amino acid sequence using Clustal-X (Ver.2.0) sequence aligning tool and aligned sequences were viewed for determination of the conserved positions by Bioedit (Ver 7.0.8) sequence alignment viewing tool.

#### 3.8.3 Structural prediction of Sub3 of *T. rubrum* UM1 protein

**a. Primary, secondary structure prediction**

The parameter of different conformational forms of protein was assessed. The protein parameters such as total and differential content of the amino acids and its properties were predicted by Protparam – ExPASy server (http://expasy.org/tools/protparam.html).

The secondary structure of the aminoacid sequence was predicted using online two dimensional (2D) prediction tool Phyre2, the top performing servers in the CASP international blind trials of structure prediction in homology modelling and remote fold recognition (http://sbg.bio.ic.ac.uk>phyre2).
The three dimensional structure (3D) of the protein was obtained by submitting the amino acid sequences to Swiss Model Server (http://swissmodel.expasy.org/workspace).

b. Pockets, Binding site Residues and Multiplicity

The Raptor X-binding (http://raptorx.uchicago.edu/BindingSite), a webserver that predicted the binding sites of a protein sequence along with the pockets and multiplicity of the protein sequence.

c. Ligand binding site

The 3DLigandSite server (http://www.sbg.bio.imperial.ac.uk/3dligandsite) identifies the homologous structures with bound ligands which were superimposed onto the protein structure to predict a ligand binding site.

d. Domain analysis

The prodom database provided protein domain families generated from the SWISS-PROT database by automated sequence comparisons through the alignment of query sequences with homologous ProDom domain families and links to the SWISS-MODEL server (http://www.expasy.ch/swissmod/SWISS-MODEL.html) for homology based 3D domain modelling where possible.

e. Phosphorylation site prediction

The serine, threonine and tyrosine phosphorylation sites were predicted by the neural network predictions produced by NetPhos 2.0 server.

3.8.4 Functional prediction of Sub3 of T. rubrum UM1 protein

Various functional analysis of Sub3 protein was performed by CombFunc, a Gene Ontology (GO) based server (http://www.sbg.bio.ic.ac.uk/~mwass/combfunc/) which runs multiple analyses to obtain data that can be associated with protein function.

3.9 MOLECULAR MODELLING OF Sub3 PROTEIN OF T. RUBRUM UM1

3.9.1. Sequence Analysis

The Sub3 protein sequence (389 aa) was compared in the BLAST searching (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using blastp algorithm, which aligned the target sequence with the homolog template sequences against PDB. The BLAST results showed very low identity and query coverage with protein
sequence in PDB database. (ex: 25% identity with 21% query coverage for the 2R5N_A). Thus, molecular threading was performed to model Sub3 protein.

3.9.2. Molecular Modeling of Sub3 Protein

All computational studies were carried out on CentOS v6.4 linux platform with Intel®Core™ i7-4770 CPU @ 3.40 GHz × 4 processors along with the installed software package Schrodinger, LLC, New York, 2015 and GROMACS v. 4.6.3. Robetta Server was used to model a protein structure with the low identity protein sequence as it provided both the ab initio and comparative models of protein domains (Raman et al., 2013). Domains without a detectable PDB homolog were modeled with Rosetta de novo protocol (Simons et al., 1997). Alignments were clustered and comparative models are generated using the Rosetta CM protocol.

3.9.3. Model Validation

All the four different modeled structures were validated using the SAVES server v4 (http://services.mbi.ucla.edu/SAVES/). The stereo chemical quality of the Sub3 protein structure and its overall residue-by-residue geometry was assessed by Ramachandran Plot analysis provided by PROCHECK. The final models were validated by inspecting the backbone conformation of the structures by analyzing the phi (φ) and psi (ψ) torsion angles. The PyMOL program was utilized for an interactive visualization and analysis of molecular structures.

3.10 VIRTUAL SCREENING OF LIGAND COMPOUNDS AGAINST TARGET Sub3 PROTEIN

3.10.1. Protein Preparation Wizard

The modeled protein was prepared using Protein Preparation Wizard in Maestro (Maestro, version 10.1, Schrodinger, LLC, NewYork, NY, 2015). Hydrogen molecules were added and unwanted water molecules were removed from the protein molecule. The energy was minimized until the average root mean square deviation (RMSD) of the non-hydrogen atoms reached 0.3 Å. Partial charges were assigned according to OPLS-2005 force field.

3.10.2. Active Site Prediction

The active site of the modeled protein was investigated using the SiteMap, version 3.4 Schrodinger, LLC, NewYork, NY, 2015 which helped to find one or more regions in the protein surface that may be suitable for binding ligands to the
receptor. Contour maps were generated using hydrophobic and hydrophilic regions. The hydrophilic maps were further divided into donor, acceptor and metal binding regions. The overall properties were calculated and SiteScore was generated. The average SiteScore for a promising binding site was 1.0. It was based on weighted sum of several properties included in this program (Halgren et al., 2009).

3.10.3. Receptor Grid Generation:

The receptor grid was generated for the prepared protein molecule using Glide program of Schrodinger software. The position of grid box is set as X = -15.16 Å; Y = 27.47 Å; Z = 7.78 Å; axis with radius ≤ 2.0 Å and the van der Waals radii of receptor atoms as 1.00 Å with a partial charge cut-off 0.25 Å to soften the potential for non-polar part of receptor (Krishnasamy et al., 2015). The top SiteScore from the site map prediction was considered for the grid generation.

3.10.4. Virtual Screening Workflow

Virtual screening (VLS) was carried out to find novel and potential leads suitable for the particular target molecule using Virtual Screening Work Flow module in Glide program of Schrodinger software. Initially, the modeled structure was screened against different databases such as Maybridge (93,148), Chembridge (2,64,776) and Allium database (Vitas ML) (35,218). The compounds were prepared by LigPrep (LigPrep, version 3.3, Schrodinger, LLC, New York, NY, 2015). The screened molecules were filtered using Lipinski’s rule of five, and ADMET properties. Finally, the molecules which pass all the filtrations were subjected to molecular docking study to find the potent inhibitors of Sub3 by Glide program (Glide, version 6.6, Schrodinger, LLC, New York, NY, 2015).

Glide docking was performed with three accuracy levels such as High Throughput Virtual Screening (HTVS), Standard Precision mode (SP) and Extra Precision mode (XP). Best inhibitors were selected based on high docking score. The selected compounds can be act as potential lead molecule against Sub3.

3.10.5. Hierarchical Virtual Ligand Clustering

The probabilistic hierarchical clustering was performed to find the most likely near-native confirmations for each complex. The distance metric used to cluster each ligand was the RMSD of its atom coordinates versus all the other ligands already in cluster. If the simulation converges, then the largest cluster with lower internal variance (denoted as the target cluster) was likely the cluster that contains
more near-native conformations. The ligand confirmation with the highest degree of
belonging (centroid) to the target cluster was selected as the predicted near-native
confirmations.

3.10.6. Absorption, distribution, metabolism, and excretion (ADME) Property
Screening prediction by using Qikprop

The QikProp v 3.0 tool from Schrodinger helps to screen the molecules for
their drug like property by using Lipinski’s rule of five. QikProp predicted physically
significant descriptors and pharmaceutically relevant properties of the lead
compounds, either individually or in batches which was essential to ensure drug-like
pharmacokinetic profile while using rational drug design. All the analogs were
neutralized before being used by Qikprop. The preferable properties which were
taken into consideration were evaluated.

3.11 MOLECULAR DYNAMIC SIMULATIONS

Molecular Dynamics (MD) simulations were performed for the both
modeled protein and selective protein ligand complex. MD simulation was
performed to gain an enhanced relaxation and a correct arrangement of the atoms
as well as to refine the side chain orientation of Sub3 model and complex, by
applying GROMOS 96 43a1 force field. All the MD simulation process was
performed using GROMACS v4.6.3. The topology and coordinate file for modeled
protein were generated in GROMACS format. Topology files and other force field
parameters for Ligand were generated using the PRODRG program (Schuttelkopf
and Aalten, 2004). The effects of inhibitor on the modeled protein was examined a
MD simulation for the protein-ligand complex. Hence, the preferred compounds
resulted from the docking were selected as a representative for MD simulation. To
neutralize the system, 22 Chloride ions was added. The full system was subjected
to 50,000 ps MD simulation at 310 K temperature and 1 bar pressure. The Particle
Mesh Ewald (PME) method for long-range electrostatics, a 14 Å cutoff for van
der Walls interactions, a 12 Å cutoff for coulomb with updates every 10 steps, and
the Lincs algorithm for covalent bond constraints were used in this study. Further,
the RMSD of backbone structure, RMSF of carbon alpha, number of hydrogen
bonds and radius of gyration (Rg) were calculated between the trajectories
generated at 300 K to investigate the flexible nature of protein and protein-ligand
complex.
3.11.1. MM-PBSA

For binding free energy calculation of protein-ligand complex Molecular Mechanics – Poisson Boltzmann Surface Area (MM-PBSA) was used (Kumari et al., 2012). Combined with molecular dynamics (MD) simulations MM-PBSA can also incorporate conformational fluctuations and entropic contributions to the binding energy. MD simulations were used to generate an ensemble of binding conformations in the presence of explicit water and further the MM-PBSA approach was used to estimate the binding energy (Parenti and Rastelli, 2012).

3.11.2. Density Functional Theory Calculation

The best screened compounds were given as input for density functional theory (DFT) calculations which were used to study electronic molecular features such as electron density, frontier molecular orbital density fields (i.e. HOMO and LUMO) and molecular electrostatic map, which can help to explain the molecular properties and also to predict the biological activity. DFT calculations were carried out in Jaguar (Jaguar, version 8.7, Schrodinger, LLC, New York, NY, 2015). The study was performed based on solvation state. Complete geometry was analyzed using DFT with Becke’s three-parameter exchange potential and the B3LYP function with basis set of 6-31G**** level via the PBF solvation (Francl et al., 1982). From the Jaguar, molecular electrostatic properties, including Molecular Electrostatic Potential (MESP), dipole moment, Lowest Unoccupied Molecular Orbital (LUMO), and Highest Occupied Molecular Orbital (HOMO) energy was computed. The electrostatic potentials were calculated over the Van der Waals contact surface area of the molecule and over the space spreading away from the molecular surface, which provides a measure of charge distribution from the point of view of an impending reagent (Kirubakaran and Karthikeyan, 2013). The overall molecular size and different charges of electrostatic potentials were represented by color coded isosurface values. The most positive electrostatic potential was indicated by deepest blue whereas the most negative was colored by deepest red. The left over color shades indicating intermediate range of reactivity.