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Exploring the promising antifungal activity of stigmasterol from *Azadirachta indica* A. Juss. kernels against black fungus

[1]S Sangeetha and [2*]S. Uma Gowrie

[1]Department of Plant Biology and Plant Biotechnology, Ethiraj College for Women (Autonomous), Affiliated to the University of Madras, Chennai- 600 008 (India)
[2]Ethiraj College for Women (Autonomous),
Affiliated to the University of Madras, Chennai- 600 008, Tamil Nadu (India)
Email: umasezhian@gmail.com

Abstract

Mucormycosis or Black Fungus is a serious third most fungal infection with high mortality reported as post COVID infection in immunocompromised patients. Rhino Orbital Cerebral Mucormycosis is a common disease in India caused by *Rhizopus oryzae* contributing to 90% of infections. Ergosterol is an essential component for fungal survival, a key enzyme lanosterol 14α-demethylase plays a major role in synthesizing ergosterol and has become a novel target for drug discovery. Novel drugs from plants are in high demand due to fungal resistance towards azoles. The objective of the present study is to analyze the potency of the five phytocompounds screened from GC-MS analysis of neem kernels against the target protein lanosterol 14α-demethylase and compared with the standard. The structure of ligands and standard were retrieved from the PubChem database. The target protein sequence was downloaded and modeled using SWISS MODEL. The stereochemical quality of the target protein was validated using the Ramachandran Plot and ERRAT. After validation, docking was carried out using PatchDock server for all the ligands and standard against the target. Among the phytocompounds docked, stigmasterol topped the list which indicates that it can act as a potential lead compound from natural resources in therapeutic application of fungal infection with minimum side effects.

Key words : *Azadirachta indica* A. Juss., Mucormycosis, Lanosterol 14α-demethylase, stigmasterol, Patch Dock.

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[1]Research Scholar, [2*]Principal

The emergence of Severe Acute Respiratory Syndrome Corona - Virus 2 (SARS-CoV-2) in 2019 at China declared as global pandemic in the year 2020 with high mortality and morbidity. As the battle of COVID-19 continued globally with a second and third wave, an uplift in the occurrence of post COVID-19 complications were reported. Among all, mucormycosis delineated as COVID-19 associated mucormycosis (CAM) by the World Health Organization (WHO) and Pan American Health Organization (PAHO) due to the prevalence of this disease during COVID-19 infection[9].

Mucormycosis is an uncommon and life threatening fungal infection that affects COVID-19 patients severely with a prevalence of nearly 80 times higher in India than globally. Immunosuppression and Diabetes mellitus are considered as common risk factors for mucormycosis. India has the second largest population with Diabetes mellitus[31]. The primary reason for mucormycosis in COVID-19 patients as a post COVID complication is due to low oxygen level (hypoxia), steroid-induced hyperglycemia, new-onset hyperglycemia, diabetic ketoacidosis (DKA), metabolic acidosis, increased iron levels and decreased phagocytic activity of white blood cells (WBC) due to immunosuppression by SARS-CoV-2 or by extensive use of steroids[20]. There is no genetic link between COVID-19 and mucormycosis, they are immunologically interconnected. COVID-19 infection decreases the amount of immune cells that maintain the homeostasis of the immune system such as CD4+ and CD8+ T cells, which leads to increased susceptibility to fungal infection. A combination of COVID-19 and Diabetes Mellitus may be the reason during the pandemic for the huge prevalence of mucormycosis in India than in other developed countries[12].

Mucormycosis or Black fungus is an angioinvasive fungal infection caused by the genus *Rhizopus, Mucor, Rhizomucor, Cunninghamella* and *Absidia* belonging to the order Mucorales. *Rhizopus oryzae* accounts for 60% of cases in humans and 90% of Rhino Orbital Cerebral (ROCM) form which is the most common type of mucormycosis in India[34]. Almost 84.4% of affected mucormycosis patients had a history of COVID-19 which occurs in three variants where 77.6% of them are Rhinocerebral, 34.3% are Cutaneous and 3% are Pulmonary in nature[33]. Fungal spores enter the host via inhalation, ingesting contaminated food and traumatic inoculation. Depending on the site of spore deposition, disease manifestation occurs. Initially, the spores come in contact with the host tissue, then they turns into hyphal forms. Finally, it invades blood vessels and causes tissue infarction, necrosis and thrombosis[7].

In managing mucormycosis, Mycoses Study Group Education and Research Consortium (MSGERC) and the European Confederation of Medical Mycology (ECMM) prescribed surgical debridement of infected tissue if needed in addition to antifungal therapies[11]. Liposomal amphotericin B is the first therapy for treating mucormycosis with a minimum dosage of 5mg/kg/day. Isavuconazole and Posaconazole were used as adjunctive therapies[26]. In India, the difficulty in managing mucormycosis is majorly due to i) discontinuity in the treatment protocol ii) financial constraints - affected individuals not able to

afford Liposomal amphotericin B which is very expensive[23]. Modern therapeutics involves several drawbacks such as drug resistance, acute and chronic side effects, less clinical efficiency and effect on non-target cells. In order to overcome these effects, there is an urge around the world to find novel and efficient antifungal drugs with cost-effective and reduced side effects[13]. 80% of the global population relies on plant-based medicines as a source of primary health care reported by the World Health Organization (WHO) and the significance of phytocompounds in the field of medicine was clearly depicted where 25% of drugs approved by Food and Drug Administration (FDA) are plant based[30].

With this concept in mind, we present the use of the indigenous medicinal plant *Azadirachta indica* for the treatment of mucormycosis due to its proven antifungal properties. *A. indica* is a multipurpose tree species belonging to the family Meliaceae. International Scientific Community included neem in the top ten list of medicinal plants for "Sustainable development of the planet and for the health of living beings"[35]. More than 150 biologically active compounds have been isolated from various parts of the tree[25]. Different parts of neem exhibit various pharmacological activities such as anti-bacterial, anti-fungal, anti-viral, antimalarial, antioxidant, anti-cancer, anti-hyperglycemic, antipyretic, anti-inflammatory and immunostimulant. It is estimated that 2,50,000 higher plant species are distributed on earth but only 15% of plant's phytochemicals were characterized[3]. This lacuna is due to the requirement of extensive resources, time and workforce for creating a new drug which is an intricate process even in today's scientific world. Therefore, the use of *in-silico* techniques in the drug development process helps to find out the direct interaction of phytocompounds with the target protein.

Hence, the objective of the present study is to attempt computational screening of bioactive compounds of neem kernels from GC-MS analysis against the target protein lanosterol 14 α-demethylase of *Rhizopus oryzae*. Lanosterol 14α-demethylase is an essential enzyme in the fungal life cycle that plays a crucial role in the synthesis of ergosterol which maintains the cell membrane integrity of fungi and has become a novel target for drug discovery[18].

Collection, Processing and Preparation of extract :

Mature fruits of neem were collected from Ethiraj College for Women Campus, Chennai, Tamil Nadu, India (Latitude: 13.064231°; Longitude:80.258806°) and deseeded. Seeds were manually cleaned to remove the debris immediately after collection, air-dried in shade and dehulled to separate the kernels for further extraction procedures. The kernels of *Azadirachta indica* in the ratio 1: 10 (w/v) were extracted using HPLC grade methanol by maceration technique for GC-MS analysis[6].

Gas Chromatography-Mass Spectrometry (GC-MS) analysis :

The methanolic extract of neem kernels was subjected to GC-MS analysis at SRM Central Instrumentation Facility, SRM University, Kattankulathur to identify the bioactive compounds. The samples were

analyzed using a Model 7890B-GC coupled with 5977A MSD (Agilent, USA). The spectrum of the components was compared with the database of the spectrum of known components stored in the GC-MS NIST (2011) library[14].

Ligand preparation :

Five phytocompounds were identified and selected as ligands from the GC-MS analysis of the methanolic extract of neem kernels. The structure of the selected ligands was retrieved from PubChem database (https: //pubchem.ncbi.nlm.nih.gov/) in SDF format. Then, the structures were converted into PDB format using Open Babel software (version 2.3.1)[19] for docking against the target protein. Similarly, the structure of the standard was also retrieved which was used as a positive control.

Target preparation :

The sequence of the target protein Lanosterol 14α-demethylase was retrieved from the National Center for Biotechnology Information database (NCBI) (ACCESSION: EIE87079.1). Till date, the crystal structure for Lanosterol 14α-demethylase has not been documented. To obtain a 3D structure for docking, the FASTA sequence was submitted to SWISS MODEL for modeling. Then, the stereochemical and overall quality of the modeled 3D structure was analyzed using PROCHECK[17] and ERRAT tool to validate the protein structure before docking.

Molecular Docking :

Docking of ligands and standard against the target protein was carried out using PatchDock server (https://bioinfo3d.cs.tau.ac.il/PatchDock/). Ligand and receptor molecules were uploaded in PDB format for docking. The best docked structure was downloaded, saved as a PDB file and viewed using Chimera in order to study the interaction of the docked complexes.

Gas Chromatography-Mass Spectrometry (GC-MS) analysis :

The chromatogram of methanolic extract of neem kernels revealed several peaks, of which five phytocompounds were selected based on their probability range above 60% and promising biological activities for docking against the target protein. The selected phytocompounds are summarized in Table-1.

Structure of ligands and Standard :

The chemical structure of phytocompounds such as Hexadecanoic acid methyl ester, Palmitic acid, Methyl Stearate, Oleic acid and Stigmasterol retrieved from the PubChem database were shown in Figure 1. Similarly, the structure of standard Isavuconazole was also retrieved and depicted in Figure 2.

Structure and Validation of target protein :

The structure of target protein Lanosterol 14α-demethylase was modeled using SWISS MODEL (Figure 3) and validated using PROCHECK (Ramachandran Plot), ERRAT prior to docking to confirm the accuracy and reliability of the structure. Ramachandran Plot revealed 91.6% residues in most favoured regions, 8.0% residues in additional allowed regions, 0.0% in generously allowed regions and 0.4% in disallowed regions for the modeled

protein structure (Figure 4). A good quality model contains most torsional angles in the allowed regions and expected to have over 90% in the most favoured regions. Since 91.6% of residues are present in most favoured regions, it is concluded as a quality model and can be used for molecular docking studies. Overall quality factor of the target protein was 95.35% analyzed through ERRAT (Figure 5). Good high-resolution structures generally produce values around 95% or higher thereby suggesting that the structure is of good quality.

Molecular docking :

Docking studies were carried out using PatchDock server for all five ligands and standard against the target protein. Results had revealed that the phytocompounds docked against the target exhibited varied docking scores given in the order as follows, Stigmasterol > Oleic acid > Methyl Stearate > Hexadecanoic acid methyl ester > Palmitic acid (Figure 6). Among the ligands docked, Stigmasterol topped the list with the docking score in comparison with the standard Isavuconazole docking score. Based on the docking score, the compounds exhibited their binding affinity with the target. This confirms that the Stigmasterol from neem kernels has the potential to inhibit the enzyme Lanosterol 14α-demethylase with good binding affinity responsible for the production of ergosterol which is essential for fungal survival. The docking interaction of all the ligands and standard against the target protein using PatchDock server were shown in Figure 7.

Mucormycosis is the third most common invasive fungal infection that affected COVID-19 patients severely as a secondary infection[32]. The rise of mucormycosis in India during the COVID storm is mainly due to immune suppression and Diabetes mellitus. The U.S Food and Drug Administration (FDA) approved the first triazole drug Isavuconazole to treat mucormycosis after a VITAL trail as a substitute to Posaconazole and Amphotericin B due to its lack of availability and toxicity. Posaconazole an another azole that is less effective than Isavuconazole leading to treatment failure where the MIC values of posaconazole were found to be 2-4 folds lower than those of Isavuconazole for Mucorales species[8,24].

Isavuconazole acts by inhibiting the Lanosterol 14α-demethylase enzyme which is responsible for converting lanosterol to ergosterol. Ergosterol plays a crucial role in maintaining membrane fluidity, permeability and the proper functioning of membrane proteins. By inhibiting the ergosterol biosynthesis pathway via the fungal cell membrane's CYP450 enzyme, the integrity of the fungal cell membrane gets altered, affecting its morphology and growth. Accumulation of cytotoxic sterol reduces ergosterol production which leads to fungal cell death. The major drawback of Isavuconazole is its resistance like other azoles which includes ERG3 gene mutation which impairs the azole-mediated cell membrane disruption and mutation of the gene encoding the target enzyme (ERG11)[28].

Considering the side effects of the azole, researchers have explored the efficacy of phytocompounds as Lanosterol 14α-demethylase inhibitors. The present study focuses on the virtual investigation of five major phytocompounds from neem kernels

Table-1. GC-MS analysis of methanolic extract of neem kernels

S. no	Name of the Compound	Retention time (minutes)	Molecular formula	Molecular weight (g mol^{-1})	Compound nature	Biological activities
1.	Hexadecanoic acid, methyl ester	12.97	$C_{17}H_{34}O_2$	270.5	Fatty acid methyl ester	Antioxidant, Anti-inflammatory, Hypocholesterolemic[5].
2.	Palmitic acid	14.32	$C_{16}H_{32}O_2$	256.42	Saturated fatty acid	Antibacterial, Antifungal, Antioxidant, Hypocholesterolemic, Anti-androgenic[1,2].
3.	Methyl Stearate	16.73	$C_{19}H_{38}O_2$	298.5	Fatty acid methyl ester	Antibacterial and Antifungal[1].
4.	Oleic acid	17.63	$C_{18}H_{34}O_2$	282.5	Unsaturated fatty acid	Reduces cholesterol level and blood pressure, anti-inflammatory effects on autoimmune disease, anticancer, Improves immune system[29].
5.	Stigmasterol	31.22	$C_{29}H_{48}O$	412.7	Unsaturated Phytosterol	Antimicrobial, Antioxidant, Anti - inflammatory, Anti-hypercholesterolemic, Antidiabetic, Anti-osteoarthritic, and Immunomodulatory[15].

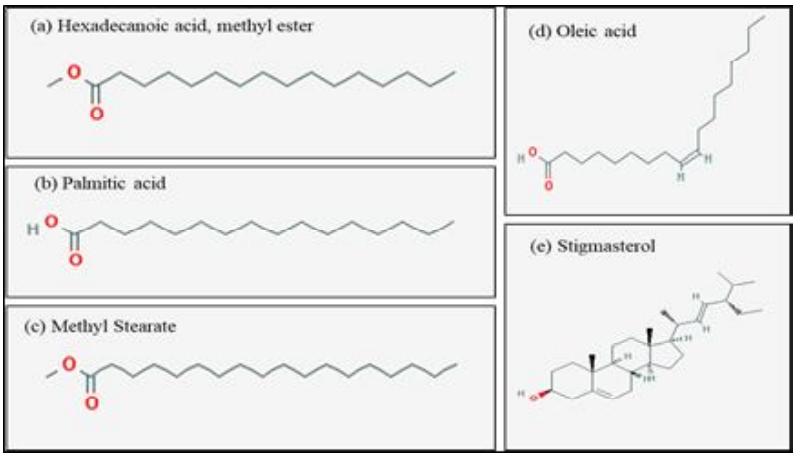

Fig. 1 (a-e): Structure of ligands present in the methanolic extract of neem kernels

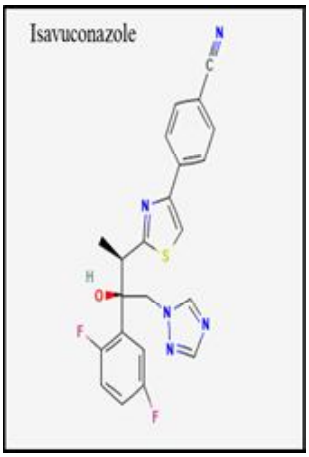

Fig. 2: Structure of Standard

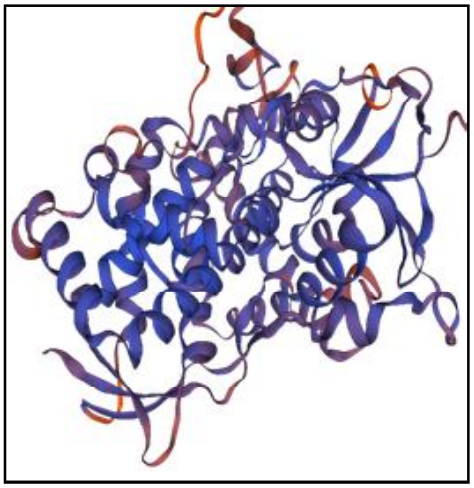

Fig. 3: Structure of target protein Lanosterol 14α-demethylase

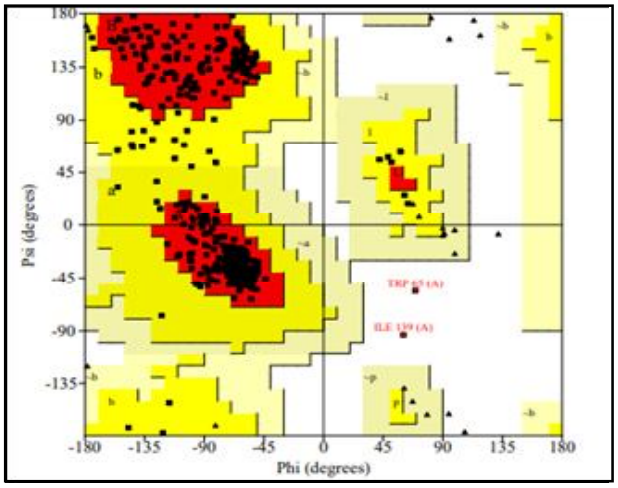

Fig. 4: Ramachandran Plot

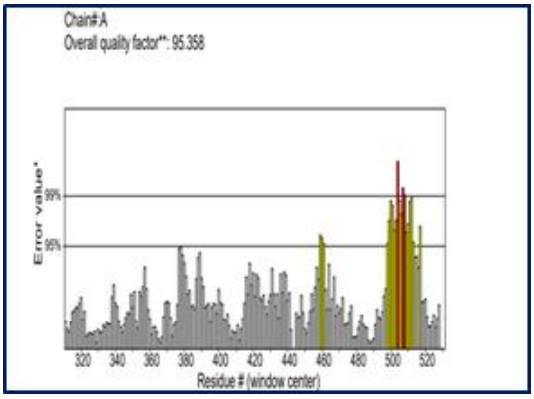

Fig. 5: ERRAT analysis

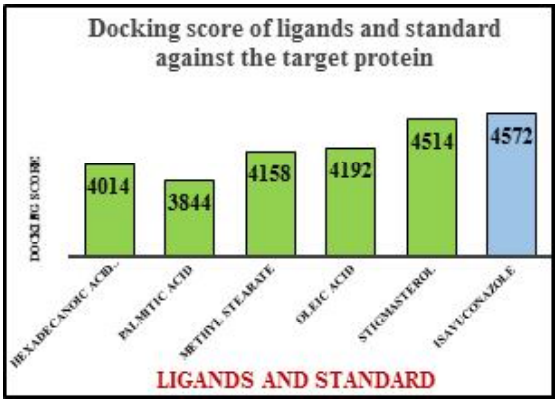

Fig. 6: Docking score of ligands and standard against the target protein Lanosterol 14α- demethylase using PatchDock server

against the black fungus. Among the phytocompounds docked, Stigmasterol exhibited a higher docking score of 4514 with an ACE value of -366.47 Kcal/mol in comparison with the standard Isavuconazole docking score 4572 and ACE value -362.70 Kcal/mol. The ACE value of Stigmasterol was found to be on par with the standard Isavuconazole revealing its higher binding affinity with the target protein.

Atomic Contact Energy (ACE) value is a good indicator to find out the efficiency of the lead molecule with the target protein[27]. From the results, it was evident that the stigmasterol can act as a potential ligand by inhibiting the target protein similar to the inhibition mechanism followed by FDA-approved triazole Isavuconazole due to its biological properties.

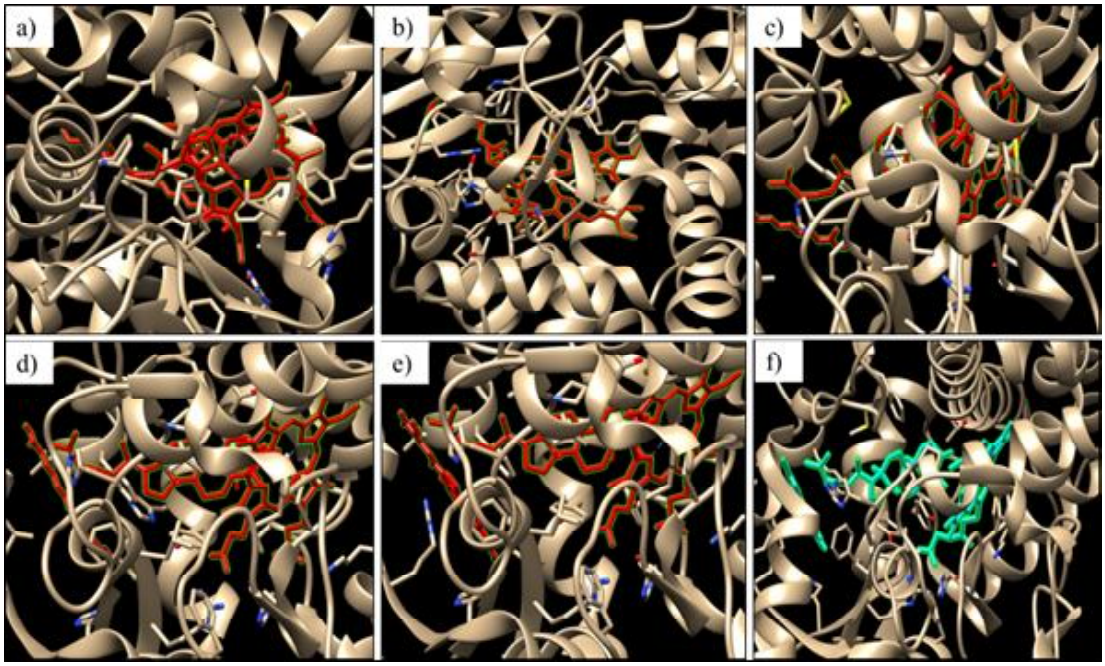

Fig. 7: Docking interaction of a) Hexadecanoic acid methyl ester b) Palmitic acid c) Methyl Stearate d) Oleic acid e) Stigmasterol and f) Isavuconazole against the target protein Lanosterol 14α- demethylase using PatchDock server.

In addition to its inhibition potential, Stigmasterol also helps in improving the immune system and controlling the diabetes of an individual which is a boon for the mucormycosis treatment where immune suppression and diabetes play a key role in the susceptibility of the COVID-19 patients. Previous experimental approaches on cyclophosphamide-induced immunosuppression in mice exhibited an effective immunomodulatory effect of stigmasterol by improving various hematological parameters and stimulating non-specific immune response[4]. Based on the previous reports, the antidiabetic activity of stigmasterol is due to the regeneration of β-cells of Langerhans of the pancreas leading to the secretion of insulin thereby controlling the blood glucose level[21].

Similarly, literature-based search for phytcompounds from neem and turmeric were reported. Initially, 256 compounds were found and Quercetin was identified as the most active compound from both the plants and provided an insight into their efficacy against mucormycosis through a three way approach - first by boosting the immune system, second via inhibiting necrosis in the affected tissues and finally as iron chelating agent imposing an antifungal effect on COVID Associated Mucormycosis (CAM). Neem targets more genes in various pathways compared to turmeric through functional network analysis and molecular docking approaches[9].

The structure of 25 flavonoids with

antifungal activity was retrieved from the PubChem database to check its binding affinity against the target protein Lanosterol 14α-demethylase, of which phloretin derived from apple tree leaves was found to be the best candidate molecule with a binding affinity of -7.8 Kcal/mol. Absorption, Distribution, Metabolism and Excretion (ADME) results of phloretin revealed a bioavailability score of 0.55 which implies phloretin can act as a good antifungal drug in the future[10].

Polyphenols such as Rutin, Quercetin, Kaempferol, Vanillic acid, Ferulic acid and Catechin were identified and quantified using HPLC analysis from eight selected plants to exhibit its antifungal potential against the target protein 14α- demethylase and nucleoside diphosphokinase (NDK) via *in-silico* studies. Rutin, kaempferol and quercetin were identified as common polyphenols among the selected plants. Rutin revealed the highest affinity of -9.4 K_d value against fungal 14α-demethylase and -8.9 K_d value against nucleoside diphosphokinase than the other phytocompounds[16].

Eugenol, a phenol-like aromatic phytocompound investigated for its antifungal activity against *Rhizopus oryzae* via *in-vitro* analysis, including ergosterol quantification to test inhibition of ergosterol production mediated antifungal action and to confirm *in vitro* findings, molecular docking and Molecular Dynamics (MD) simulation were performed with Lanosterol 14α-demethylase of *R. oryzae.* Eugenol showed a 26±1 mm zone of inhibition against the isolated *R. oryzae* through agar well diffusion assay compared to clotrimazole (29±2 mm). *In-silico* analysis of eugenol with the target protein, a key enzyme in the ergosterol pathway, strengthens the *in vitro* findings with the binding value (ΔG_{Bind}) of - 48.34 Kcal/mol[22].

Thus, the results of the present study support the previous investigation on natural resources which has revealed promising evidence that phytochemicals can inhibit the growth of fungi and can act as potent inhibitors against the target protein.

The design of new antifungal molecule is a worldwide priority, mainly for fungal diseases like “Mucormycosis” which develop resistance against existing medications and treatments. More than 300 million people are reported to suffer from serious fungal infections globally. The severity of fungal infections elevated the need to identify antifungal drugs from natural sources for the treatment and prevention of infection. From the present study, we conclude that stigmasterol is effective and able to impede the growth of fungal infection by replacing synthetic antifungal drugs. As per our knowledge, this is the first report of stigmasterol from neem kernels inhibiting the target protein Lanosterol 14α-demethylase responsible for ergosterol production. Thus, there is a need for *in vitro and in vivo* studies to confirm the efficacy of stigmasterol against mucormycosis.

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References :

1. Agoramoorthy, G., M. Chandrasekaran, V. Venkatesalu and M.J. Hsu (2007). *Brazilian Journal of Microbiology*. *38*: 739-742.
2. Anwar, P.Z, U.G. Sezhian and A. Narasingam (2020). *International Journal of Pharmaceutical Sciences and Research*. *11*: 5553-5560.
3. Asghar, H. A, S. Q. Abbas, M. K. Arshad, A. Jabin, B. Usman and M. Aslam (2022). *Scholars International Journal of Traditional and Complementary Medicine*. *5*(3): 47-64.
4. Bakrim, S., N. Benkhaira, I. Bourais, T. Benali, L. H. Lee and N. El Omari (2022). *Antioxidants*. *11*: 1912.
5. Belakhdar, G., A. Benjouad and E. H. Abdennebi (2015). *Journal of Materials and Environmental Science*. *6*(10): 2778-2783.
6. Bendigeri, S., G. Das, K. Shrman, S. Kumar, R. K. Khare and S. Sachan (2019). *International Journal of chemical studies*. *7*(4): 126-131.
7. Bhadra, A., M. S Ahmed, M. A Rahman and S. Islam (2021). *Bangabandhu Sheikh Mujib Medical University Journal*. *14* (COVID 19 Supplement): 51-56.
8. Chatur, V. M., S. G. Walode, M. Rudrapal, G. Sanjog, S. Doshi and G. A. Shrawani (2021). *Systematic Reviews in Pharmacy*. *12*(9): 490-495.
9. Datta, S., I. Sarkar, G. Sen and A. Sen (2022). *Journal of Biomolecular Structure and Dynamics*. *28*: 1-4.
10. George, S. C., R. Shaji, N. Kuriakose, J. P. Tom and A. Mechoor (2022). *VSRD International Journal of Biotechnology and Pharmaceutical Sciences*. *11* (Special issue): 94-98.
11. Ghosh, D., S. Dey, H. Chakraborty, S. Mukherjee, A. Halder and A. Sarkar (2022). *Clinical Epidemiology and Global Health*. *15*: 101013.
12. Hussen, N.H., A.H. Hasan, J. Jamalis, S. Shakya, S. Chander and H. Kharkwal (2022) *Computational Toxicology*. *24*: 100247.
13. Jadhav, A. K., P. K. Khan and S. M. Karuppayil (2020). *International Journal of Applied Pharmaceutics*. *12*: 18-30.
14. Karunanithi, B and K. T. Maria (2015). *International Journal of Engineering Research and Applications*. *5*(5): 32-37.
15. Kaur, N., J. Chaudhary, A. Jain and L. Kishore (2011). *International Journal of Pharmaceutical Sciences and Research*. *2*(9): 2259-2265.
16. Khanzada, B., N. Akhtar, M. K. Okla, S. A. Alamri, A. Al-Hashimi and M. W. Baig (2021). *Molecules*. *26*: 7164.
17. Kumar, P and A. Arya (2018). *Pathfinder Research and Training Foundation*. 1-7.
18. Madanagopal, P., N. Ramprabhu and R. Jagadeesan (2022). *Bulletin of the National Research Centre*. *46*(1): 1-21.
19. O'Boyle, N. M., M. Banck, C. A. James, C. Morley, T. Vandermeersch and G. R. Hutchison (2011). *Journal of cheminformatics*. *3*(1): 1-4.
20. Pippal, S. K., D. Kumar and L. Ukawat (2021). *Indian Journal of Otolaryngology and Head & Neck Surgery*. *23*: 1-7.
21. Poulose, N., A. Sajayan, A. Ravindran, A. Chandran, G. B. Priyadharshini and J. Selvin (2021). *Frontiers in Nutrition*. *8*: 694362.
22. Prajapati, J., P. Rao, L. Poojara, D. Acharya, S.K. Patel and D. Goswami (2023). *Current*

Microbiology. *80*: 47.

23. Prakash, H and A. Chakrabarti (2021). *Microorganisms*. *9*: 523.
24. Rawlani, S. S., A. Siddiqui, M. Reza, S. Chelkar, T. Rani and H. K. Roy (2021). *International Journal of Medical and Dental Research*. *1*(2): 1-8.
25. Reddy, I. V and P. Neelima (2022). *International Journal of Economic Plants*. *9*(1): 59-63.
26. Riad, A., A. A Shabaan, J. Issa, S. Ibrahim, H. Amer and Y. Mansy (2021). *Journal of Fungi*. *7*: 837.
27. Roy, A., P. Rajagopal and L. Thangavelu (2020). *Bioinformation*. *16*(10): 748.
28. Sahu, R. K., M. M. Salem-Bekhit, B. Bhattacharjee, Y. Almoshari, A. M. Ikbal and M. Alshamrani (2021). *Antibiotics*. *10*: 1079.
29. Sales-Campos, H., P. Reis de Souza, B. Crema Peghini, J. Santana da Silva and C. Ribeiro Cardoso (2013). *Mini reviews in medicinal chemistry*. *13*(2): 201-210. 25.
30. Sangeetha, S and S. U Gowrie (2021). *Indian Journal of Natural Sciences*. *12*(68): 34770-34780.
31. Sengupta, I and T. Nayak (2022). *Mycology*. *32*(3): 101257.
32. Shah, N. N., Z. Khan, H. Ahad, A. Y. Elderdery, M. N. Alomary and B. Atwah (2022) *Journal of Infection and Public Health*. *15*: 1299-1314.
33. Shahi, A and S. K. Mishra (2021). *Drug Discovery*. *15*(36): 221-230.
34. Singh, A. K., R. Singh, S. R Joshi and A. Misra (2021). *Diabetes & Metabolic Syndrome: Clinical Research & Reviews*. *15*: 102146.
35. Tiwari, N (2022). *Asian Journal of Pharmacognosy*. *7*(3): 51-60.