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In-silico Characterisation and Binding Affinity Evaluation of Hypothetical Proteins from *Trichophyton verrucosum* against Azole Antifungals and Natural Compounds

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Abstract

Trichophyton verrucosum is a pathogenic dermatophyte known to cause skin infections, particularly among individuals in close contact with livestock, such as farmers and veterinarians. **Aim:** This study presents an *in-silico* approach to identify and characterise novel therapeutic targets by focusing on functionally uncharacterized hypothetical proteins (HPs) from *T. verrucosum*. These HPs may play key roles in the pathogen's biology and represent promising candidates for antifungal drug development. **Materials and Methods:** In the initial phase of the study, previously published data included 8028 proteins retrieved from NCBI, among which 6227 were annotated as hypothetical proteins (HPs). Conserved domain analysis revealed functional motifs in 4432 of these HPs. Non-homology screening against the human proteome yielded 1648 fungal-specific proteins, from which 51 essential proteins were predicted using the Geptop server. These essential proteins were further analysed for physicochemical properties, subcellular localisation, and structural features. **Results:** Most were cytoplasmic (~74.5%) and showed diverse isoelectric points, hydrophilic character, and amino acid compositions. Structural models were generated using the Phyre2 server, and eight stable proteins were selected based on ProtParam analysis for docking studies. Molecular docking with Ketoconazole, Clotrimazole, Terbinafine, and phytocompounds (Eugenol, 1,8-Cineole, Camphor) revealed strong binding affinity, particularly for Ketoconazole with XP_003018087.1 and XP_003018908.1.

Major Findings: These findings suggest promising therapeutic targets in *T. verrucosum* and provide a basis for future structure-based antifungal drug development.

Keywords: Hypothetical Proteins, Ketoconazole, Physicochemical Characterisation, Phytocompounds, Structure-based Drug Discovery

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1 Introduction

Fungal infections are increasingly recognised as a major global health challenge, affecting both healthy individuals and those with compromised immunity. These pathogens are responsible for a wide range of diseases, from mild skin conditions to severe, life-threatening systemic infections. Among the major fungal pathogens, dermatophytes represent a distinct group of fungi known for infecting the skin, hair, and nails of humans and animals. In recent years, a widespread outbreak of dermatophytosis has been observed in India and several neighbouring countries within the subcontinent. The infection spreads mainly through direct human contact and is often associated with recurrent and treatment-resistant cases, emphasising the urgent need for better control and therapeutic approaches. The World Health Organisation (WHO) reports dermatophytosis affects 20-25% of the

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world's population[1,2]. Dermatophyte infections are among the most common fungal diseases, affecting both humans and animals, such as horses and dogs. These fungi can colonise 30 to 70 percent of the human population without causing symptoms, contributing to their silent transmission and widespread prevalence[3].

The growing incidence of dermatophytosis and the emergence of treatment-resistant cases underscore the continued public health burden of superficial fungal infections. Dermatophytic fungi cause a variety of skin diseases, including athlete's foot (clinically termed tinea pedis), jock itch (tinea cruris), and ringworm (tinea capitis or tinea corporis, depending on the area of the body infected)[4]. Dermatophytosis is primarily caused by a group of keratinophilic filamentous fungi classified under three closely related genera—*Trichophyton*, *Microsporum*, and *Epidermophyton*—collectively referred to as dermatophytes. Among these, *Trichophyton verrucosum*, a zoophilic species, has been frequently reported in regions such as Southern Europe and the Middle East[5]. This species is recognised as the predominant etiological agent of dermatophytosis in cattle, although it can occasionally infect other domestic animals and humans. Notably, in recent years, there has been a steady rise in the number of human infections attributed to *T. verrucosum*, a trend that appears to correlate with increased exposure in agricultural settings, particularly in areas where cattle farming is prevalent. This emerging pattern highlights the zoonotic potential of *T. verrucosum* and underscores the need for enhanced surveillance and control strategies in both veterinary and human health domains. Moreover, this fungal infection in humans can be considered as an occupational disease[6]. Individuals such as veterinarians, livestock technicians, and farmers are at higher risk of *T. verrucosum* infection due to frequent contact with infected cattle. In humans, the infection often causes acute inflammatory skin lesions. Constitutional symptoms like lymphadenopathy and fever may also develop[7]. In recent years, the incidence of *Trichophyton verrucosum* isolation from individuals with mycotic infections has shown a consistent increase, which is closely associated with exposure to cattle farming environments. The frequency of *T. verrucosum* isolation among other dermatophytes species is relatively high in Greece (1.8%), France (1. 53%), and Poland (1%), compared to studies from Italy (0.04%), the Czech Republic (0.01%), and Germany (0%)[8].

In many organisms, more than 30% of proteins are classified as hypothetical proteins (HPs) because their functions are still unknown. These proteins may help fill important gaps in our understanding of the genome and protein function. Using *in-silico* methods to study HPs can predict their 3D structures, which may reveal new domains, biological pathways, and protein interactions. This information can also help identify possible biomarkers and drug targets. Notably, in fungal infections like *Trichophyton verrucosum*, functionally uncharacterized hypothetical proteins lacking similarity to the human proteome offer interesting fungus-specific treatment targets and are likely involved in essential fungal biological processes. Targeting these proteins may allow for selective antifungal intervention by disrupting fungi-specific mechanisms while limiting host cross-reactivity and off-target consequences. Several bioinformatics databases and tools have been successfully used to annotate the functions of hypothetical proteins in different pathogenic microorganisms[9,10].

In our previous study (Gaur *et al*., 2020), we utilised various computational tools and databases, including NCBI, NCBI-CD, and the NCBI-BLASTp, to identify and preliminarily characterise a set of hypothetical proteins (HPs) from the genome of *Trichophyton verrucosum*. Through this approach, we classified these HPs based on conserved domain analysis, essentiality, and non-homology, thereby laying the foundation for further functional exploration[11,12]. In this study, hypothetical proteins (HPs) from *Trichophyton verrucosum* were analysed using targeted in silico approaches. Functional annotation, subcellular localisation, secondary structure, and active site predictions were performed to assess their biological roles. Molecular docking was conducted with synthetic antifungals and selected phytocompounds to evaluate their therapeutic potential. Importantly, while our previous work was limited to target identification, the present study advances this by evaluating the druggability of these proteins through structural and docking analyses, supporting their potential as selective antifungal targets in *Trichophyton verrucosum.*

2. Material and Methods

2.1 Identification of Essential Proteins and Physicochemical Characteristics

The analysis continued by evaluating essential proteins from the previously identified non-homologous protein set. Protein essentiality was assessed using the Geptop server

based on phylogenetic profiling and orthology mapping, and proteins with Geptop scores ≥ 0.24 were selected as essential candidates. The shortlisted essential proteins were then analysed for their physicochemical properties using the EMBOSS PepStat tool, including assessments of aromatic and aliphatic content, polarity, and the acidic or basic nature of their amino acid composition[11,13].

2.2 Integrated Analysis of Protein Localisation, Function Prediction

Cellular and subcellular localisation were computed to provide information about the proteins' possible biological roles and functional relevance. Cellular localisation was predicted using TMHMM[14], HMMTOP[15], and TOPCONS[16]. Subcellular localisation was predicted using PSORTb version 3.0.3 (https://psort.org/psortb/). PSORTb employs a multi-classifier framework derived from PSORT I and analyses key sequence features, including signal peptides, transmembrane helices, and homology, to predict protein localisation[17].

2.3 Model Quality Assessment and Structural Integrity Verification

Three-dimensional structures of 51 hypothetical proteins were predicted using the batch processing mode of Phyre2. This tool employs advanced remote homology recognition techniques to construct structural models of proteins lacking experimental characterisation. Phyre2 provides an integrated web-based platform for predicting protein structure, assessing potential function, and evaluating the impact of mutations. The focus of Phyre2 is to provide biologists with a simple and intuitive interface to state-of-the-art protein bioinformatics tools[18,19]. Further, these models were validated using PROCHECK. The Ramachandran plot for the model was built using this program[20]. Also, EXPASY ProtParam was employed to classify the stability of modelled structures. EXPASY ProtParam gives a detailed result about the molecular weight, theoretical isoelectric point (PI), the total number of positively and negatively charged residues, molecular formula, total number of atoms, the instability index, aliphatic index, and grand average of hydropathicity (GRAVY)[21].

2.4 Molecular Docking

Molecular docking is a computational approach used to predict the binding conformation of ligand molecules within the active site of a target protein. This technique helps assess the binding affinity and interaction energy between the ligand and receptor, providing insights into their potential biological activity. Six drug compounds, including Terbinafine, Clotrimazole, Itraconazole, Ketoconazole, 1,8-Cineole, Camphor, and Eugenol, were selected as potential ligands based on their known antimicrobial properties and relevance in targeting fungal infections. Molecular docking was conducted via AutoDock Vina, which implements an iterated local search global optimisation technique. The centre coordinates of the grid box were x = 82.097, y = 45.214, and z = 150.716. Docking simulations were conducted using the default exhaustiveness value of 8, generating 9 binding modes per ligand. All water molecules and solvent atoms were removed from the protein structures, and polar hydrogen atoms were added to prepare the proteins for docking. The receptor was kept rigid, while ligands were treated as flexible to explore potential binding conformations. AutoDock assessed the binding poses based on calculated binding free energy (ΔG). The interaction plot representing the H-bond interactions of the protein-ligand complex was generated using PyMOL v 3.0.2 (Schrödinger, Inc.)[22,23].

3. Results

As reported in our earlier study (Gaur *et al.,* 2020), a systematic *in-silico* screening approach was applied to the *Trichophyton verrucosum* proteome to identify potential therapeutic targets. The complete proteome comprising 8028 proteins was retrieved from the NCBI database, of which 6227 were annotated as hypothetical proteins (HPs). Functional annotation using the NCBI Conserved Domain Database (CDD) identified 4432 HPs containing conserved domains, suggesting potential biological significance. To ensure fungal specificity and eliminate human homologs, these 4432 proteins were screened against the human proteome using BLASTp, resulting in 1648 non-homologous proteins. The outcomes of this initial screening formed the basis for the current study, in which selected essential HPs were subjected to further essentiality, physicochemical, structural, and functional characterisation.

These non-homologous proteins were subsequently analysed for essentiality using the Geptop server, which employs phylogenetic profiling and orthology data for essential gene prediction. This led to the identification

of 51 essential proteins, representing a refined list of candidate targets essential for the survival of the fungal organism and absent in humans. The EMBOSS PepStat tool was used to analyse the aromatic and aliphatic properties of the protein sequences, along with the average composition of polar and non-polar amino acids and the overall acidic or basic nature of each sequence. A comprehensive analysis was conducted on 51 essential non-homologous fungal proteins to evaluate their physicochemical and compositional attributes. The theoretical isoelectric points (pI) ranged from 4.10 to 11.61, indicating a broad spectrum of acidic and basic proteins, with approximately half of them displaying basic pI values (>7.0). The molecular weights varied significantly, from 9983.47 Da to 225313.01 Da, reflecting diversity in sequence length and structural complexity. The GRAVY (Grand Average of Hydropathicity) scores were predominantly negative (from -1.066 to 0.234), suggesting that the majority of proteins are hydrophilic in nature. The aliphatic index ranged between 21.04% and 38.02%, and aromatic content spanned from 4.07% to 18.10%, indicating variation in side chain composition and potential thermostability. Non-polar residues accounted for 43.57% to 63.26%, while polar residues comprised 36.74% to 56.43%, reflecting a balanced hydrophobic–hydrophilic character favourable for solubility and biological interactions. Charged amino acids constituted 19.33% to 34.75% of the sequences, with basic residues ranging from 10.12% to 24.46% and acidic residues from 6.64% to 18.55%. This physicochemical characterisation offers valuable insights into the structural and functional diversity of the essential fungal proteins, providing a strong foundation for structure-based antifungal drug development.

In this analysis, three transmembrane helix prediction servers, TMHMM, HMMTOP, and TOPCONS, were employed to evaluate the presence and topology of transmembrane helices (TMHs) across 51 proteins. The TMHMM server predicted TM helices in a limited number of proteins, identifying a few as potential single- or multi-pass membrane proteins. HMMTOP showed a broader range of TMH predictions, suggesting a higher number of transmembrane regions across several proteins, including a few with up to 7 TMHs, indicating likely multi-pass membrane proteins. TOPCONS, as a consensus tool, offered more conservative predictions, with only a handful of proteins showing TMHs, highlighting its stringent criteria. Notably, only a small subset of proteins displayed consistent predictions across all three tools. Proteins such as XP_003019836.1 and XP_003024152.1 showed consistent TMH predictions, indicating a high likelihood of membrane localisation. In fungi, sterols such as ergosterol are essential components of the cell membrane, maintaining its structure, fluidity, and proper functioning of membrane-associated proteins, including enzymes and transporters. TMH-containing proteins might be easily accessible and pharmacologically important targets for clinically used antifungals, especially CYP51 inhibitors, as they interfere with the membrane-associated sterol production pathways[24]. Overall, the combined use of these three prediction tools provided a more comprehensive and comparative assessment of potential membrane protein topology.

To predict the subcellular localization of the selected protein sequences, the PSORTb server was employed. Out of the 51 proteins analyzed, a substantial majority (n=38, ~74.5%) were predicted to be localized in the cytoplasm, indicating a dominant intracellular role, likely related to essential metabolic or regulatory functions. Four proteins (~7.8%) were identified as cytoplasmic membrane-associated, suggesting potential involvement in transport or signal transduction pathways. Interestingly, only one protein (~2%) was predicted to be extracellular, which may reflect a role in host interaction or environmental signaling. Notably, ten proteins (~19.6%) were classified under unknown localization, highlighting possible limitations of the current prediction model or indicating the presence of novel or less-characterized localization signals. These findings provide valuable insight into the potential biological roles of these proteins and support downstream functional annotation efforts. Of these, 2 proteins were found to have signal peptides as well. Signal peptides have an essential role in carrying information related to protein secretion, disease diagnosis and immunization processes[25,26].

3D structures are prepared using phyre2 web server. Following the validation of structure and assessment of stability for the modelled protein structures, eight proteins demonstrated stable conformations. These stable protein models were subsequently selected for molecular docking studies. The functional roles defined for these proteins provided a rational basis for exploring their binding affinity with both synthetic antifungal agents and phytocompounds, offering further insights into their potential as therapeutic targets. The predicted functions of the analysed hypothetical proteins from *Trichophyton*

verrucosum suggest their significant involvement in essential metabolic and cellular processes (Figure 1).

Several proteins were found to participate directly in amino acid biosynthesis, indicating their role in fundamental cellular functions. One protein stimulates a reaction critical for recycling thiazole (Thz) into the thiamine biosynthesis pathway, highlighting its potential role in cofactor regeneration. Another protein catalyses a key step in riboflavin biosynthesis, which is vital for microbial metabolism. Class I aldolases among the identified proteins are multifunctional enzymes that catalyse reactions across various metabolic pathways, enhancing biochemical flexibility. Additionally, proteins involved in amino acid transport and metabolism were noted, pointing toward their relevance in nutrient assimilation and intracellular homeostasis. One protein was predicted to catalyse the interconversion between 3-phosphoglycerate (3PGA) and 2-phosphoglycerate (2PGA), indicating its involvement in glycolysis or gluconeogenesis. The presence of chorismate synthase suggests a critical role in the synthesis of aromatic amino acids. Furthermore, TIM-like beta/alpha barrel domain-containing proteins were identified, which are essential for cargo transport, signalling, and membrane biogenesis[27].

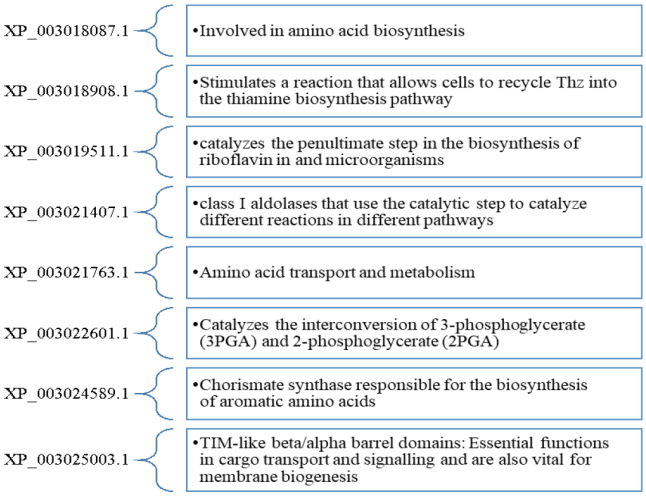


Figure 1. Predicted functional roles of selected hypothetical proteins from *T. verrucosum*.

The molecular docking results obtained using AutoDock, as presented in Table 1, highlight the binding affinities of standard antifungal agents (Terbinafine, Clotrimazole, and Ketoconazole) and selected phytocompounds (1,8-Cineole, Camphor, and Eugenol) against key fungal proteins with crucial biological functions.

Among the tested compounds, Ketoconazole consistently exhibited the highest binding affinities, particularly against XP_003018087.1 (-10.0 kcal/mol) and XP_003018908.1 (-9.1 kcal/mol), which are involved in amino acid biosynthesis and thiamine recycling, respectively. Table 2 presents the detailed binding affinities of Ketoconazole with the selected proteins, along with information on their predicted binding pockets.

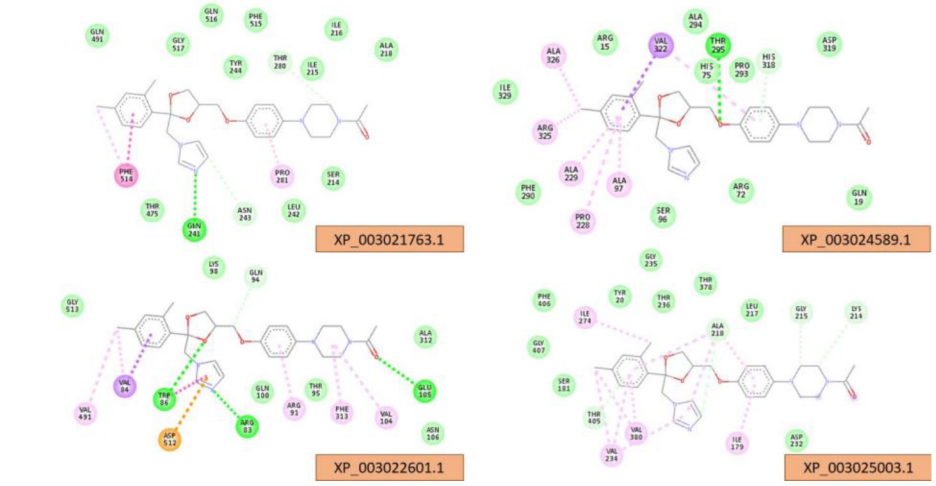
Molecular docking representation of ketoconazole binding pockets across selected protein targets. The figure (Figure 2 (a) (b)) illustrates the key amino acid residues involved in interactions with ketoconazole, categorised as hydrogen bonds (green) and hydrophobic interactions (pink/purple). Binding affinity values (in kcal/mol) and interacting residues were identified through *in-silico* docking analysis, highlighting the ligand's preference for specific hydrogen-bonding and hydrophobic pocket environments across different protein models. Clotrimazole and Terbinafine also showed effective interactions, with notable binding to proteins such as XP_003021763.1, responsible for amino acid transport and metabolism, and XP_003024589.1, a chorismate

Table 1. Binding affinities of antifungal drugs and phytocompounds against key fungal proteins predicted by AutoDock

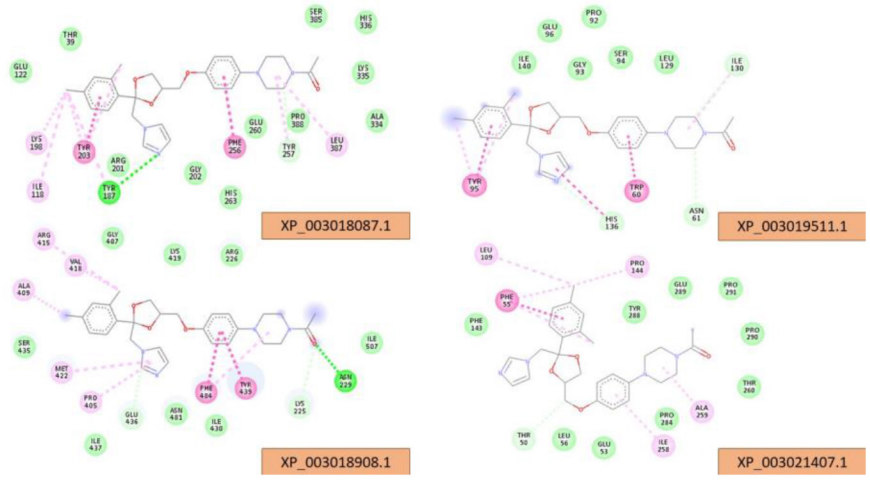
S.No.	NCBI Reference Sequence	Terbinafine	Clotrimazole	Ketoconazole	1,8-Cineole	Camphor	eugenol
1.	XP_003018087.1	-8.5	-8.8	-10	-6.6	-5.3	-5.8
2.	XP_003018908.1	-7.2	-7.4	-9.1	-6.3	-5.6	-5.6
3.	XP_003019511.1	-6.1	-6.4	-7.5	-5.6	-5.3	-5.2
4.	XP_003021407.1	-7.6	-7	-8.2	-5.4	-5.2	-5.3
5.	XP_003021763.1	-6.6	-8.2	-8.7	-7	-6	-5.6
6.	XP_003022601.1	-6.4	-6.4	-8	-4.9	-4.5	-4.9
7.	XP_003024589.1	-6.9	-7.4	-8.9	-5.6	-5.1	-5.7
8.	XP_003025003.1	-6.2	-7	-7.1	-5.8	-4.8	-5.6

Table 2. Predicted binding pocket residues of Ketoconazole in selected fungal proteins based on molecular docking analysis

SL No.	NCBI Reference Sequence	Binding Affinity (kcal/mol)	Key Binding Pocket Residues
1	XP_003018087.1	-10	PHE55, LEU109, PRO144, THR50, LEU56, GLU53, TYR288, PRO284, ILE258, ALA259
2	XP_003018908.1	-9.1	PHE514, GLN241, THR475, PRO281, ASN243, TYR244, THR280, LEU242
3	XP_003019511.1	-7.5	VAL84, TRP86, ARG83, ASP512, GLU105, PHE313, THR95, ARG91, GLN100
4	XP_003021407.1	-8.2	VAL322, ALA229, ALA97, ARG15, PRO228, HIS75, THR295, PRO293
5	XP_003021763.1	-8.7	ILE274, VAL380, VAL234, THR405, PHE406, ALA218, ASP232, ILE179
6	XP_003022601.1	-8	TYR203, TYR187, ARG201, PHE256, GLU260, PRO388, LEU387
7	XP_003024589.1	-8.9	ARG415, VAL418, PRO405, PHE484, TYR439, ASN229, ASN481, GLU436
8	XP_003025003.1	-7.1	ALA294, ILE329, ARG325, ALA326, PHE290, PRO228, SER96, HIS318



(a)



(b)

Figure 2 **(a) (b).** Binding pocket visualisation of ketoconazole with target proteins. Key hydrogen bonds (green) and hydrophobic interactions (pink/purple) are highlighted based on docking results.

synthase involved in the biosynthesis of aromatic amino acids. On the other hand, phytocompounds showed relatively lower binding affinities. Among them, Eugenol exhibited the best interaction, particularly with XP_003025003.1, a protein containing TIM-like beta/alpha barrel domains, which play essential roles in cargo transport, signalling, and membrane biogenesis. 1,8-Cineole and Camphor, however, demonstrated weaker binding profiles across most targets. Additional protein targets, such as XP_003019511.1 (involved in riboflavin biosynthesis), XP_003021407.1 (a class I aldolase participating in diverse catalytic steps), and XP_003022601.1 (catalysing the interconversion of 3-phosphoglycerate and 2-phosphoglycerate), were also evaluated, revealing a consistent trend of higher binding with synthetic antifungal agents.

These results underscore the superior binding efficacy of azole-based antifungal drugs over natural phytocompounds in targeting essential fungal metabolic pathways. However, the moderate binding of compounds like Eugenol suggests potential for further structural optimisation. Overall, this docking analysis provides valuable insights into protein-ligand interactions and may guide the rational design of novel antifungal agents targeting vital fungal enzymes.

4. Discussion

Fungal infections caused by *Trichophyton verrucosum* remain a clinical challenge, particularly among individuals with frequent animal contact. Despite the increasing availability of antifungal agents, treatment failure and recurrence are often reported, highlighting the need for novel molecular targets and improved therapeutic options. This study focused on exploring hypothetical proteins (HPs) as potential therapeutic candidates using a robust *in-silico* approach. We prioritised 1648 non-homologous HPs based on domain conservation and lack of similarity to the human proteome, ensuring target specificity. We further refined these candidates by identifying 51 essential proteins using the Geptop server. These essential proteins are likely critical to fungal survival, making them attractive targets for antifungal development.

Expanding on our earlier research (Gaur *et al.*, 2020), 1648 hypothetical non-homologous proteins were prioritised based on domain conservation and lack of similarity to the human proteome in order to ensure target selectivity. Molecular weight, isoelectric point, hydropathy, and amino acid content varied widely, according to physicochemical investigation; the majority of proteins showed hydrophilic properties, which are suggestive of good solubility. The presence of signal peptides and transmembrane helices in a subset indicates possible roles in membrane-associated processes and host-pathogen interactions. The majority of proteins are cytoplasmic, according to subcellular localisation prediction, whereas very few are extracellular or membrane-bound, indicating functional variety among key targets. Functional annotation connects 8 hypothetical proteins to vital metabolic processes, such as amino acid biosynthesis, thiamine recycling, glycolysis, and riboflavin metabolism. TIM-like β/α barrel domains are an additional sign of potential enzymatic functions.

Molecular docking analyses indicated the therapeutic potential of the selected targets, with ketoconazole and 1,8-cineole exhibiting strong predicted binding affinities toward XP_003018087.1 (amino acid biosynthesis) and XP_003018908.1 (cofactor regeneration). Proteins involved in glycolysis (e.g., XP_003022601.1), riboflavin biosynthesis (e.g., XP_003019511.1), and chorismate synthesis (e.g., XP_003024589.1) emerged as particularly promising due to their biological relevance and strong drug binding profiles. Terbinafine and Clotrimazole showed favourable binding profiles that reflected their clinical effectiveness in dermatophytosis, but Camphor and Eugenol showed relatively lower binding affinities than conventional antifungal agents. These findings suggest that natural compounds, with structural optimisation, may serve as potential scaffolds for novel antifungal agents. Docking analyses also suggests key binding pocket residues that stabilize ligand–protein interactions, providing a basis for future structure-based drug design.

Collectively, these findings highlight the importance of exploring functionally uncharacterized proteins for antifungal target discovery. The computational strategy used in this study, including essentiality analysis and molecular docking, follows established approaches commonly used in antimicrobial drug discovery[28,29]. Targeting essential metabolic pathways is already a recognised antifungal strategy[30]. In our study, synthetic antifungals showed stronger predicted binding, while phytocompounds such as Eugenol showed moderate interactions, suggesting possible use in combination therapies. However, these results are based on computational predictions and should be considered hypothesis-generating. Further validation through experimental studies (e.g., gene knockout, expression

analysis, and in vitro binding assays) will be necessary to confirm the biological roles and druggability of the proposed targets. Nevertheless, the present *in-silico* findings provide a solid foundation for rational antifungal drug discovery focused on *Trichophyton verrucosum*.

5. Conclusion

This *in-silico* investigation effectively narrowed down fungal hypothetical proteins to a subset of essential, non-human homologous candidates suitable for antifungal targeting. Physicochemical characterisation and membrane topology analyses enhanced understanding of their structural and functional diversity. Molecular docking results confirmed the superior binding affinity of azole drugs, particularly Ketoconazole, toward critical fungal enzymes, emphasising their therapeutic relevance. Although natural phytocompounds showed comparatively lower affinity, compounds like Eugenol demonstrated moderate binding, indicating scope for chemical modification and development as alternative antifungal agents. Overall, this integrative computational study lays the groundwork for future experimental validation and drug development aimed at combating fungal infections through targeted inhibition of essential fungal proteins.

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Author Contributions

Nishant Gaur contributed to the methodology, molecular docking studies, data analysis, and first manuscript drafting. Nayyar Parvez contributed to Study supervision data interpretation, and manuscript review. Kalpana Nagpal contributed to project administration, conceptualization, validation of results, manuscript review, critical revision, and final approval of the manuscript.

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