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Research Article

GC-MS Analysis and *In-silico* Docking Study of Active Antifungal Components of *Entada rheedei* Spreng. (Seeds)

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ABSTRACT

Entada rheedei Spreng., is a liana or a climber belonging to the family Fabaceae and is widely distributed in tropical and subtropical areas. The seeds of *E. rheedei* Spreng. has been found to contain important phytoconstituents such as phenolics, thioamides and saponins. In this study, we investigated the antifungal properties of *E. rheedei* Spreng. and imply *in-silico* methods to study its bioactive phytoconstituents. The aqueous extract of the seeds exhibited significant antifungal inhibitions against *Aspergillus flavus* and *A. fumigatus*. GC-MS analysis reveals the presence of 13 bioactive compounds that could be potent fungal inhibitors. Subsequently, *in-silico* Molecular docking analysis recognised benzoic acid, 2, 4-bis (trimethylsilyloxy)- trimethylsilyl ester as the active antifungal constituent of the aqueous extract with a docking score of -8.0570 and -9.4564 kcal/mol against *A. flavus* and *A. fumigatus* respectively. The *in-silico* studies were further backed by 100 ns molecular dDynamics simulation studies. This study can thus lead to the production of potent plant-based antifungal drugs.

INTRODUCTION

The phytomedicines are part of our culture and were followed as traditional or indigenous knowledge practices. However, modern medicines replaced them from our lifestyle. Though there are tremendous developments in the field of modern medicine, laterally there are many new challenges arising. Over time, the demand for the herbal-based formulation for curing these diseases is increasing. India being a country with rich bio resources has plenty of herbal formulations and many plants as well as tree parts were widely used for curing many diseases in different forms.[1] Plant kingdoms are rich sources of secondary metabolites, many of which have been used for medicinal purposes.[2] Nagaland, a north-eastern state of India is still yet to be explored scientifically. One such area is the district Tuensang of Nagaland inhibited by the indigenous Chang Naga tribe. Tuensang district shares an international border with the country Myanmar on the eastern sector and lies between 26°142 N latitude and 94°492 E longitudes.[3] Traditional use of plants still prevails in many areas of Tuensang district despite the continuous advancement of modern medicine.

Many villagers depend on local practitioners and used locally available medicinal plants as a substitute of modern medicine.[4] *Entada rheedei* Spreng. is one such plant commonly found in the Tuensang district of Nagaland which is believed to have valuable medicinal properties. *E. rheedei* Spreng., is a woody climber shrub of the Family Fabaceae inhabitant to most tropical countries including India.[5]

The presence of bioactive phytochemicals in this plant may account for its various medicinal properties and its extensive use in traditional medicine. Previous investigations have shown that several *Entada* species

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contain saponins in considerable amounts. Four phenolics: protocatechuic acid, protocatechuic acid methyl ester, 1,3,4-trihydroxybenzene glucoside, phaseoloidin, three thioamides: entadamide A, entadamide A-β-d-glucopyranoside, entadamide C, and two saponins: rheedeioside A and rheedeioside B were isolated from Ethanol extract.[6] Previous phytochemical studies on the genus *Entada* revealed the presence of saponins,[7,8] thioamides, Sulfur-containing amides from Entada phaseoloides[9,10] and phenylacetic acid derivatives.[11]

A number of phenolics, thioamides and saponins were isolated and characterized from various extracts of the seed.[12] Md. Abu Sufian and coworkers isolated Entadatamide A from the ethyl acetate extract.[13] Tyrosine O-glucoside and dopamine 3-O-glucoside have also been isolated from the seeds of Entada.[14] Two triterpenoid saponins have also been isolated from the seed kernels of *E. rheedii.*[15] The presence of these phytochemicals enhances interactions with the biological system in the form of herbal drugs.[16] Medicinal plants can form an excellent source for the derivation of newer drugs. The knowledge base on folk medicinal practitioners can in this instance form an invaluable source on which further scientific studies may be based, for the folk medicinal practices that dates back to centuries ago. They also have proved to be a rich source of new active compounds which are less toxic and less costly when compared to synthetic drugs. Tobacco made from the seeds of this plant has been reported to cause vivid dreaming and, for this reason, the plant is commonly known as African dream herb or snuff box sea bean.[17] *E. rheedei* has various medicinal uses, including the treatment of jaundice, diarrhea,[18] musculoskeletal problems[19] and mumps.[20] The seeds are used by folkloric medicinal practitioners, locally known as narcotic, emetic, febrifuge, alexiteric and antiperiodic. Triterpenes isolated from the seed of *E. rheedei* have antiproliferative and antioxidant activity.[21,22] Infusion of *E. rheedei* bark was used in Tanzania to cure scabies. Similarly, pains and itch are mitigated by the bark and seeds of *E. rheedei* in South-East Asia.[23]

The present study will help the industry to produce herbal drug with less side effect, economically affordable and more effective in the treatment of various diseases. Owing to the various medicinal properties of *E. rheedei*. Antibacterial studies[1,24] have been done extensively, however, antifungal studies have not been done on the plant under study to the best of our knowledge. Hence, this study focuses mainly on the antifungal analysis of the plant extracts in various solvents, GC-MS analysis, and molecular docking analysis, which will help in understanding the action of the phytoconstituents against the target fungus.

Materials and Methods

Chemicals, plant collection and extraction

Petroleum ether and methanol (99%) were purchased from Merck, India. Potato dextrose agar medium, Amphotericin B and antimycotic solution were purchased from Hi media, India. Plant material (seeds) of *E. rheedei* were collected from Tuensang district of Nagaland. The botanical material was identified by Dr. N. Odyuo, Scientist-E and deposited at the Botanical Survey of India, Eastern Regional Centre, Shillong, with voucher no. BSI/ERC/Tech/2022-23/319. The seeds were washed with distilled water and air-dried at room temperature for 3 weeks. The seeds were ground into uniform powder with the help of an electric grinder. In 10 g of the powder was used for Soxhlet extraction with 300 ml of solvents (methanol, petroleum ether & water). The extraction with each solvent was performed at about 10°C higher than the boiling point of the solvent, and the extraction was allowed to be carried out until the solvent in the extraction chamber became colorless. The extracts were filtered, and solvents were evaporated using a rotary evaporator. The dried extracts were stored in centrifuge tubes at 4°C for further use.

Test for antifungal activity

Four species of fungus were selected for the antifungal activity test *viz., Candida albicans, Aspergillus niger, A. flavus and A. fumigatus,* with disk diffusion method.[25] The potato dextrose agar medium was prepared by dissolving 20 g of potato infusion, 2 g of dextrose and 1.5 g of agar in 100 mL of distilled water. The dissolved medium was autoclaved at 15 lbs pressure at 121°C for 15 minutes. The autoclaved medium was mixed well and poured onto 100 mm petri-plates (25–30 mL/plate) while still molten. Petri plates containing 20 mL potato dextrose agar medium was seeded with 72 hours culture of fungal strain *(Candida albicans, A. niger, A. flavus and A. fumigatus).* Wells were cut and different concentration of sample 1 (water) and Sample 2 (methanol) (500, 250, 100 and 50 µg/mL) was added. The plates were then incubated at 28°C for 72 hours. The anti-fungal activity was assayed by measuring the diameter of the inhibition zone formed around the wells. Amphotericin B was used as a positive control. The values were calculated using Graph Pad Prism 6.0 software (USA).[26]

Gas chromatography-mass spectrometry (GC-MS) analysis

Gas Chromatography-Mass Spectrometry with column SH-RXi-5Sil MS was performed with SHIMADZU (model: QP2020) at Trichy Research Institute of Biotechnology Pvt. Ltd., Tamilnadu. The details of the GC-MS experiment conditions are listed in Table 1.

Absorption, distribution, metabolism and excretion (ADME) analysis

Lipinski rule-of-five was applied to the 20 hits acquired from GC-MS analysis for ADME analysis.[27] The SDF structure files of the compounds were downloaded from Pubchem and ADME analysis was performed using the web-based server program, supercomputing facility for bioinformatics and computational biology, IIT Delhi.[28]

Table 1: GC-MS analysis conditions

Column Oven Temp – 50°C	Column Flow – 1.20 mL/min
Injection Temp – 250°C	Purge Flow – 3.0 mL/min
Split ratio – 10	Linear velocity – 39.7 cm/sec
Pressure – 68.1 kPa	Injection mode – Split
Total Flow – 16.2 mL/min	Flow Control Mode – Linear velocity

Molecular Docking

Molecular docking analysis was performed using MOE2015[29] software program. In performing the docking analysis, triangle matcher method was used with an induced fit refinement model. London dG scoring method was used to generate the first 30 poses and GBVI/WSA method was used to generate the 5 best poses. The docking results were analyzed using biovia discovery studio.[30] Amphotericin B is used as the reference drug in comparing the docking results. PDB ID:4YNU[31] was taken as the enzyme structure of *A. flavus*. The N-Myristoyltransferace enzyme is found to be essential for the development of spore and sclerotia and also regulates aflatoxins, a series of secondary metabolites secreted by the fungus. Inhibition of the enzyme could deter potent functions of the fungus.[32] As details of the active site of the enzyme are not available, MOE was used to generate the active site for the enzyme. For *A. fumigatus*, PDB ID:2VF5[33] is considered for the docking analysis. 2VF5 is a glucosamine-6-phosphate synthase enzyme and studies have revealed that inhibition of this enzyme in the cells of fungi, even for a short period of time, results in induction of the lysis, agglutination, and certain morphological changes.[34] Twelve amino acid residues of 2VF5 (VAL399, GLN348, LYS603, GLY301, ALA602, ALA400, THR352, THR302, SER303, SER349, SER347 and CYS300) were taken as the active site.[35]

Molecular Dynamic Simulation

Molecular dynamic simulation studies were performed on the top binding ligands of both the target proteins using NAMD.[36] Topology files for the ligands were prepared using CHARMM-GUI,[37] a web-based program, and the topology files of the protein was prepared using VMD.[38] Solvation box was added using molecular dimension with a box padding of 0 (min) and 5 (max) in the X, Y and Z directions. CHARMM36m force field[39] was used to run the simulation in NAMD. Periodic boundary conditions were applied and long-range electrostatic interactions were computed using Particle Mesh Ewald (PME) algorithm.[40] Langevin dynamics was used at a temperature of 310K and simulations were ran for 100 ns. The trajectories of the MD simulation output file was investigated for RMSD, RMSF and Hydrogen bonding analysis. All the analysis results were plotted using Qtgrace.

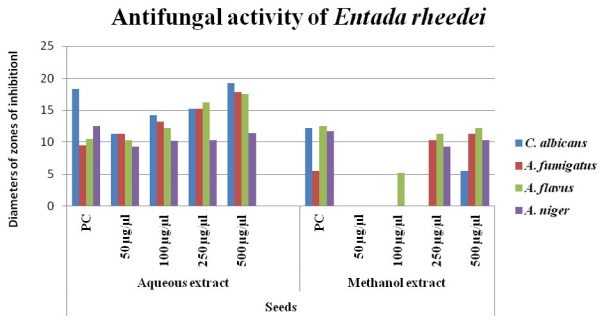

Fig. 1: *The graphical representation of the antifungal activity of aqueous* extract and methanol extract of *E. rheedei*

RESULTS

Antifungal Activity

The crude seed extracts in water and methanol solvent were tested for antifungal activity against four fungi *viz.*; *C. albicans, A. niger, A. flavus and A. fumigatus. The graphical representation of the antifungal activity of the seed is provided in Fig. 1 (Antifungal test in supplementary Fig. 1).* The graph for both the aqueous and methanol extracts of the seeds exhibited similar pattern of increased antifungal activity with increase in the concentration of the extract. The aqueous extract showed extensive antifungal activity against all the four test fungal strains at all the concentrations of the extract i.e.; 50, 100, 250 and 500 µg/µL. However, in the case of the methanol extract no antifungal activity was reported against any of the four test organisms at a concentration of 50 µg/µL, no activity against *C. albicans*, *A. fumigatus* and *A. niger* at a concentration of 100 µg/µL and no antifungal activity against *C. albicans* at 250 µg/µL concentration. The antifungal activity of the aqueous extract against *A. fumigatus* showed very interesting and promising results with the zones of inhibition higher than the positive control at all the four concentrations of the extract i.e.; 11.25 mm at 50 µg/µL, 13.25 mm at 100 µg/µL, 15.25 mm at 250 µg/µL and 17.75 mm at 500 µg/µL while that of the positive control was 9.5 mm Fig. 2(A). Similar trend was observed against *A.flavus* except at the concentration of 50 µg/µL where the zone of inhibition of 10.25 mm was recorded which was slightly less than that of the positive control of 10.5 mm, while extensive antifungal activity was exhibited where the zones of inhibition recorded were 12.25 mm at 100 µg/µL, 16.25 mm at 250 µg/µL and 17.5 mm at 500 µg/µL all of which were greater than the positive control of 10.5 mm Fig. 2(B).

However, in the case of *A.niger*, the antifungal activity was comparatively lower than the other three test organisms as the zones of inhibition recorded were all lower than the positive control. In the methanol extract of the seeds, the antifungal activity against *C. albicans*, *A. flavus* and *A.niger* were not very significant because at lower concentrations no activities were recorded and at higher concentrations though antifungal activities were observed none of the zones of inhibition were reported to be more than that of the positive control. Comparatively, we find that the

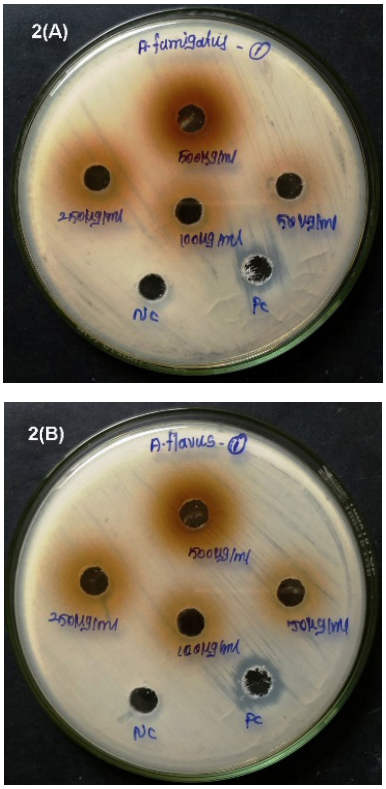

Fig. 2: (A) Antifungal activity of aqueous extract of seeds against *A. fumigatus*, (B) Antifungal activity of aqueous extract of seeds against *A. flavus.*

aqueous extract of the seeds showed very significant and extensive antifungal activity than the methanol extract. However, the promising results obtained from the antifungal activity assay of the seed extracts itself produces immense information about the possibility of the presence of significant bioactive compounds in the plant *E. rheedei*.

Gas Chromatography-mass Spectroscopy (GC-MS) Analysis

GC-MS data of the aqueous extract reveals a total of 13 compounds arising from 20 peaks as shown in Table 2 (GC-MS spectra in supplementary Fig. 2). The major phytoconstituents included dimethylsulfoxonium formylmethylide (86.5% peak area), 1,2-dimethyl-benzene (2.92% peak area), Bis(2-ethylhexyl) and phthalate (2.7% peak area).

The 13 compounds obtained from GC-MS were analysed for drug-likeness by screening with Lipinkski rule-of-five. The analysis report is listed in Table 3. Among 13 compounds only 7 compounds were chosen based on their ADME properties. Other phytoconstituents were rejected due to the absence of hydrogen donor/acceptor and high LOGP value.

Molecular docking analysis

Validation of the docking protocol was performed by considering the protein structure 4YNU. The conformation and interactions of the native ligand *(D-glucose-1,5-lactone)* in the XRD structure were compared with the conformation and interactions of the same ligand after docking at the same site. The 2D images and 3D images of the interactions and orientations are shown in Fig. 3. In the two complexes, the ligand is seen to have hydrogen bonding interactions with the same amino acid residues (Tyr53, Glu413, Arg501, Asn503, His505 and His548) at the site (Figs 3A & 3B). There are slight differences in orientations of a docked ligand with that of the XRD data (Fig. 3C). However, since the major hydrogen bonding interactions between the native ligand and the docking site are conserved the docking protocol can be considered as valid.

According to GC-MS analysis, dimethylsulfoxonium formylmethylide, is the major component of the aqueous extract of *E. rheedei* and is reported to be the major component in many plants with antimicrobial properties.[41–44] But according to the docking results,

Table 2: GC-MS analysis of aqueous extract

Peak	*Retention time*	*Area %*	*Height %*	*Name*
14.056		86.5	87.45	Dimethylsulfoxonium formylmethylide
2	4.213	2.92	2.17	1,2-dimethyl-Benzene
3	4.384	1.7	1.27	Xylene
4	4.786	0.58	0.58	Xylene
5	4.938	1.21	1.65	2-Ethoxyethyl acetate
6	5.195	0.3	0.34	Dimethyl sulfone
7	13.98	0.25	0.28	Dodecamethyl-Cyclohexasiloxane
8	16.189	0.6	0.66	Pentadecane
9	18.299	0.41	0.44	Octadecane
10	20.304	0.3	0.33	Octadecane
11	22.201	0.17	0.19	Pentadecane
12	28.411	0.13	0.14	Benzoic acid,2,4-bis(trimethylsiloxy)-, trimethylsilyl ester
13	30.472	0.23	0.25	Cyclononasiloxane, octadecamethyl-
14	32.384	0.26	0.26	Cyclodecasiloxane
15	34.185	0.39	0.38	Cyclodecasiloxane
16	34.727	0.22	0.22	2-methyl-5-nitro-1H-indole
17	34.977	2.7	2.43	Bis(2-ethylhexyl) phthalate
18	35.878	0.5	0.41	Tetracosamethyl-Cyclododecasiloxane
19	37.461	0.35	0.3	Tetracosamethyl-cyclododecasiloxane
20	38.975	0.27	0.24	Tetracosamethyl-cyclododecasiloxane

Table 3: ADME Screening of 13 phytoconstituents of aqueous extract

Compounds	*Mass (< 500 Dalton)*	*Hydrogen bond donor (< 5)*	*Hydrogen bond acceptors (< 10)*	*LOGP (>5)*	*Molar Refractivity (40–130)*
2-Ethoxyethyl acetate	312	5	6	-0.053101	77.145782
2-methyl-5-nitro-1H-indole	176	1	2	2.384520	49.690094
1,2-dimethyl-Benzene	106	0	0	2.303440	35.915997
Benzoic acid,2,4-bis(trimethylsiloxy)-, trimethylsilyl ester	320	0	4	2.147600	89.311485
Bis(2-ethylhexyl) phthalate	390	0	4	5.910781	121.071976
Dodecamethyl-Cyclohexasiloxane	468	0	6	1.561201	121.787964
Octadecamethyl-Cyclononasiloxane	702	0	9	2.341800	182.682312
Dimethyl-sulfone	94	0	2	0.741600	20.811996
Dimethylsulfoxonium-formylmethylide	120	0	2	0.397200	32.253494
Octadecane	254	0	0	7.267802	85.219963
Pentadecane	212	0	0	6.097501	71.368973
Tetracosamethyl-Cyclododecasiloxane	312	5	6	-0.053101	77.145782
Xylene	106	0	0	2.303440	35.915997

Table 4: Docking scores of the interactions of phytoconstituents with *A. fumigatus* and *A. flavus*, and interacting active site amino acids

Compounds	*A. fumigatus*		*A. flavus*	
	Docking score (Kcal/mol)	*Interacting amino acids*	*Docking score (Kcal/mol)*	*Interacting amino acids*
2-methyl-5-nitro-1H-indole	-5.4486	LYS 603, SER 401, GLU 396, CYS 300	-5.9578	GLU 36, ALA 37, THR 234, ARG 279, TRP 63, ALA 275, GLY 12
Benzoic acid, 2, 4-bis (trimethylsilyloxy)-trimethylsilyl ester	-8.0570	GLY 301, ALA 602, LEU 601	-9.4564	ALA 275, TRP 63, THR 89, ALA 82, PRO 506
Bis(2-ethylhexyl) phthalate	-7.3479	LEU 484, SER, 604, VAL 605, VAL 399, ALA 602, CYS 300	-8.6141	ALA 277, GLY 276, PRO 506, LEU 278, ALA 82, PHE 504, LEU 549, HIS 505, VAL 550, HIS 548
Dimethyl sulfone	-3.9272	SER 347, GLN 348, SER 604	-4.1504	HIS 548, LEU 549, ASN 93
Dimethylsulfoxonium formylmethylide	-4.4093	GLN 348	-4.7657	ALA 37, GLY 12, ALA 275, GLU 36, ARG 279
Dodecamethyl-cyclohexasiloxane	-7.3081	SER 604, VAL 399, LEU 601, LEU 484, VAL 605, CYS 300	-6.8451	GLY 276, TYR 65, ARG 279, TRP 63, LEU 278, THR 89, ALA 82, PRO 506, ALA 538, LEU 549, VAL 550, THR 15, LEU 553
Tetracosamethyl-cyclododecasiloxane	-8.0863	HIS 493, GLY 301, LEU 484, LYS 487, CYS 300, LEU 601, TYR 304, ILE 326, VAL 605	-8.1551	ARG 501, PHE 57, LEU 55, TYR 53, GLY 54, HIS 403, VAL 79
Amphotericin B	-7.5305	ASN 640, SER 401, GLN 348, GLU 329	-8.3174	TYR 53, SER 333, GLY 331, ASN 49, ASN 499, TRP 495

Dimethylsulfoxonium formylmethylide, showed only a decent docking score of -4.4093 and -4.7657 kcal/mol against *A. fumigatus* and *A. flavus,* respectively Table 4. On the other hand, Benzoic acid, 2, 4-bis (trimethylsilyloxy) - trimethylsilyl ester, produced the best docking score of -8.0570 and -9.4564 kcal/mol against *A. fumigatus* and *A. flavus,* respectively, which is more than the reference drug. The reference drug, amphotericin B, also gave good docking scores of -7.5305 and -8.3147 kcal/mol against *A. fumigatus* and *A. flavus,* respectively. Tetracosamethyl-cyclododecasiloxane also gave a better docking value (-8.0863 kcal/mol) than the reference drug against *A. fumigatus*. Bis(2-ethylhexyl) phthalate produced a docking score of -8.6141 kcal/mol which is also better than the reference drug against *A. flavus*.

The interaction of Benzoic acid, 2, 4-bis (trimethylsilyloxy)-trimethylsilyl ester with *A. fumigatus* is highly stabilized by hydrogen bonding with ALA602 and carbon-hydrogen

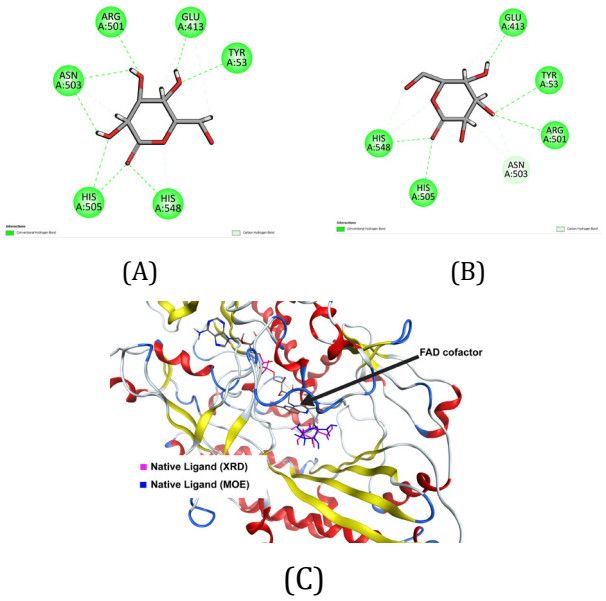

Fig. 3: 2D and 3D images of native ligand (D-glucose-1,5-lactone) with protein (4YNU) and cofactor (FAD) (A) 2D image of Ligand-4YNU complex taken from PDB (B) 2D image of Ligand-4YNU complex after docking (C) 3D image of Ligand-4YNU complex showing different orientations of the native ligand.

bonding with GLY301 and LEU601 (Fig. 4). Its interaction with *A. flavus* is stabilized by hydrogen bonding with THR89, carbon-hydrogen bonding with ALA275, Pi-Lone pair interactions with ALA275 and a couple of alkyl and Pi-akyl interactions (Fig. 5). Tetracosamethyl-cyclododecasiloxane does not show any hydrogen bonding interaction at the active site of *A.* fumigates but the interactions are stabilized by multiple alkyl and pi-alkyl interactions (Fig. 4). Bis(2-ethylhexyl) phthalate, produces good binding scores against *A. flavus* but there is an unfavorable acceptor-acceptor interaction with PHE504. The reference ligand amphotericin B, produced 4 and 5 hydrogen bonding interactions with *A. fumigatus* (Fig. 4) and *A. flavus* (Fig. 5), respectively.

Although dimethylsulfoxonium formylmethylide is the major phytoconstituents according to GC-MS analysis, molecular docking analysis reveals that benzoic acid, 2, 4-bis (trimethylsilyloxy)-trimethylsilyl ester could be the most potent antifungal components among the phytoconstituents.

Molecular Dynamics Simulation Analysis

The results of MD simulations are displayed in Fig. 6. The root mean square deviation of a system reflects the

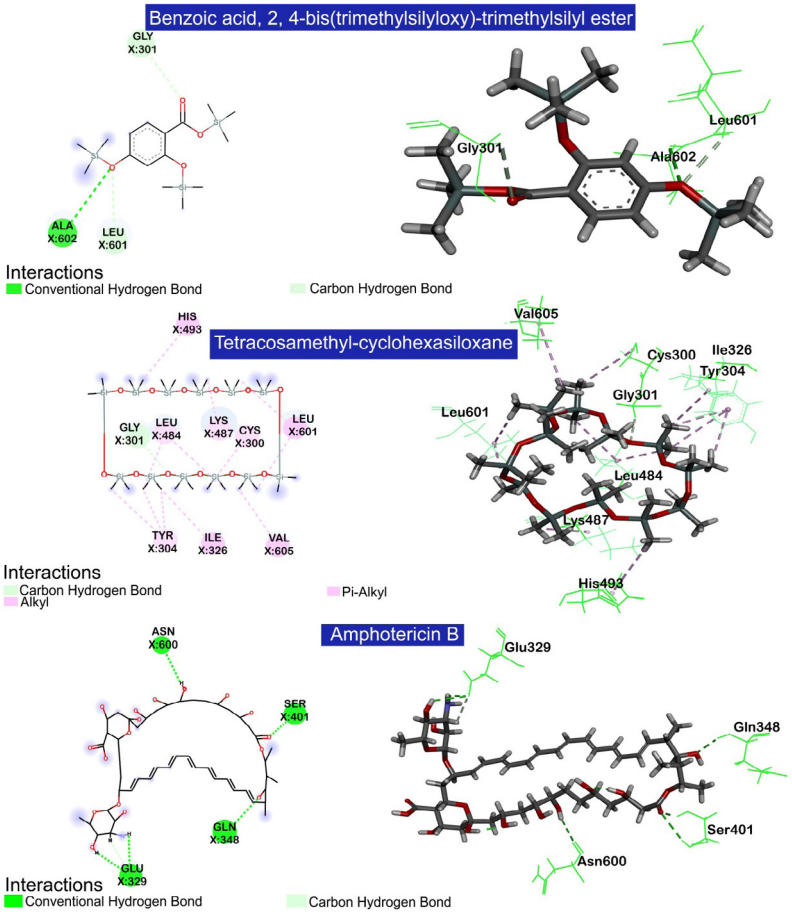

Fig. 4: 2D and 3D interaction diagram of the top scoring compounds and the reference compound against *A. fumigatus*

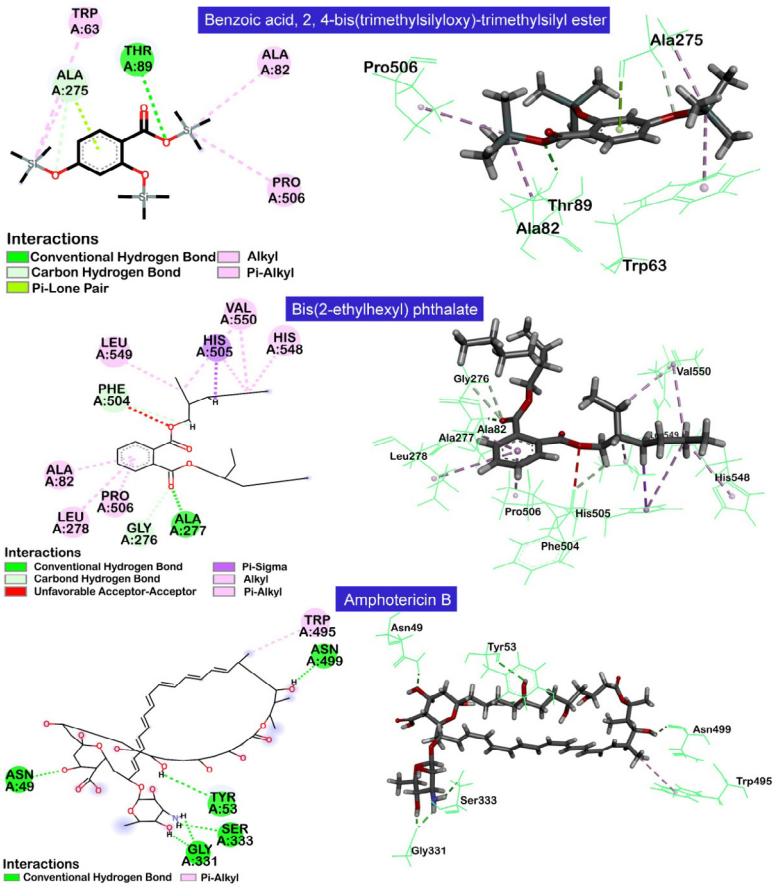

Fig. 5: 2D and 3D interaction diagram of the top scoring compounds and the reference compound against *A. flavus*.

stability of the protein-ligand complex amidst internal fluctuations and motions during the simulation. The RMSD of ligand-*A. fumigatus* complex (Fig. 6A) shows higher deviations for amphotericin B-*A. fumigatus* complex. The complex of benzoic acid, 2, 4-bis (trimethylsilyloxy)-trimethylsilyl ester with *A. fumigatus* shows stable RMSD values throughout the simulation period, but has slightly higher RMSD values around 65 to 85 ns. The complex with tetracosamethyl-Cyclododecasiloxane ligand exhibits stable RMSD values till 65 ns, but increases beyond 65 ns. The RMSD plots correspond to the docking scores with benzoic acid, 2, 4-bis (trimethylsilyloxy)-trimethylsilyl ester as showing the highest docking score and the most stable RMSD. The average RMSD values are below 2 Å, which is considered as fairly good, except for Amphotericin B-*A. fumigatus* complex which has higher average RMSD. The RMSF values of the carbon backbone around the pocket regions of the ligand-*A. fumigatus* complexes (Fig. 6C) are quite low except for C-600, C-601, C-602 and C-605 which have high RMSF values in all the complexes. These high RMSF values could be correlated to the rise in RMSD values in all the ligand-*A. fumigatus* complexes. The hydrogen bonding analysis of the ligand-*A. fumigatus* complexes are shown in Fig. 6E. In the case of benzoic acid, 2, 4-bis (trimethylsilyloxy)-trimethylsilyl ester the hydrogen bonding interaction with Ala602 is quite consistent from 20 to 85 ns, but disappears beyond 85 ns. For tetracosamethyl-cyclododecasiloxane there is no evidence of any strong hydrogen bonding interaction beyond 7 ns. In the case of amphotericin B the four hydrogen bonds are slightly stable across 100 ns simulation.

The RMSD, RMSF and hydrogen plot of ligand-*A. flavus* complexes are shown in Fig. 6. The complexes with benzoic acid, 2, 4-bis (trimethylsilyloxy)-trimethylsilyl ester and Amphotericin B (Fig. 6B) show stable RMSD values beyond 20 ns simulation with average RMSD value of 1.3Å. The complex with bis(2-ethylhexyl)phthalate also shows quite stable RMSD beyond 20 ns, but has a slightly higher average RMSD of 1.7Å. The RMSF of the complex with benzoic acid, 2, 4-bis (trimethylsilyloxy)-trimethylsilyl ester show low RMSF values around C63, C82, C89, C275 and C506, which indicates stable structure of the protein backbone at the pocket site. In the complex with Bis(2-ethylhexyl) phthalate, all the

interacting amino acid residues (Fig. 5) at the pocket site have how RMSF values, indicating stable structure of the carbon backbone at the pocket site. In the case of the complex with Amphotericin B, the RMSF values of the carbon backbone of the interacting amino acid residues at the docking site are low, except for C49 and C53 which

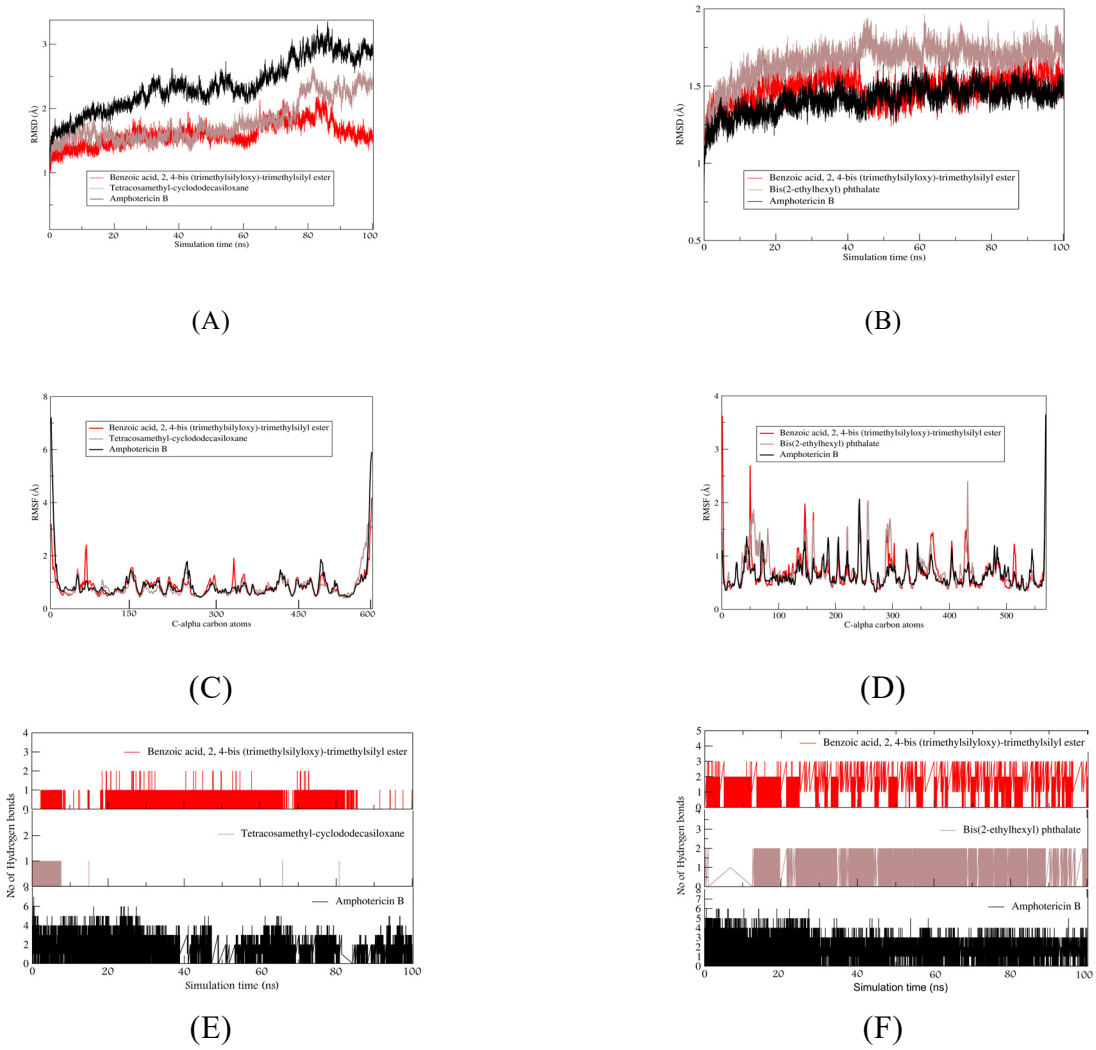

Fig. 6: Molecular Dynamics Simulation analysis (A) RMSD of ligand-*A. fumigatus* complex (B) RMSD of ligand-*A. flavus* complex (C) RMSF of ligand-*A. fumigatus* complex (D) RMSF of ligand-*A. flavus* complex (E) Hydrogen bond analysis of ligand-*A. fumigatus* complex (F) Hydrogen bond analysis of ligand-*A. flavus* complex.

show slightly higher average RMSF of about 0.7 Å. For the analysis of hydrogen bonds (Fig. 6F) the complex with benzoic acid, 2, 4-bis (trimethylsilyloxy)-trimethylsilyl ester has 2-3 hydrogens bonding interactions across 100 ns simulation time. The complex with bis(2-ethylhexyl) phthalate has two hydrogen bonds that are quite stable between 12 to 100 ns. The complex with Amphotericin B shows six hydrogen bonding interactions among which three of them are stable across 100ns simulation time. The MD simulation studies reveals better and stable interactions of the ligand- *A. flavus* complexes as compared to ligand- *A. fumigatus* complexes.

Discussion

The seeds of *E. rheedei* spreng. collected from tuensang district of Nagaland, India were used for Soxhlet extraction with methanol and water as the solvents. The plant aqueous extracts showed significant antifungal activity against *A. fumigatus* and *A. flavus* as compared to the reference drug, amphotericin b. Gas chromatography-mass spectrometry analysis of the aqueous extract exhibited 13 phytoconstituents of which dimethylsulfoxonium formylmethylide was the major component of the extract. The 13 compounds were analyzed for drug-likeness by screening with Lipinski rule-of-five after which 7 compounds were chosen based on their ADME properties. This was followed by a molecular docking study on the 7 compounds. Benzoic acid, 2, 4-bis (trimethylsilyloxy)-trimethylsilyl ester, exhibited the best docking score against *A. fumigatus* and *A. flavus* which most probably accounts for the significant antifungal activity of the seeds of *E. rheedei* spreng. MD simulation studies of 100 ns also

confirm the stability of the ligand-protein complexes. RMSD, RMSF and hydrogen bonding analysis of the trajectory files reveals that the ligand-*A. flavus* complexes are more stable, which correlates to their higher docking scores, as compared to ligand-*A. fumigatus* complexes. Also, since no research has been reported on benzoic acid, 2, 4-bis (trimethylsilyloxy)-trimethylsilyl ester as a potent antifungal activity component, further in-vitro analysis can be carried out in the future to confirm the conclusion made in this paper and more fungal strains can be selected as test organisms to study the antifungal activity of *E. rheedei* Spreng.

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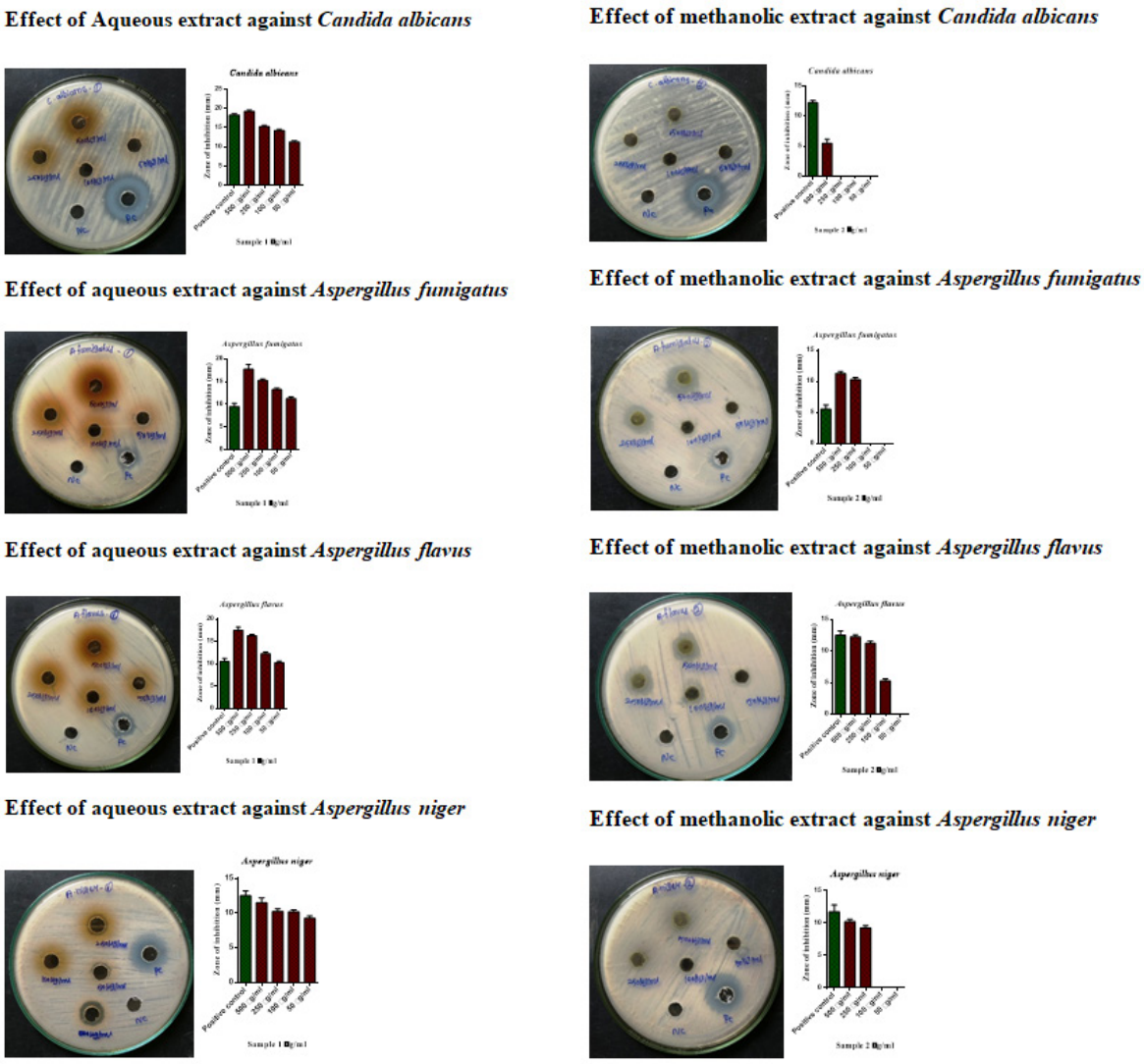

Supplementary Fig. 1: Antifungal screening of aqueous and methanolic extract against the fungal strains: *Candida albicans, Aspergillus fumigatus, Aspergillus flavus* and *Aspergillus niger*