IN VITRO AND IN SILICO APPROACH TO EVALUATE THE ANTI-DERMATOPHYTIC ACTIVITY OF *ENICOSTEMMA LITTORALE* BLUME

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ABSTRACT

Dermatophytes are the causative agents of superficial skin infections, dermatophytoses, which are capable of invading and digesting keratin. The current usage of synthetic drugs leads to either side effects in human or resistant fungal varieties due to prolonged use. Hence the present study aims at finding leads from phytochemicals to target the enzymes involved in maintaining fungal cell wall integrity, namely, 1, 3 β -D glucan synthase and chitinase, important enzyme having a role in morphogenesis. Docking analysis was carried out for the above targets with already reported phytochemicals of *Enicostemma axillare* (syn. *littorale*) Blume and compared with the specific synthetic drugs to evaluate their efficacy as fungal inhibitors using Glide software. The *in vitro* study was carried out for *E. littorale* extract against *Microsporum gypseum*. The significant results were observed with the petroleum ether extract of the aerial parts of *E. littorale* at a concentration of 3000 μ g/ml.

Keywords: *Enicostemma littorale*, *Microsporum gypseum*, *in silico* analysis, antifungal and antidermatophytic assay.

INTRODUCTION

Dermatophytic infections (Tinea) are caused by the group of organisms which are categorized under three genera, namely, *Trichophyton*, *Microsporum* and *Epidermophyton* and the infection can also be chronic in case of immune compromised persons¹. The available drugs, first, the naturally occurring antibiotics such as polyenes and echinocandins, second, the synthetic drugs allylamines, azoles and fluorinated pyrimidines are not only producing side effects but also the fungal pathogens are becoming resistant to these drugs. The dermatophytic infections are mostly superficial that grow on human skin, hair and nails but are attached with social stigma which forces to explore the alternate treatment for curing it. Depending upon the place of infections there are different types of Tinea. Tinea corporis or ringworm is the annular scaly lesions with central clearing that generally occurs on the trunk, extremities or face with pustules or follicular papules on the border of the lesion. Tinea barbae involves the skin, coarse hairs of the beard and moustache area whereas tinea faciei occurs in the no bearded area of the face. Itching, burning, round or annular red patches are associated with it. Tinea manuum is infection on one or occasionally both hands and often occurs in patients with tinea pedis. The palmar surface is diffusely dry and hyperkeratotic. Tinea cruris frequently called “jock itches” is a dermatophytic infection of the groin² and Tinea unguium is of the nail³. From ancient period, medicinal plants are in use for common treatments in different parts of the world. Usage of medicinal plants demonstrates the promising and potential effects in the treatment against many human diseases, in particular the plant components act as therapeutic agents. Microbiologists are also interested in exploring the phytochemicals as antimicrobials owing to a more safer, reliable and effective product⁴. *Enicostemma littorale* Blume (Synonym-*Enicostemma axillare* subsp. *littorale* (Blume) A. Raynal) commonly known as Chota-Kirayat or Chota-Chirayata is a glabrous perennial herb that belongs to family Gentianaceae. It is traditionally used as antidiabetic, urinary astringent, antiperiodic, anthelmintic, anti-inflammatory, laxative and

carminative and also possesses antioxidant, hypolipidemic, antimicrobial, antinociceptive, antiedematogenic and antitumor activities⁵. The present study focuses on evaluating the antifungal efficacy of the medicinal plant *E. littorale* through *in silico* and *in vitro* approaches. The phytochemicals of *E. littorale* that have been already reported were docked with the selected fungal targets and the binding efficiency were observed and compared with the binding efficiency of available drugs. Distinctly the fungal cell walls are unique with chitin, β -1, 3 and β -1, 6 glucans, mannoproteins in their cell wall. Therefore the enzymes 1, 3- β D-glucan synthase (GS) and chitinase which are involved in the synthesis and repair of cell wall components were chosen as drug targets in the present study. GS, a glucosyltransferase enzyme catalyzes the transfer of sugar moieties in the formation of glycosidic bonds to generate the β - glucans. In *Candida* sp. the FKS1 catalytic subunit of GS was found likely to be an essential protein than the FKS2 subunit⁶ and therefore, the GS component of FKS1 subunit was considered in this study. The chitinase is the hydrolytic enzyme that cleaves the β -(1, 4) glycosidic bond of chitin and it is found to express in archaea, prokaryotes and eukaryotes. Chitinase, ChiA1 has role in fungal cell wall repair and morphogenesis, disruption of which leads to the altered hyphal growth rate and germination⁷. Generally the chitinases in plants and other chitinase producing organisms are utilized to protect themselves from the fungal infection where as in fungus it is also reported to have specific role in morphogenesis during the mother-daughter separation⁸ and hence the chitinases are considered as a potent target.

MATERIALS AND METHODS

In vitro analysis

Isolation of keratinophilic geophilic dermatophyte- Hair-bait technique

Isolation of dermatophyte *Microsporum gypseum* from soil sample was done using hair-bait technique⁹. Soil sample was collected from Thoppampatti area of Coimbatore District, India around the cattle farm. About 50 g of soil was placed

into empty sterile petri dish and baited with sterilized human hair, cut into small pieces. The soil was moistened with 5-10 mL of sterilized distilled water containing ampicillin (0.1 µg/mL) and cycloheximide (actidione) (0.1 µg/mL). The baited samples were incubated at 25°C in the dark and moistened at intervals of 3-4 days to prevent the soil from drying. The plates were inspected daily for up to 5 weeks before being discarded. The presence of keratinophilic fungi was confirmed by low power microscopic examination. Fragments of colonized hair were inoculated onto slopes of SDA containing cycloheximide and ampicillin and were incubated for 2 weeks at 25°C.

Inoculum preparation

Autoclaved SDA medium was poured in the sterilized petridishes and solidified, in which the spores of *M. gypseum* are swabbed using sterilized swabs and incubated at 28°C for 5 days and the grown mycelial mat before sporulation was further used for preparing inocula.

Plant material preparation

The aerial parts of *Enicostemma littorale* Blume were collected from Edappadi of Namakkal district, India during the rainy season of February to March of 2011. The collected plants were washed in tap water and shade dried for three to four weeks. Shade dried plant materials were made into dry, coarse powder using a mixer grinder and used for extract preparation. The extraction of shade dried, powdered aerial parts of *E. littorale* plant was carried out in soxhlet apparatus using five different solvents in the order of their polarity (petroleum ether < hexane < chloroform < ethyl acetate < methanol). About 50 g of the powdered plant material was placed in a porous cellulose thimble and the extraction was carried out to obtain crude extract. The crude extract was taken in three concentrations (3000 µg/mL, 2000 µg/mL and 1000 µg/mL) and considered for antidermatophytic assays.

Antidermatophytic assay

Antidermatophytic activity was determined using poisoned food technique¹⁰. The prepared inoculum was punched aseptically with a sterile cork borer of 6 mm diameter. The fungal discs are then placed on the agar medium impregnated with the prepared plant extracts at desired concentrations and incubated in room temperature. Colony diameter was recorded on successive days by measuring the two opposite circumference of the colony growth. Percentage inhibition of mycelial growth was evaluated by comparing the colony diameter of poisoned plate (with plant extract of conc. 3000, 2000, 1000 µg/mL) and non poisoned plate (with DMSO) were compared with negative control containing only SDA medium and positive control containing standard drug ketoconazole (0.1 mg/mL conc.) and the experiment was done in triplicate. Percentage of inhibition was calculated using the formula

$$\% \text{ Mycelial inhibition} = \frac{\text{Mycelial growth (control)} - \text{Mycelial growth (treatment)}}{\text{Mycelial growth (control)}} \times 100$$

In silico analysis

The fungal cell wall target chitinase (type-A) (PDB ID: 2XVP) was retrieved from Protein Data Bank and since the structure of 1, 3, β-D glucan synthase (GS) was not available, the sequence was retrieved from Uniprot database and modelled using online tool I-Tasser. Based on the C-score

value, the best modelled protein structure was energy minimized using SPDB Viewer and validated using SAVS online server to observe the quality of the structure through Ramachandran plot. The active site of chitinase were obtained from the literature and for GS, it was predicted using Q-site finder, in which the energetically favourable probe sites are clustered according to their spatial proximity and clusters are then ranked according to the sum of interaction energies for sites within each cluster¹¹. The 3D structure of phytoconstituents of *Enicostemma littorale* - apigenin, genkwanin, isovitexin, swertisin, saponarin, gentianine, enicoflavine, swertiamarin, quercetin, catechin, betulin, vanillic acid, ferulic acid, syringic acid, protocatechuic acid and *p*-coumaric acid and synthetic drugs ketoconazole, allosamidin, azetozolamide were retrieved from Pub Chem database.

Protein and ligand preparation

Docking of the selected targets with the respective ligand molecules was carried out using Glide module of Schrodinger suite. The proteins were pre-processed and minimized by generating the missing loops and residues and by removing all water molecules from the structure for generating heteromeric states. Charges and atom types were assigned. The ligand structures were subjected for ligand preparation to produce possible conformers of the ligand molecule based on ionization states, tautomers, stereo chemistries and ring conformations. Partial charges were assigned for both protein targets and ligand molecules according to OPLS_2005 force field. Receptor grid was generated covering the active site residues of the protein.

Docking

Docking was carried out using "Extra precision" (XP) mode of ligand docking option in glide module. Force field used was "OPLS_2005" and rest of the parameters was set to default. The docking results were interpreted using XP visualizer and evaluated based on the Glide score and bonding interactions.

RESULTS

In vitro Analysis

The keratin degrading geophilic dermatophyte was isolated using hair-baiting technique. After 4 weeks of incubation, the plate was observed with mixed number of microorganisms grown on hairs and were microscopically observed for its morphological characteristics and the pure cultures were obtained. The isolated keratinophilic fungus had the orange-brown reverse pigment, deep cream to tawny-buff to pale cinnamon coloured red surface of macroconidia which are large spindle shaped, 4-6 celled with verrucae on the walls. The shape, size and cell wall features of macroconidia are important characteristics for species identification. Based on the microscopic observation of morphological features and colony characteristics in culture tube, the organism was confirmed as *Microsporum gypseum*. The rate of inhibition was calculated with anti dermatophytic assay and petroleum ether extract was observed with maximum inhibition of 76 % at 1000 µg/mL concentration which gradually increases to 79 % and 85 % at 2000 µg/mL and 3000 µg/mL, respectively. Following petroleum ether, hexane extract was observed with 67 % of inhibition at 3000 µg/mL.

Table 1: Docking Analysis with Chitinase (Chia1) Enzyme

S. No.	Compound Name	G-score	No. of H-bonds	H-bond length	Interaction residues
1	Saponarin	-12.0	11	2.618 2.608, 2.291 2.240 2.226, 2.040 2.015 2.012, 1.989 1.949 1.928	TRP 312 (H) ... (O) ASN 233 (H) ... (O) (H) ... THR 278(O) (H) ... GLN 37(O) ALA 124 (H) ... (O) (H) ... GLN 230(O) TYR 232 (H) ... (O)
2	Swertiamarin	-8.2	6	2.349 2.184 2.017, 1.934 1.880 1.648	(H) ... GLN 37(O) ASN 233 (H) ... (O) (H) ... GLU 174(H) TRP 312 (H) ... (O) (H) ... ALA 279(O)
3	Isovitexin	-8.1	6	2.552 2.315 2.269 2.138 1.875 1.737	(H) ... GLU 174(O) ALA 124 (H) ... (O) ASN 233 (H) ... (O) (H) ... GLU 174(O) TRP 312 (H) ... (O) (H) ... ALA 279(O)
4	Swertisin	-7.9	4	2.585, 2.092 1.987 1.742	(H) ... GLU 174(O) ALA 124 (H) ... (O) ASN 233 (H) ... (O)
5	Catechin	-6.70	5	1.725 1.794 1.830 1.867	(H) ... ASP 225(O) (H) ... ASP 225(O) ARG 189(H) ... (O) (H) ... ASN 193(O)
6	Allosamidine (Synthetic Drug)	-9.94	9	2.633 2.006 2.069 2.179 2.207 2.280 2.455 2.597 2.616 2.623	(H) ... ASP 225(O) (H) ... GLN 37(O) (H) ... GLN 37(O) ARG 189(H) ... (O) ASN 76 (H) ... (O) ASN 40 (H) ... (O) ASN 193 (H) ... (O) ASN 193 (H) ... (O) (H) ... ASP 226(O) ASN 193 (H) ... (O)

Table 2: Docking Analysis with 1, 3 B-D Glucan Synthase

S. No.	Compound Name	G-score	H-bond	H-length	Interaction residues
1	Swertiamarin	-11.6	3	1.796 1.916 2.241	(H) ... MET 122(O) (H) ... CYS 124(O) (H) ... TYR 130(O)
2	Quercetin	-10.0	2	1.804 2.054	(H) ... MET 122(O) (H) ... ILE 141(O)
3	Catechin	-8.83	3	2.113 2.359 2.445	(H) ... LEU(O)143 (H) ... CYS(O)124 (H) ... ILE(O)141
4	Apigenin	-8.4	2	1.881 2.111	TYR 88 (H) ... O (H) ... ILE 141(O)
5	Genkwanin	-8.2	3	1.772 2.048 2.470	LEU 143(H) ... O (H) ... ILE 141(O) MET 120(H) ... O
6	Ketoconazole (Synthetic Drug)	-1.9	1	2.377	LEU 143 (H) ... (O)

Figure 1: Percentage inhibition of plant extracts on *M. gypseum* growth

Figure 2: Interaction between Saponarin and Chitinase

Methanolic extract exhibited very less inhibitory action and both ethyl acetate and chloroform extracts were observed with moderate activity against *M. gypseum* and the effect of DMSO on growth was negligible (Figure 1).

In silico Analysis

Chitinase (PDB ID: 2XVP) is an apoprotein of 310 amino acid length from *Aspergillus fumigatus* which has two chains A and B and complexed with a phosphate ion. 1, 3 β -D glucan synthase was modelled, due to the unavailability of the 3D structure and related homologs. The GS sequence with 240 amino acid (Uniprot Acc. No.: E5QZJ7) was modelled using I-Tasser and the modelled structure was based on the multiple templates 1K47A, 3MKSA, 1CT9A, 1KKHA, 1YT3A, 1QL5A, 3EAYA, 2XWUB and 2FNAA. I-Tasser predicted 5 models, of which, the model scoring least C-score was considered as top most model. First model was selected with C-score value -3.875, RMSD was calculated at 1.731 with template 1K47A and the energy was minimized to -6283.78 Kcal/mol using Swiss PDB Viewer. The validation of the modeled GS protein structure was observed in Ramachandran plot which showed 84.3 % of amino acids in the favourable region, 10.3 % in additional allowed region, and 2.7 % in both general allowed region and in disallowed region.

Active Sites of Fungal Targets

The active site residues and the surrounding hydrophobic residues of the chitinase (CHIA1) enzyme selected through the literature survey were ASP 172, GLU 174, TYR 232, GLN 230, TRP 312, TYR 125, ALA 124, GLN 34, GLN 207, ASP 170, PHE 60 and ASN 76¹². The active site of modelled 1, 3 β -D glucan synthase was predicted using online tool Q-site finder which predicted ten pockets of binding sites. Among them the first site was chosen for the study which consists of residues such as TYR 88, LEU 89, TRP 91, PHE 92, LEU 93, SER 118, THR 119, MET 122, TRP 129, TRP 130, ILE 133, VAL 134, LYS 140, ILE 141, VAL 142, LEU 143, GLY 144, LEU 145, MET 146, TYR 147, LEU 156, MET 160, ILE 163, ILE 164, TRP 185.

Docking Analysis

The selected fungal targets, 1, 3 β D glucan synthase and chitinase, were docked with the reported phytocompounds of *E. littorale* and compared with the respective synthetic drugs. The docking of chitinase enzyme (2XVP) with reported phytocompounds were compared with the specific synthetic drugs such as ketaconazole, acetazolamide and allosamidine. Among the phytocompounds docked with chitinase, flavonoids such as saponarin and swertiamarin were observed

Figure 3: Interaction between Swertiamarin and 1,3 β glucan synthase

with least G-score comparing to the phenolic acids such as vanillic acid, syringic acid, protocatechuic acid, ferullic acid and *p*-coumaric acid whereas the only flavonoid to have -4.6 as G-score was apigenin. However, the synthetic drugs allosamidine, acetazolamide and ketaconazole were observed with G-score of -9.94, -7.30 and -6.0, where allosamidine showed hydrogen bond interactions mostly with polar uncharged residues ASN at 40, 76 and 173 positions, 2 interactions with GLN 37 and one with polar negatively charged residue ASP226 and the bond length were observed in the range of 2-2.6 Å. The significant G-score obtained by phytocompounds and synthetic drug were tabulated (Table 1) along with the interacting residues. The interaction of the saponarin with chitinase is shown in Figure 2. The docking with GS was found to exhibit least G-score of -11.6 with swertiamarin forming 3 hydrogen bond interactions with the hydrophobic residues MET 122, TYR 130 and with CYS 124 whereas quercetin with G-score of -10.0 formed hydrogen bonding with MET 122 and ILE 141. Catechin, apigenin and genkwanin were observed with -8.83, -8.4 and -8.2 as G-score and the three compounds were found to interact commonly with ILE 141. The phenolic acids were observed with lesser G-score than the flavonoids and the number of hydrogen bonds and inter atomic distances of each interaction are tabulated (Table 2). Swertiamarin (Figure 3) exerts best G-score value in comparison with the synthetic drug where ketaconazole showed -1.9 and one interaction with hydrophobic residue LEU 143.

DISCUSSION

Through *in vitro* studies, the antidermatophytic potency of the extracts was evaluated. The activity was observed in the order petroleum ether > hexane > ethylacetate > chloroform > methanol and also the effect of DMSO on the *M. gypseum* growth was recorded at 1-5 % inhibition in the culture medium which is quite lesser than the inhibition rate of 20-50 % observed in *T. tonsurans* by Chan¹³. Hence the DMSO effect is considered negligible in the present study. There are reports already available showing the hexane extracts with better antimicrobial activity. The hexane, chloroform extracts of *Ocimum gratissimum* were proved effective against *M. canis*, *M. gypseum*, *T. rubrum* and *T. Mentagrophytes*¹⁴. The hexane leaf extracts of *Cupressus lusitanica* was fractioned using column chromatography in which the fraction 1 and 3 exhibited fungistatic potency with respective MICs 250 and 125 μ g/mL. The chemical composition of both the fractions were analysed by gas chromatography and gas chromatography-mass spectrometry and where the fraction 3 was rich in hydrocarbon sesquiterpenes (45.4 %) and relatively high amount of diterpenes and lipidic derivatives

whereas the fraction 1 possess only hydrocarbon monoterpenes with α -pinene and sabinene as major components¹⁵. Since the phytocompounds are the decisive reason behind for the plants various potential activities, the phytocompounds of *E. littorale* that are already reported are evaluated for its inhibitory activity mainly against the chitinase and 1, 3 β -D glucan synthase enzymes. Chitinase and 1, 3 β -D glucan synthase are the enzymes involved in the maintenance and repair of the cell wall^{16,17}. The docking analysis of the phytocompounds with chitinase enzyme showed favourable interactions. In this case, if the interactions are strong would ultimately lead to the cell wall inhibition and cell death. And also the interactions of various compounds with the target were found to be with the reported active site residues¹². GLU 174, ASP 172, TRP 312 and ALA 124 are the most conserved residues were found mostly interacting with the ligand molecules for the inhibition of chitinase. Flavonoid saponarin was observed to score least Glide score value of -12.0 forming 11 hydrogen bond interactions with the hydrophobic residues TRP 312, ALA 124 and TYR 232. The protein was observed to form hydrogen bond with other polar uncharged residues ARG 233, THR 278 and GLN 37. Of all compounds, flavonoids as a whole exhibited a better binding activity except apigenin and genkwanin. Phenols and phenolic acids exhibited the lowest binding affinity in the present study. Allosamidine is a strong AfchiA1 inhibitor¹⁸ and also has the least glide energy among synthetic drugs. However, the synthetic drugs allosamidine, acetazolamide and ketaconazole were observed with G-score of -9.94, -7.30 and -6.0 and the residues involved in the binding were GLN 37, ARG 189, ASN 76, ASN 40, ASN 193. Compared to allosamidine (G-score: -9.94; 9 interactions), saponarin (a flavonoid) exhibited better binding as evident via glide score of -12.0 and 11 interactions. Swertiamarin, a major component of *E. littorale*¹⁹ was found best to interact with GS by sharing three H-bonds with residues MET 122, CYS 124, and TYR 130 with G-score value of -11.6. On comparing with the synthetic drug ketaconazole with -1.9 as G-score, the other phytocompounds scored less. In spite of swertiamarin, other compounds like quercetin, catechin, apigenin, genkwanin and saponarin, which are flavonoids, were also observed to have low G score values with GS. In this *in silico* study, the flavonoids²⁰ from *Enicostemma littorale* were found to be highly responsible for the antifungal activity. Of the various flavonoids found in *E. littorale*, saponarin and swertiamarin (secoiridoid glucoside) can be sorted as a good antifungal agent against both fungal enzymes considered in this study, eventually targeting the disruption of the fungal cell wall and cell death. Though saponarin and swertiamarin of the plants show differential activity against each of the protein targets, they bind with more affinity than the compared available present day drugs. Natarajan and Natarajan²¹ reported that the ethanolic, ethyl acetate and hexane extracts of the *Acacia concinna* pods exhibited significant antidermatophytic property and also possessed the cell wall toxicity suggesting that the major component 'saponin' a detergent in *A. concinna* may be the reason for its fungicidal activity. According to Bowman and Free²², chitin is the minor but is an important component contributing for the fiber insolubility and for regeneration of the cell wall, whereas the β -1, 3 glucan-chitin complex is the major constituent of the inner wall. Hence these two compounds, saponarin and swertiamarin, can be considered for further analysis to develop as potential leads. Further studies can be carried out

to isolate the specific phytocompounds and can be studied for their potency to inhibit the cell wall through *in vitro* analysis. According to Rajendraprasad²³ who investigated the antidermatophytic effect of *Psoralea corylifolia* revealed that the plant seed extracts especially methanolic extract to show good inhibition, thereby the chromatographic analysis was carried out and found the active flavonoid a lipophilic compound possessing a -OMe group in the para position of β -phenyl ring was responsible for antifungal activity. Flavonoids of roots and leaves *Lantana camara* was reported to be fungicidal²⁴. Therefore, from the *in silico* and *in vitro* study, it is expected that flavonoids might have influence over the anti-dermatophytic activity of *E. littorale*.

CONCLUSION

The *in-vitro* and *in-silico* analysis shows *E. littorale* to be a potent anti dermatophytic plant. The active constituents of the plant possibly act against the dermatophytes by attacking the enzymes, viz. 1, 3 β -D glucan synthase and chitinase important to maintain their cell wall integrity. Therefore, more work is required to establish our plant of interest as an important drug source.

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