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Drug Repositioning for Dermatophyte Infections: Assessing Efficacy and Dermal Safety

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Abstract

This study explores the antidermatophytic potential and acute dermal toxicity of approved drugs, addressing the challenge of fungal resistance to existing therapies. Current antifungal treatments involve azoles, polyenes, allylamines, and echinocandins and other miscellaneous drugs. However, the rising resistance to these options prompted us to investigate alternative therapies using both *in silico* and *in vitro* methods, focusing on sterol 14α-demethylase- a key enzyme targeted by antifungal azoles. We selected antiepileptic drugs with similar pharmacophores as potential ligands. These compounds were sourced from PubChem, while the target proteins were obtained from the Protein Data Bank. Molecular docking and Molecular Dynamics (MD) simulations were performed using the Prescience *In Silico* Solution Suite (version V 2.1.0) to compare the binding efficiency of the selected ligands with two proteins: Human lanosterol 14-alpha-demethylase (PDB ID: 3LD6) and fungal lanosterol 14-alpha-demethylase (PDB ID: 5V5Z). Among the tested compounds, Nirvanol, Mephytoin, and Phenytoin exhibited effective binding to both proteins. Notably, Phenytoin demonstrated an average RMSD of 0.198 for the human protein and 0.146 for the fungal protein, with interaction energies of -146.05 kJ/mol and -191.64 kJ/mol, respectively. *In vitro* assays revealed that Phenytoin's minimum inhibitory concentrations were 2.5 mg/ml for *Microsporum canis*, 10 mg/ml for *Trichophyton rubrum*, and 1.25 mg/ml for *Epidermophyton floccosum* - significantly higher than standard antifungals like Itraconazole and Ketoconazole (0.01-0.02 mg/ml). Additionally, skin sensitization and acute dermal toxicity tests were conducted to assess safety. The findings suggest that Phenytoin, despite its liver-related side effects, has potential as an antifungal agent when used alongside bioactive compounds to mitigate toxicity.

Major Findings: This study evaluated the antifungal potential of antiepileptic drugs, identifying Phenytoin as a promising ligand targeting lanosterol 14-alpha-demethylase. Phenytoin demonstrated an average RMSD of 0.198 for the human protein and 0.146 for the fungal protein, with interaction energies of -146.05 kJ/mol and -191.64 kJ/mol, respectively. In vitro assays revealed that Phenytoin's minimum inhibitory concentrations were 2.5 mg/ml for Microsporum canis, 10 mg/ml for Trichophyton rubrum, and 1.25 mg/ml for *Epidermophyton floccosum,* significantly higher than standard antifungals like Itraconazole and Ketoconazole (0.01-0.02 mg/ml). Despite higher MICs than standard antifungals, Phenytoin showed acceptable dermal safety, suggesting its potential use with bioactive compounds to reduce toxicity.

Keywords: Acute Dermal Toxicity, Dermatophytic Fungus, Docking, Drug Repurposing, Fungal Resistance

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1. Introduction

Dermatophytosis (commonly called '*Tinea*') is a fungal infection that affects keratinized tissues like the skin, hair, and nails. It is caused by a group of fungi called dermatophytes, which are filamentous (thread-like) in structure. The Dermatophytes invade and feed on keratin, a protein found in these keratinized tissues, leading to the characteristic symptoms and manifestations of Tinea infections. It constitutes a significant proportion of infective Dermatoses worldwide, especially in developing countries. But over the last 5-6 years, the prevalence has alarmingly increased in some parts of the world, particularly in Southeast Asia. Although the initial reports of these atypical patterns, combined with reduced responsiveness to standard antifungal therapy, came from India and other countries of Southeast Asia, reports are emerging from west Asia, notably the United Arab Emirates, and Europe. Its gradual global march has been documented in several other countries like Iraq, Iran, Germany, Estonia, Switzerland, Finland, Poland, Cambodia, *etc*[1,2]. Recent prevalence of dermatophytosis in India ranges from 36.6-78.4%[3]. A major cause of this rapidly unfolding epidemic appears to be the rampant use of irrational combinations of potent topical corticosteroids, antifungal, and antibacterial agents. The unregulated sale and erratic, unsupervised use of topical steroids and Fixed-Dose Combination creams (FDC) containing steroids are considered prime culprits in the unprecedented epidemic of recalcitrant dermatophytosis on the Indian subcontinent as well as in Middle Eastern countries.

Further subsequent rise in immunocompromised patients including diabetic, cancer, HIV patients, and organ transplant recipients has accelerated the occurrence of fungal infection[4]. The most prevalent form of infection caused by fungi is superficial mycoses, belonging to the genus *Trichophyton, Microsporum,* or *Epidermophyton*. Individuals with impaired immune systems are susceptible to deeper infections[5]. In underdeveloped nations, where mycoses are common, dermatophytosis has a significant impact on hospital and community health care expenses[6]. Dermatophyte infections are highly prevalent worldwide, with estimates suggesting that one-fifth to one-quarter of the global population is affected by these fungi. These microorganisms exert their pathogenic effects by infiltrating and proliferating within the keratinized layers of the human body, including the skin, hair, and nails. Their ability to colonize and thrive in these keratinized tissues is a hallmark of dermatophyte infections. Dermatophytosis is a serious public health issue that can occasionally have an impact on a patient's quality of life due to the physical symptoms and detrimental psychological effects associated with dermatophytic infections. Dermatophytic infections are typically long-lasting due to their slow growth and are challenging to cure as they remain in superficial layers of the infecting site.

In recent years, drug repositioning has gained popularity. In contrast to conventional drug development methods, this approach is effective, cost-effective, and risk-free. The repurposing strategy is used to explore the potentials of existing drugs for other properties by using *in-silico* techniques (Molecular Docking, MD simulations) by exploring the FDA-approved drugs. Reductions in development time and expense are among the benefits that this approach offers over de novo drug development. Especially for resistance to drugs towards fungal species, this approach may significantly speed up the development of new antifungal medicines. Extensive use of available drugs and development of drug resistance towards the available pharmacotherapy is necessary to develop new antifungal drugs that have high efficacy and low toxicity[7]. Repurposing Anti-Epileptic Drugs (AEDs) for dermatophyte skin infections is novel because it explores an untapped therapeutic potential. AEDs, traditionally for neurological conditions, may offer new ways to combat increasing antifungal resistance[8]. This strategy allows for a faster, more cost-effective development of new treatments, as AEDs have established safety profiles. Their unique mechanism of action could also expand the antifungal arsenal and provide new insights for future drug development, addressing an urgent clinical need.

2. Materials and Methods

2.1 Docking Studies

2.1.1 Selection of Desired Protein

The desired proteins were obtained from a Protein Data Bank (PDB). The 3D structures for human 'Lanosterol 14 - alpha demethylase proteins with PDB ID: 3LD6 is shown in Figure 1a with Uniport ID: Q1680. Ketoconazole forms a complex with human lanosterol 14-demethylase (CYP51) crystal structure. Whereas 3D structures for fungal 'proteins with PDB ID: 5V5Z (Figure 2a, Uniport ID: P10613) further the reported resolution of 5V5Z

protein was 2.90 Å while the resolution of 3LD6 protein was 2.80 Å. Based on protein template 3D structure was determined using PRinS3 (Prescience *in silico* solution suite) version V 2.1.0. cast P images of both the proteins were as per Figure 1c.

2.1.2 Selection of Potential Ligand

Potential ligands were selected based on a similar pharmacophoric group as in standard drugs**.** The PubChem database was used for the collection of ligands in SDF format from as shown in Tables 1 and 2. These compounds were prepared for docking, using PRinS3 software version V 2.1.0.

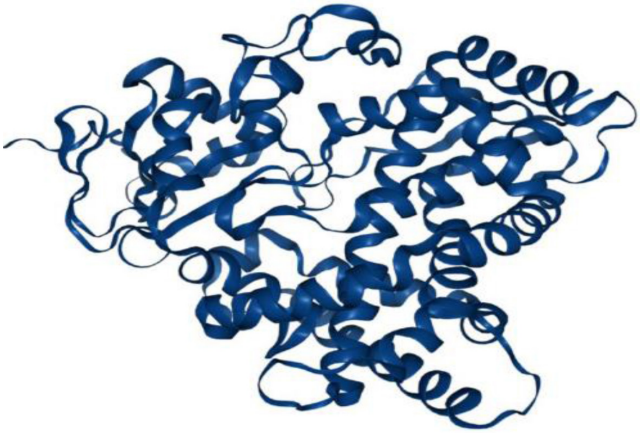


Figure 1(a). 3D structure of Human Lanosterol 14- alpha demethylase (PDB ID: 3LD6).

2.1.3 Pre-processing of Proteins

BioIn application from PRinS3 was used to prepare protein for docking. Processes like the addition of missing residues in protein, loop refinement, deletion of water molecules, and addition of hydrogen atoms were done using BioIn. The protein was downloaded in PDB file format.

2.1.4 Prediction of Binding Sites

The binding sites were found with the help of literature in two studies; the researchers have discovered binding sites for 3LD6 where the ligand was Epifriedelanol[10] and Methylphloroglucinal derivatives respectively[11]. *Epifriedelanol* is a natural plant compound derived from

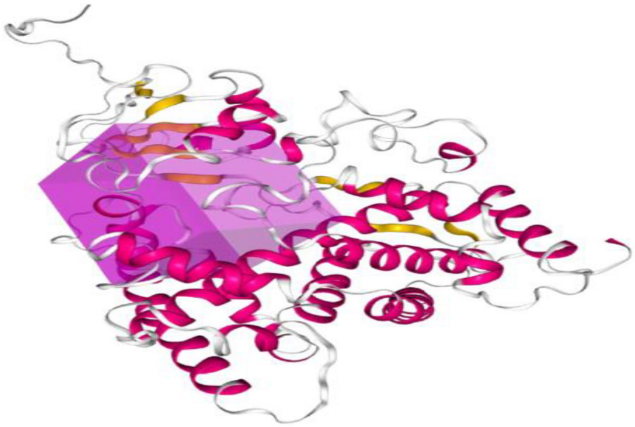


Figure 1(b). Binding sites of 3LD6 inside a grid box.

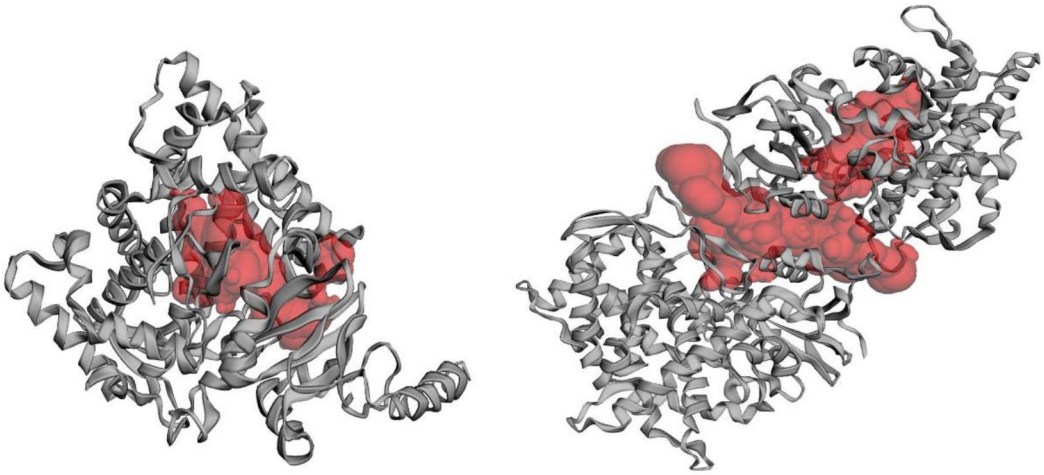


Figure 1(c). castP images of proteins (PDB ID: 3LD6 and 5V5Z).

Table 1. List of selected ligands

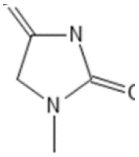
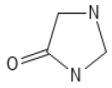
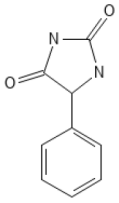
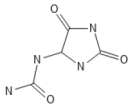
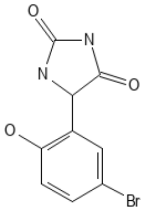
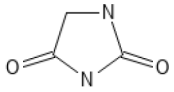
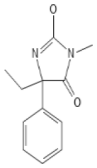
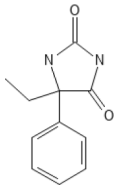
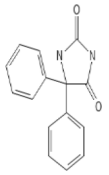
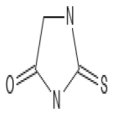
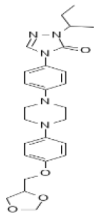
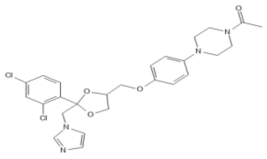
S. No.	Ligand Name	PubChem ID	Molecular Formula and Molecular Weight	Chemical Structure
1.	1-Methylhydantoin	PubChem CID: 69217	$C_4H_6N_2O_2$ MW: 114.1g/mol	
2.	4-Imidazolidinone	PubChem CID: 1502063	$C_3H_6N_2O$ MW: 86.09g/mol	
3.	5-Phenylhydantoin	PubChem CID: 1002	$C_9H_8N_2O_2$ MW: 176.17g/mol	
4.	Allantoin	PubChem CID: 204	$C_4H_6N_4O_3$ MW: 158.12g/mol	
5.	5-Bromo hydantoin	PubChem CID: 15716895	$C_3H_3BrN_2O_2$ MW: 178.97g/mol	
6.	Hydantoin	PubChem CID: 10006	$C_3H_4N_2O_2$ MW: 100.08g/mol	
7.	Mephytoin	PubChem CID: Compound CID: 4060	$C_{12}H_{14}N_2O_2$ MW: 218.25g/mol	
8.	Nirvanol	PubChem CID: 91480	$C_{11}H_{12}N_2O_2$ MW: 204.22g/mol	
9.	Phenytoin	PubChem CID: 1775	$C_{15}H_{12}N_2O_2$ MW: 252.27g/mol	
10.	Thiohydantoin	PubChem CID: 1274030	$C_3H_4N_2OS$ MW: 116.14g/mol	

Table 2. List of standard drugs

S. No.	Ligand Name	PubChem ID	Molecular Formula and Molecular Weight	Chemical Structure
1.	Itraconazole	Compound CID: 55283	$C_{35}H_{38}Cl_2N_8O_4$ MW: 705.6g/mol	
2.	Ketoconazole	Compound CID: 456201	$C_{26}H_{28}Cl_2N_4O_4$ MW: 531.4g/mol	

Guazuma ulmifolia and phloroglucinols demonstrated potent antifungal properties. Collectively the binding sites for 3LD6 was identified as 379Ile, 378Met, 487Met, 131Tyr, 135Thr, 134Leu, 382Arg. Similarly, for 5V5Z, the ligands that have been studied are coumarin derivatives and 3-sitosterol hydrogen phthalate which is a potential antifungal agent[12]. 380Phe, 118Tyr, 508, 304Ile, 131Ile, 132Tyr, 139Leu, 150Leu, 471Ile, 476Ala, 475Phe, 143Lys are the binding sites that were identified for 5V5Z Fungal Lanosterol 14 alpha-demethylases. The binding sites of protein 3LD6 inside a grid box and of protein 5V5Z are depicted in Figure 1(b) and 2(b) respectively.

2.1.5 Ligand and Protein Interactions

Ligand and protein optimization were done by using X-ESS (multi ligand enhanced sample screening). The target proteins were taken from the protein data bank in PDB format and SDF files were taken as input for ligands. Active site docking was performed by specifying the amino acid residue number of binding sites. For the docking process, the Lamarckian Genetic Algorithm (LGA) was used along with 100 GA runs to obtain statistically improved data.

2.2 Molecular Dynamics (MD) Simulation

Molecular Dynamics (MD) simulation studies are used to predict the binding poses and binding affinities of small molecules with the target proteins also helpful in the assessment of binding stability and any conformational changes in ligands during binding with proteins in our studies lowest binding energy was considered for MD simulation[11]. X-ESS application from PRinS3 was used to run simulations. The MD simulation time was set to 50ns.

2.3 *In-vitro* Studies

2.3.1 Procurement of Fungal Strain and Drug Sample

To evaluate antidermatophytic activity the three species of *Dermatophytes* were used and obtained from obtained from microbial type culture collection, Chandigarh *T. rubrum* (MTCC ID:3272), *E. floccosum* (MTCC ID: 7880) and *Microsporum canis* (MTCC ID: 3270). The fungal strains were cultivated on Sabouraud dextrose agar media (Difco, BD Biosciences) and incubated at 28° C. The marketed preparation phenytoin sodium (Manufactured by Zydus Healthcare Ltd.) was used for the evaluation of their antifungal potency and Itraconazole (Metro Chem API Private Limited, Sunder Nagar, Hyderabad) and ketoconazole (Arti Drugs Limited Manufacturer of Bulk drugs and Chemicals) taken as standard drugs.

2.3.2 Antifungal Sensitivity Test (AST)

The antifungal activity of samples was evaluated against the microbes, *T. rubrum*, *E. floccosum,* and *M. canis*. The activity was evaluated using the Disc Diffusion Method. The Muller Hin-ton Agar (MHA) medium was made in compliance with the standard protocol provided by Hi-media, which calls for dissolving 38 g of the medium in 1 liter of water. The media is subsequently autoclaved using an autoclave (Gentek India Pvt. Ltd.) at 121° C and 15 psi for 15 minutes. Following the sterilization procedure, aseptic procedures were used to transfer the culture media (20 milliliters per plate) into sterile glass Petri dishes placed in a laminar airflow (Toshiba, India). After allowing the plates to set in laminar, the media was

injected with a fungal culture dressed in liquid broth. A sterile glass rod was used to evenly disseminate 100µl of the culture broth into the media. Discs were inserted into medium plates using sterile forceps ten minutes after spreading. Following the samples diffusion into the media, the plates were paraffin-sealed and incubated for 24 and 48 hours at 37º C. 6 discs were used in the plates; three of the discs contained varying concentrations of the given sample, 2 of the discs contained 300 ppm concentrations of Itraconazole and Ketoconazole (taken as positive control), and one disc contained DMSO (taken as negative control) The clear zone surrounding the well, known as the zone of inhibition, it was observed on the fourth day of inhibition, and the diameter of each of these zones was calculated in millimeters[13,14].

2.3.3 Minimum Inhibitory Concentration (MIC)

Sabouraud dextrose broth was used to determine the lowest inhibitory concentration of the samples. The broth was made following standard composition and sterilized by autoclaving. Ten test tubes were used in the experiment, with the decreasing order of drug concentration *i.e.*, from highest to lowest, media control (containing broth and without test organism), and growth control (containing test organism and without test material). Next, 100µl of a fungal culture of the strain of fungal being studied that had been growing for 24 hours was put into each tube that held the test sample and medium. After the fungal culture was added, the tubes were plugged and incubated at 25º C for 48 hours. Following incubation, turbidity in the medium is a sign of fungal growth. Using a UV-visible Double Beam Spectrophotometer (PC-based Double Beam UV-vIS Spectrophotometer 2201). The optical density of each tube was quantified. The MIC value for this set of analyses is established by identifying the antifungal medication's minimum concentration at which there is no discernible turbidity. The tubes showing no turbidity were then spread on sabouraud dextrose agar plates to determine the minimum fungicidal concentrations. Plates with no colony appearance were recorded as the minimum fungicidal concentrations[15].

2.4 Skin Sensitization

The skin sensitization studies were performed using the Organization for Economic Cooperation and Development (OECD) guidelines. The OECD test guideline no. 406 was used for skin sensitization studies. The study presented was conducted at Deshpande Laboratories Pvt. Ltd. Bhopal, an ISO 9001:2008 and CPCSEA approved, Certified Drug Testing Laboratory (CPCSEA/IAEC Approval No.: CPCSEA/IAEC/DIPS/0304/1462).

2.4.1 Experimental Procedure

The temperature of the experimental animal room should be 20° C (+3° C) and the relative humidity 30-70 percent. Where the lighting is artificial, the sequence should be 12 hours light, and 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. Animals are acclimatized to the laboratory conditions for at least 5 days before the test. Before the test, animals are randomized and assigned to the treatment groups. Removal of hair is by clipping, shaving, or possibly by chemical depilation, depending on the test method used. Care should be taken to avoid

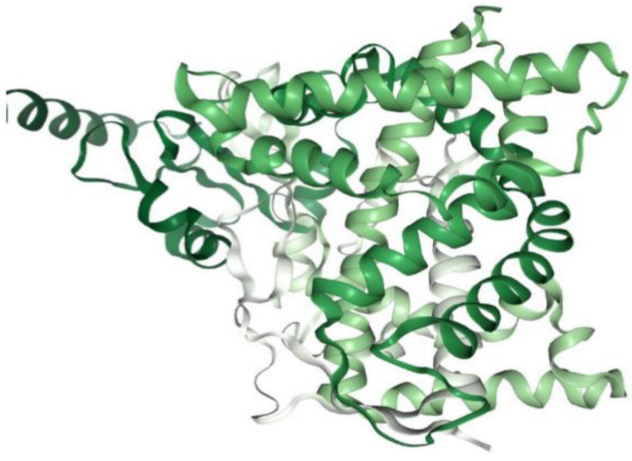


Figure 2(a). 3D structure of Fungal Lanosterol 14 alpha-demethylase (PDB ID: 5V5Z)

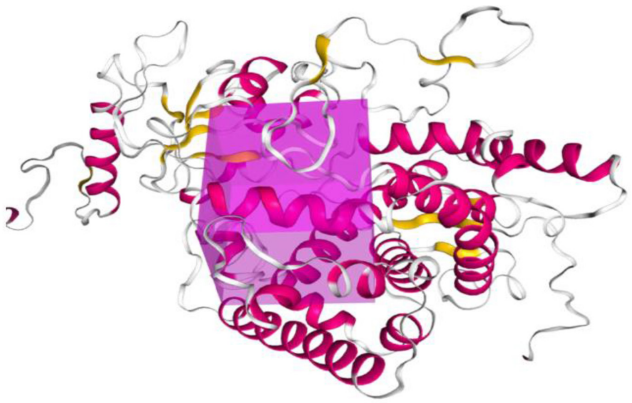


Figure 2(b). Binding sites of 5V5Z inside a grid box

a branding the skin. The animals are weighed before the test commences and at the end of the test. Three pairs of intradermal injections of 0.1 ml volume are given in the shoulder region which is cleared of hair so that one of each pair lies on each side of the midline. Approximately 24 hours before the topical induction application, if the test chemical is not skin irritant, the test area after close-clipping or shaving is painted with 0.5 ml of 10% sodiumlauryl sulfate in Vaseline, to create a local irritation. Observations are per Magnusson and Kligman grading scale[16].

2.5 Acute Dermal Toxicity

The acute dermal toxicity tests were performed using the Organization for Economic Cooperation and Development (OECD) guidelines. The presented study was carried out at Deshpande Laboratories Pvt. Ltd. Bhopal, an ISO 9001:2008 and CPCSEA approved, Certified Drug Testing Laboratory (CPCSEA/IAEC Approval No.: CPCSEA/IAEC/DIPS/0304/1462). This study used a qualitative descriptive analytical approach in experimental design. The OECD guideline for testing of chemicals- No. Test: 402, acute dermal toxicity-fixed doses procedure was the basis of the acute dermal toxicity test method that is being utilized[13]. This approach has been approved and acknowledged globally because it was chosen to optimize the study design by using the minimum number of possible experimental animals.

2.5.1 Experimental Animals

Adult female Wistar albino rats that had been nulliparous were utilized in this study. The rats were 200-300 g in weight, 8-10 weeks old, and in good health. The animals were housed in individual cages in the experimental room for a minimum of 5-6 days to allow them to acclimate. The cages had a base area of 350 cm^2 and a height of 18 cm, coated with wire and covered in husk. Around 25° C was the ambient temperature, the humidity ranged from 60 to 70%, and there was a 12-hour light or dark cycle. The animals were given 28g of commercial pellet meal per day, as well as an environment that was as little stressful as possible and unrestricted utilization of drinking water. The drugs were applied over the surface area (TBSA) of 10 % secured by porous gauze dressing and held in contact with skin for 24 hours[17].

2.5.2 Study Design

The acute dermal toxicity studies were divided into two *i.e.,* the Dose Range finding study, Primary test Figure 3(a) and then proceeds to the Main test stage Figure 3(b). Dose range finding study: If there is no information on the safety of the test items, a starting dose of 200 mg/kg is used as a single dose based on the 48 hours. Observation if there are no adverse clinical signs and mortality or morbidity. The same dose was selected for the main study. If adverse clinical signs and mortality or morbidity are observed at 200mg/kg, the dose will be reduced to 50 mg/kg. There is no toxicity seen up to 200 mg/kg in dose-finding studies, and then a 200mg/kg dose was selected for the main study. As per procedure, the main study was started with a dose of 200 mg/kg body weight[18]. The test materials Phenytoin 1% gel were applied to the exposed skin and covered with sterile gauze and surgical tape to ensure the test material was in contact with the skin for 24 hours. After 24 hours the surgical tape was removed and the remaining test material was rinsed with distilled water then observations were taken out 6, 24, 48, and 72 hours after exposure to test materials and continued up to 14 days as per OECD guidelines, 2017. The body weight was also recorded on treatment day (Day 1) and weekly once (Day 7 and Day 14)[19,20].

2.5.3 Clinical Observation

Clinical observations were done on both groups just after the exposure period. Hourly assessments get extra attention during the first 6 hours, are observed for 24 hours, and are then repeated once daily for the next 14 days. The body weight of each rat was determined, clinical evaluations were made visually, and test animals were observed to die. All information, which included toxic indicators, appearance time, toxicity duration and recovery period, body weight changes, and death time, was methodically documented and compared with the control group. It was vital to pay attention to the signs of tremors, seizures, drooling, diarrhea, lethargy, sleep, and coma. Changes in the skin, hair, eyes, mucous membranes, respiratory system, blood circulation, central and autonomic nervous systems, somatomotor activity, and respiratory system should all be noted.

Observation was generally carried out once a day in the morning because mice will become aggressive during the day. The body weight was also recorded

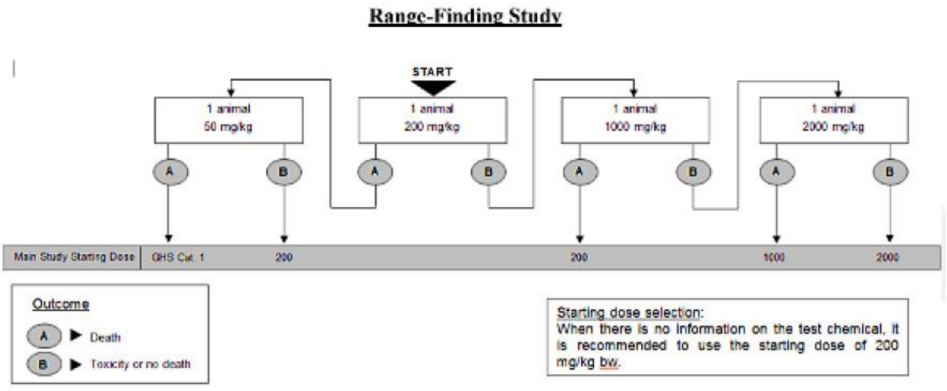


Figure 3(a) Dose range finding study (OECD, 2017)

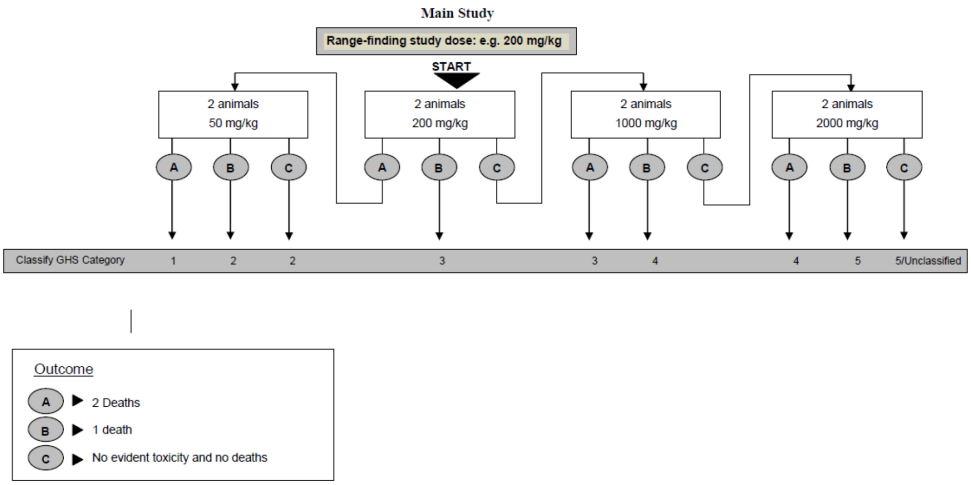


Figure 3(b) : Main evaluation stages (OECD, 2017).

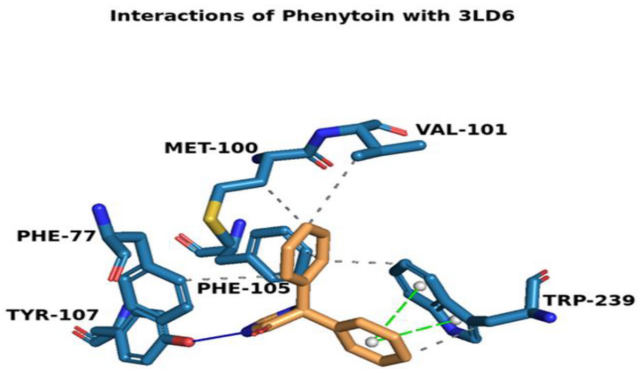


Figure 4. Interaction of phenytoin with 3LD6

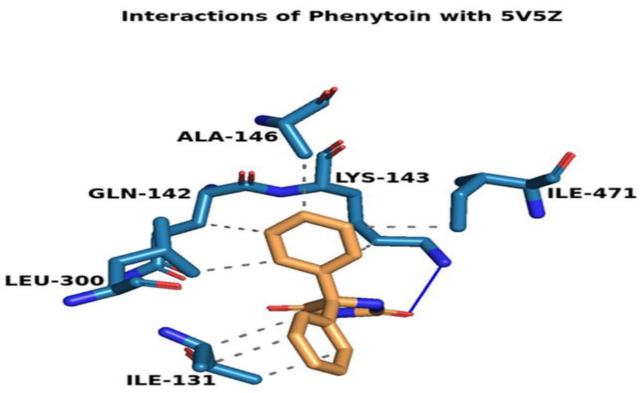


Figure 5. Interaction of phenytoin with 5V5Z.

on treatment day (Day 1) and weekly once. If animals are found to be dying, showing severe pain or signs of physical injury, the animal should be immediately sacrificed.

2.5.4 Data Analysis and Assessment of Acute Dermal Toxicity

The global harmonized system of classification and labeling of chemicals were used as the basis to determine

Table 3. Binding energy and amino acid residues of anticonvulsant and standard drugs against two protein targets

Proteins	Compounds	Binding Energy (kcal/mol)	Amino Acid Residue
3LD6	Nirvanol	-6.03	107 Tyr
	Mephytoin	-6.02	487 Met, 489 His
	Phenytoin	-7.32	107 Tyr
5V5Z	Nirvanol	-6.02	377Arg, 378 Ser
	Mephytoin Phenytoin	-6.14 -7.4	381 Arg, 463 phe, 470C ys 143 Lys
Standard drugs			
3LD6	Itraconazole	-14.07	-
	Ketoconazole	-11.88	379,489 His
5V5Z	Itraconazole	-12.23	-
	Ketoconazole	-11.24	132 Tyr, 377 His, 378 Ser

the LD50 of test preparations. A descriptive presentation of the non-parametric observation data will be made. The mean (x ± SD) is used to express the overall data. The Amended Draize test was used to classify the major irritation index. Based on a descriptive analysis of the results of the corresponding parameters, conclusions were drawn.

3. Results

3.1 Molecular Docking and MD Simulation

Based on the related pharmacophoric groups, drugs of the anti-epileptic category were selected for molecular docking and further MD simulation. Two protein targets were chosen, one as human lanosterol 14-alpha demethylase (PDB ID:3LD6) and the other fungal protein lanosterol 14-alpha-demethylase (PDB ID:5V5Z). In molecular docking calculations, each compound yields a set of nine top-scoring docked poses. Table 3 highlights the binding energy and amino acid residues of anticonvulsant and standard drugs against 2 protein targets. The interaction sites of the studied drugs and ligand-receptor contacts along with 2D representations of these interactions are demonstrated in Figures 4 and 5. Figure 6 gives a graphical representation of docking energy and docking score of complexes of selected ligands of the anti-epileptic category. The findings demonstrated that all ligands bind to the enzyme's active site and remain there for the duration of the MD simulations. The docking energy range for our desired ligands for 3LD6 was between -3.63 and -7.32 kcal/mol, whereas for 5V5Z it was between -3.37 and -7.4 kcal/mol. A cut-off value of 6.00-kcal/mol was set. The common drug molecules that fall inside the cutoff range for both proteins include Nirvanol, Mephytoin, and Phenytoin as summarized in Table 3, but out of them, phenytoin showed docking near standard drugs.

3.2 Molecular Simulation

The results are calculated based on average RMSD (Root Square Mean Deviation), Hydrogen bonds, Interaction energy, and COM (Centre of Mass) distance and summarized in Table 4.

3.3 Average RMSD (nm)

The RMSD (Root Square Mean Deviation) encapsulates the stability of the refined structure by displaying various fluctuations present in the complex during the MDS. The target's binding pocket residues Root Mean Square Deviation (RMSD) is computed by contrasting the MD equilibrated structure in the final frame of the MD simulation- with the original MD configuration. The results are shown in the RMSD *vs* ligand graph in Figure 7and for standard drugs in Figure 8.

The RMSD values are crucial for examining the equilibration of MD trajectories. The stability of each system was determined by plotting the RMSD of the core amino acid residues with and without standard drugs and phenytoin versus time (ps) Molecular dynamics performed at 50 ns. After 50 ns, a notable conformational shift was seen, which persisted until the simulation's conclusion (Figure 9).

3.4 Hydrogen Bonds

The graph displays the number of hydrogen bonds that exist between the ligand and the target. These hydrogen bonds are calculated using the angle 30° and bond length 0.35 nm criteria. All the complexes have hydrogen bonds in the range of 1-3 as in Figure 10.

3.5 Interaction Energy

The interaction energies between ligands and proteins, known as the non-bonded ones, (the van der Waals and

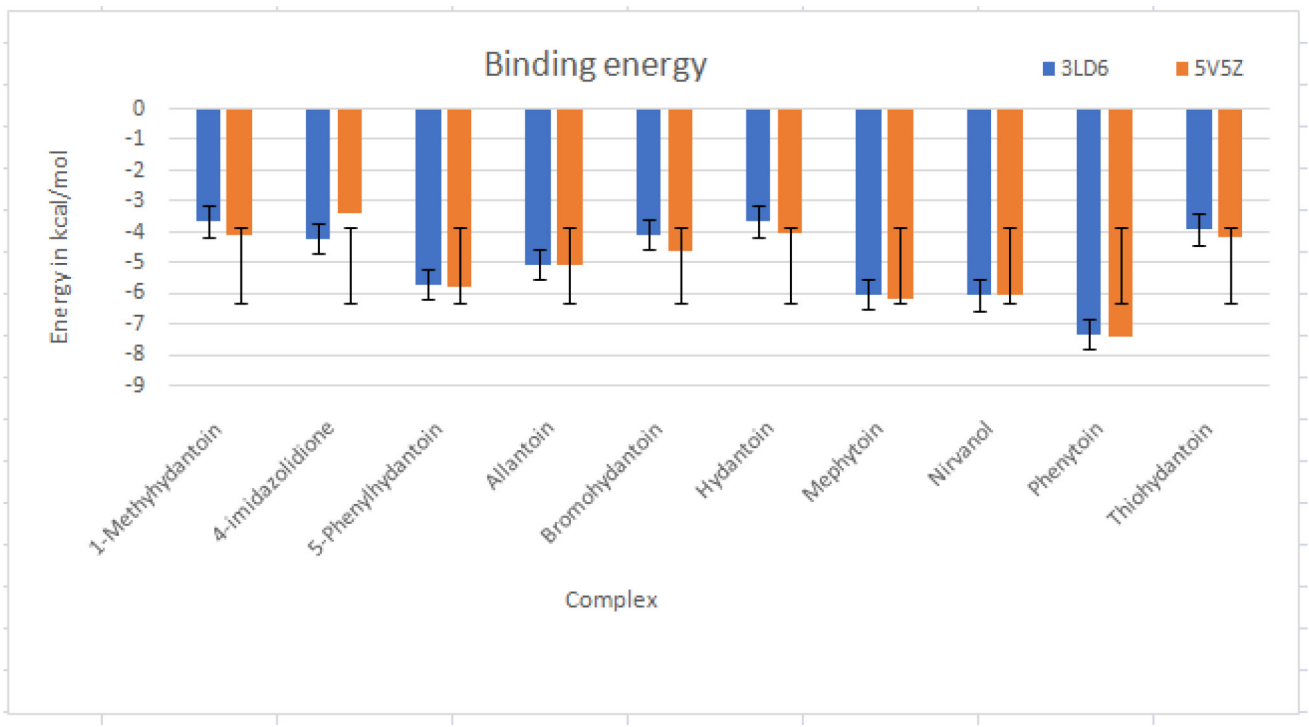


Figure 6. Graphical representation of docking energy and docking score of selected ligands of the anti-epileptic category.

Table 4. Compiled results of MD simulation of Antiepileptic drugs

Category	Protein	Drugs	Average RMSD (nm)	COM Distance (nm)	Interaction Energy(kJ/mol)			No. of H-Bond	Interacting Residues		
		Mephytoin	0.197	0.19	-50.6675	-85.863	-136.5305	2	489 HIS		489 HIS
	3LD6	Phenytoin Nirvanol	0.198 0.151	0.774 0.93	-52.6839 -13.0158	-93.3626 -57.8657	-146.0465 -70.8815	2 2	107 TYR 236 HIS	107 TYR 236 HIS	
Antiepileptic Drugs Standard Drugs	5V5Z 3LD6	Nirvanol Phenytoin Mephytoin Itraconazole	0.156 0.146 0.17 0.161	0.789 1.805 1.086 0.111	-45.8585 -108.669 -14.7906 -39.8839	-60.0417 -82.9706 -91.1838 283.918	-105.9002 -191.6396 -105.9744 -323.8019	2 3 2 2	132 TYR 143 LYS 381 ARG 135 THR	132TYR 132 TYR 381 ARG 378 MET	143 LYS

Coulomb interactions) are determined and depicted in Figure 11. This gives an idea about the strength of interaction between protein and ligand. For proteins with PDBID 3LD6 and 5V5Z, Mephytoin displayed favourable results based on the lowest interaction energy and hydrogen bonds. The interaction energy for Phenytoin was the lowest among all for both proteins.

3.6 *In-vitro* Studies

The antifungal sensitivity test was performed to determine the given sample's sensitivity towards the given sample phenytoin shows the zone of inhibition against *M. canis, T. rubrum* and *E. floccussum* tested against three concentrations the zone of inhibition shown by the

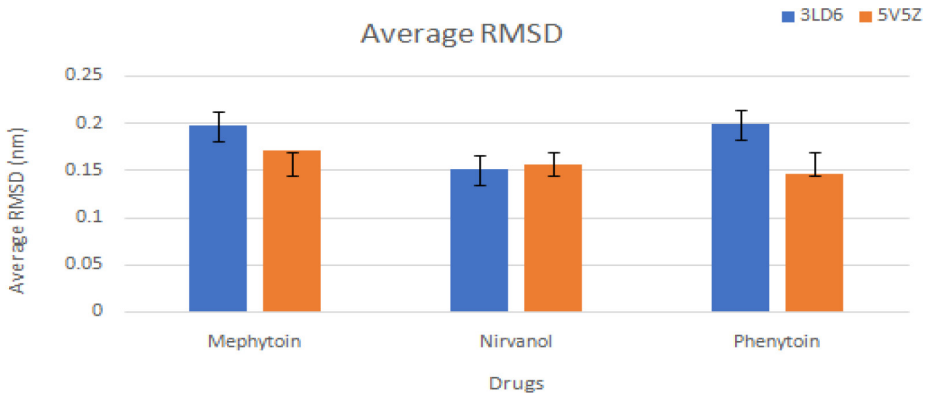


Figure 7. Graph of average RMSD (nm) of the target binding pocket residues (3LD6 and 5V5Z) with antiepileptic drugs.

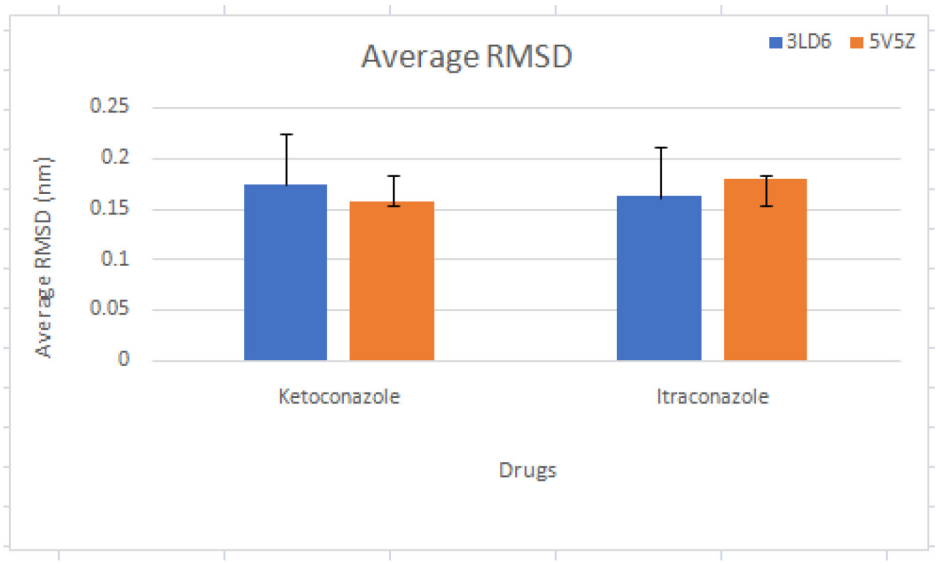


Figure 8. Graph of average RMSD the target binding pocket residues (3LD6 and 5V5Z) with Standard drugs.

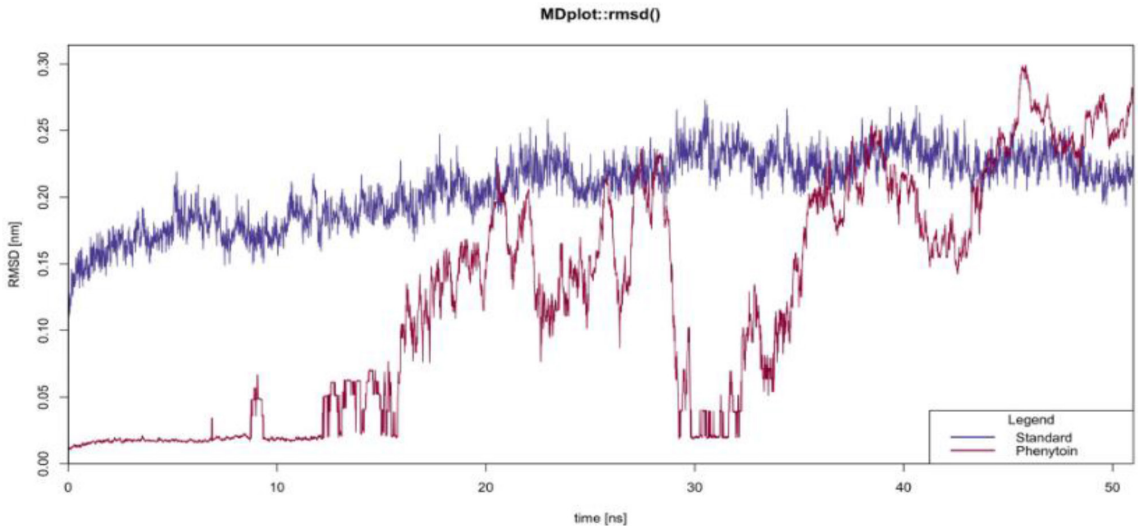


Figure 9. RMSD graph show protein ligands complex of standard drugs & phenytoin.

Table 5. Antifungal activity of phenytoin sodium tablets IP, Itraconazole and Ketoconazole (+ve control), distilled water (-ve control) against T. *rubrum, E. floccosum,* and *M. canis* using disc diffusion method

S. No.	Drug	Concentration (mg/ml)	Zone of Inhibition(mm)		
			T. rubrum	*E. floccussum*	*M. canis*
1.	Phenytoin Sodium Tablets IP	10 20 30	NIL 11.5 12.7	8.9 10.9 12.2	NIL 9.8 15.4
2.	Positive control (Itraconazole)	300 ppm	22.6	29	26.6
3.	Positive control (Ketoconazole)	300 ppm	13.9	10.1	NIL
4.	Negative control (DW)	-	NIL	NIL	NIL

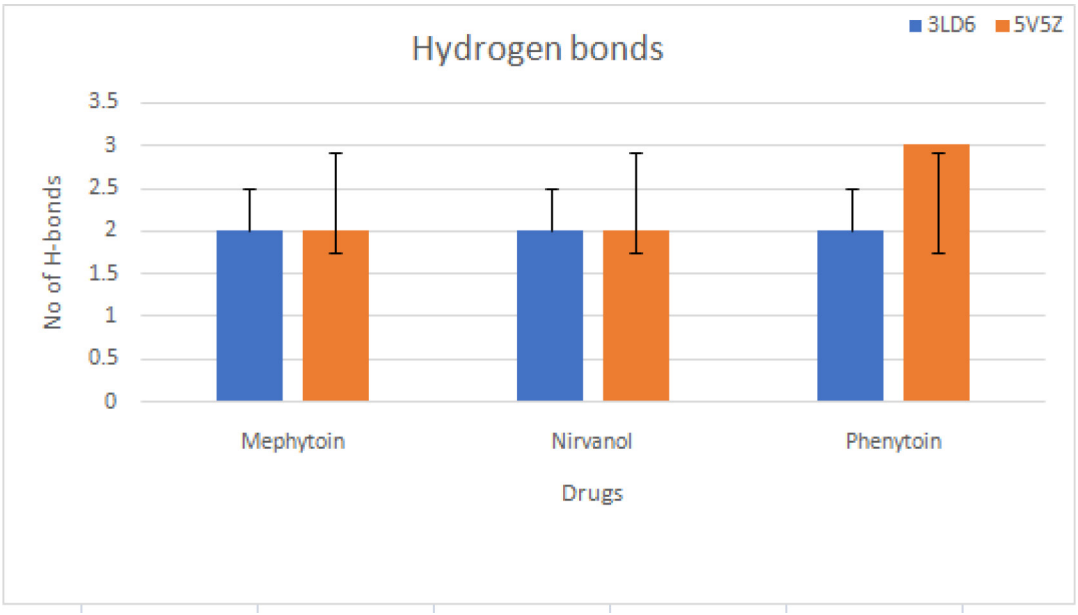


Figure 10. Graph showing the number of hydrogen bonds between the ligand and the target protein (3LD6 and 5V5Z) of antiepileptic drugs.

concentration shown by the concentrate on[9]. The results are summarized in Table 5. A clear zone of inhibition was observed by disc diffusion method by Phenytoin against all 3 fungal species *viz. E. floccussum, T. rubrum,* and *M. Canis* were observed in Figures 12,13, and 14 respectively.

The drugs showing good binding energy and docking score were selected for MD simulation and then for *in-vitro* analysis. The results were compared with the standard drugs Itraconazole and Ketoconazole. The docking energy range for the 3LD6 protein target was between -3.63 and -7.32 kcal/mol, whereas for the 5V5Z protein target, it was between -3.37 and -7.4 kcal/mol. The common drug molecules that fall inside the cutoff range for both proteins include Nirvanol, Mephytoin, and Phenytoin. The average RMSD (nm) for the 3LD6 protein target for phenytoin was found to be 0.198; COM distance (nm) was 0.774 and its interaction Energy (kJ/mol) was -146.0465 and the interacting residues were 107TYR 107 TYR. The average RMSD for the 5V5Z protein target for phenytoin was found to be 0.146; COM distance was 1.805 and its interaction energy (kJ/mol) was -191.6396 and the interacting residues were 143LYS 132TYR 143LYS.

The minimum inhibitory concentration value for Phenytoin as found against fungal strain *M. canis* was 2.5 mg/ml with *T. rubrum* 10 mg/ml and *E. floccosum* 1.25 mg/ml. The resultant minimum inhibitory concentration value for standard drugs Itraconazole and Ketoconazole against all three strains *M. canis*, *T. rubrum,* and *E. floccosum* was found in between 0.01 mg/ml - 0.02 mg/ml as summarized in Table 5.

3.7 Skin Sensitization

The skin sensitization studies were performed using a drug sample of Phenytoin 100 mg/kg dose for skin sensitization studies (Figure 15).

3.8 Acute Dermal Toxicity

The acute dermal toxicity test was conducted with an initial dose of 200 mg/kg BW. The major evaluation found no toxicity symptoms or deaths at this dose; thus it was continued. This includes the absence of signs of toxicity, weight loss, or death.

3.9 Clinical Observation

Observations includes changes in skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic, and central nervous systems, and somatomotor activity and behavior patterns. Experimental animals did not show any symptoms of toxicity such as deviations from normal activities, tremors, seizures, and diarrhoea (Figure 16). The eyes and mucous membranes as well as the respiratory system appear normal, with no abnormalities. The mortality rate was obtained at 0% because there were no deaths in the treatment and control groups.

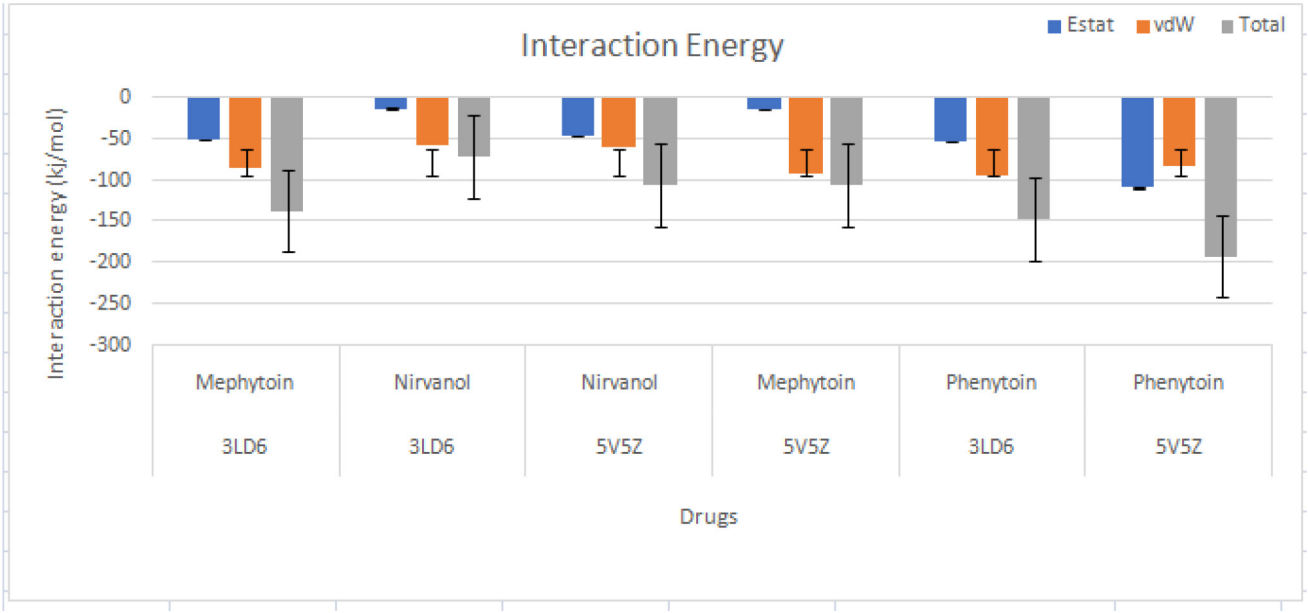


Figure 11. Graph showing interaction energy between the ligand and the target protein (3LD6 and 5V5Z) of antiepileptic drugs.

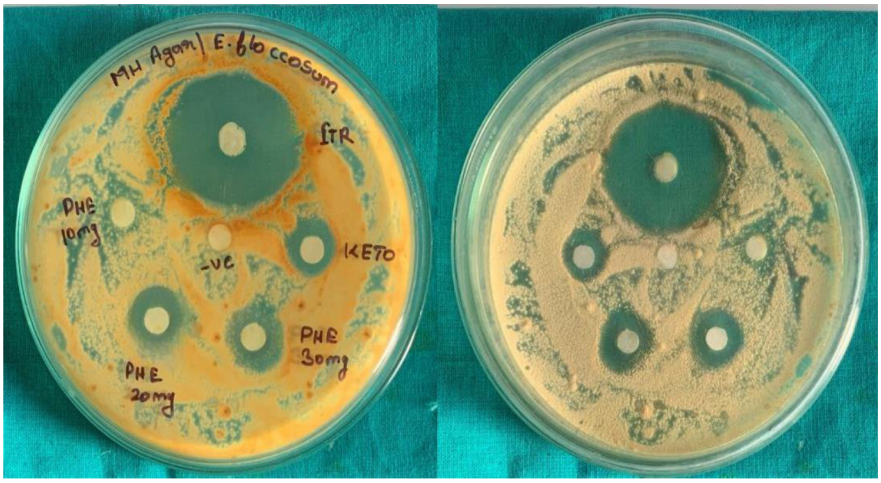


Figure 12. Antifungal Activity of sample Phenytoin Sodium Tablets IP(PHE) against *E. floccusum* using disc diffusion method.

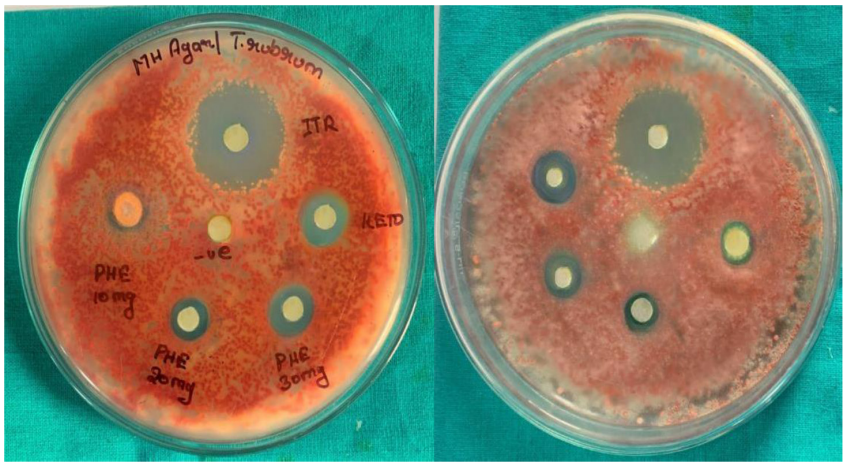


Figure 13. Antifungal activity of sample phenytoin sodium tablets IP (PHE) against *T. rubrum* using disc diffusion method.

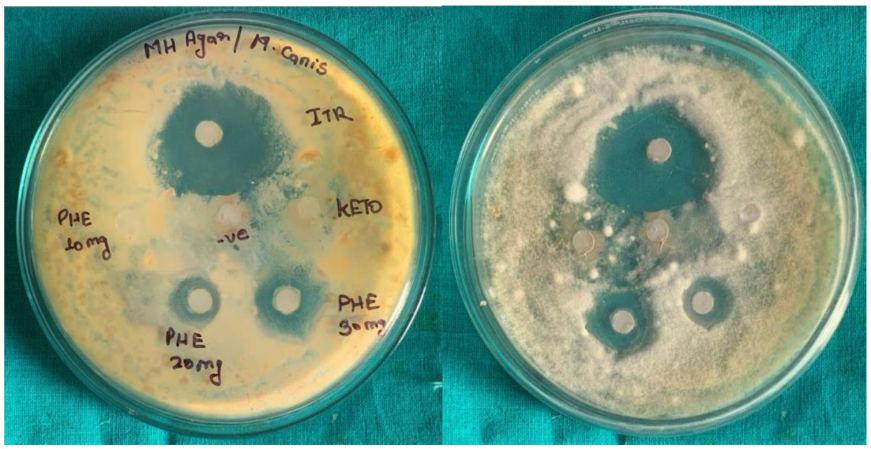


Figure 14. Activity of sample Phenytoin Sodium Tablets IP (PHE) against *M. canis* in disc diffusion method.



Figure 15. Local irritation with 0.5 ml lauryl sulphate Phenytoin



Figure 16. Control group (Simple 1% gel) and Treatment group (1% gel containing 2000 mg/kg)

4. Discussion

Cytochrome P450 (CYP450) enzymes are a diverse group of Heme- containing enzymes found in both humans and various organisms, including fungi. These enzymes play crucial roles in the metabolism of a variety of chemicals, such as endogenous molecules, medicines, and poisons. However, their specific functions and importance differ between humans and fungi, with human CYP450 enzymes being primarily involved in drug metabolism and fungi CYP450 enzymes contributing to secondary metabolite production and environmental adaptation. Drugs, steroids, fatty acids, and environmental toxins are just a few of the numerous endogenous and exogenous substrates that human CYP450 enzymes can metabolize. Fungal CYP450 enzymes are involved in the biosynthesis and degradation of fungal-specific compounds. They play a key role in the synthesis of fungal secondary metabolites, which can have various functions, including pathogenicity, defense against predators, and the adaptation of fungi to their environment. Human CYP450 enzymes, especially those found in the liver (*e.g.*, CYP3A4, CYP2D6, CYP1A2), are crucial for the metabolism of many drugs and are a major determinant of drug efficacy and safety in humans. Ergosterol is a sterol that is unique to fungi, and CYP51 (Erg11) is a member of the cytochrome P450 monooxygenase (CYP-450) super family that mediates an important step in ergosterol synthesis.

Repurposing antimicrobial drugs offers a promising avenue for treating a range of microbial diseases caused by parasites, fungi, viruses, and bacteria. This strategy presents advantages in combating antibiotic resistance and accelerating the availability of new therapies compared to the lengthy process of developing entirely novel drugs. Fungal species develop resistance through physiological adjustments or by acquiring beneficial mutations. To minimize this inevitable adaptation, it's crucial to understand the evolutionary and mechanistic factors driving the spread of resistant fungal infections. Comprehending these elements will ultimately contribute to the development of novel treatments and expand the therapeutic options for individuals worldwide suffering from fungal infections. The economic costs associated with fungal infections are still too great, even though fungal resistance is not on with bacterial resistance[20,21]. Clinical resistance to antifungal was rare during the early phases of antifungal therapy. One of the early instances of dermatophyte drug resistance to griseofulvin was documented by Michaelides *et al.* in 1961. Overcoming antifungal resistance is one of the key tactics for improving therapy for mycoses, especially considering the dearth of antifungal medications now accessible[22]. Burkhart *et al.* provided the first explanation of the function of biofilms in dermatophytosis[13]. They suggested that dermatophytes

can form biofilms *in vivo*. Fungal elements that are alive, such as hyphae, can be explained by this biofilm-forming ability and *Arthroconidia* that strongly adhere to the nail plate in cases of *Dermatophytes*, making them resistant to traditional therapies and more difficult to remove surgically[22,23]. CYP51 fungal resistance to Azoles develops primarily *via* point mutation changes, such as Y132F in *Candida albicans* which decreases drug binding affinity[24]. CYP51 overexpression together with the efflux pumps, Cdr1 and Mdr1, increases enzyme levels and decreases efficacy of the drug, resulting in actively removing the drug. Membrane changes reduce drug uptake while biofilms enhance tolerance by limiting penetration[26]. Azole resistance is common due to the same mechanisms of resistance. Fighting resistance can be achieved through combination therapy, efflux pump inhibitors, next generation azoles, such as isavuconazole, and targeting biofilms to weaken fungal defenses.

A good antifungal drug should barely affect human CYP51 enzymes. With this hypothesis we carried out docking on both human CYP51 and fungus CYP51 for better understanding and a comparison of the selectivity of drugs on fungus versus human enzymes. Antiepileptic Drugs (AEDs) are being investigated for new uses beyond epilepsy treatment[25]. This drug repurposing strategy offers a faster route to novel therapies compared to developing new medications and can help address the challenges posed by antimicrobial resistance. This approach holds the potential to tackle the increasing problem of antibiotic resistance and provide innovative strategies against infectious diseases. In the present study, we explored the potential of antiepileptic drugs as antifungal agents, specifically evaluating their effectiveness against dermatophytic fungi. Based on similar pharmacophoric groups, a select few anticonvulsant medications were chosen, and their potential to combat dermatophyte fungi was initially evaluated using *in-silico* methods. Following these computational results, the antifungal activity of phenytoin was specifically assessed against three common dermatophyte species-*M. canis, T. rubrum*, and *E. floccosum*- to determine their minimum inhibitory concentrations and perform an antifungal susceptibility test. Approved epilepsy drugs were investigated using both *in-silico* and *in-vitro* methods for their potential to inhibit sterol 14α-demethylase, a key target for antifungal azoles. The study compared the binding of these drugs to both the human (PDB ID: 3LD6) and fungal (PDB ID: 5V5Z) versions of this protein. *In-silico* analysis revealed that Nirvanol, Mephytoin, and Phenytoin exhibited binding affinities within an effective range for both proteins. Specifically, Phenytoin showed average RMSD values of 0.198 Å for the human protein and 0.146 Å for the fungal protein, with corresponding interaction energies of -146.05 kJ/mol and -191.64 kJ/mol. *In-vitro* testing determined the Minimum Inhibitory Concentrations (MICs) of Phenytoin against 3 common dermatophytes: 2.5 mg/ml for *M. canis,* 10 mg/ml for *T. rubrum*, and 1.25 mg/ml for *E. floccosum*. These values were notably higher than those observed for standard antifungal drugs like Itraconazole and Ketoconazole (0.01-0.02 mg/ml). To evaluate the safety of repurposing Phenytoin, further studies on skin sensitization and acute dermal toxicity were also conducted to rule out potential adverse effects in its new application.

5. Conclusions

The ongoing research brings forth the possible use of certain antiepileptic drugs, especially phenytoin, as antifungal agents targeting dermatophytes. *In-silico* assessment showed promising binding affinities of phenytoin to human and fungal CYP51, suggesting possible usefulness. Phenytoin was found to have antifungal activity against *M. canis, T. rubrum,* and *E. floccosum* with higher MICs than would be expected with Azole antifungals. Therefore, antiepileptic drugs, particularly phenytoin though has liver related side effects, show potential for treating dermatophyte infections and may be evaluated further for its potential role as anti-dermatophytic agent.

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