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FLAVONOIDS AS POTENTIAL DRUG AGAINST POST-COVID-19 MUCORMYCOSIS: AN *IN-SILICO* STUDY

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ABSTRACT: Covid-19 associated mucormycosis rose sharply during India's 2nd wave of coronavirus infections. The administration of immunosuppressive drugs led to increased susceptibility of patients to oppurtunistic diseases like mucormycosis. One of the causative species of mucormycosis is *Rhizopus microsporus*. For this study, we choose two chalcones and examined their ability to act as potential anti-mucormycosis agents by inhibiting the *R. microsporus* endo β-1,4-Mannanase protein. We studied their possibility to inhibit the SARS-CoV-2 main protease and RNA dependent RNA polymerase. The chalcones were docked against the proteins of interest using Autodock 4.0 followed by Molecular dynamics simulation. Our study revealed that 2', 4'-dihydroxychalcone had the best docking with the endo β-1,4-Mannanase protein with steady root mean square deviation values and showed favourable docking with the SARS-CoV-2 proteins while passing all the drug likeliness filters. Thus 2', 4'-dihydroxychalcone can be put through further verification to test its efficacy against the causative agents of mucormycosis and the Covid-19 pandemic.

INTRODUCTION: As the Covid-19 pandemic surged in India, a large number of people were getting administered immunosuppressive drugs to control the inflammatory immune responses that deteriorated the condition of the Covid-19 infected patients [1]. However, the administration of immunosuppressive drugs had resulted in the rise of opportunistic diseases leading to new infections [2]. One such opportunistic infection is mucormycosis. It had taken a grim turn during India's 2nd wave of the Covid-19 pandemic as there were over 15000 reported cases by the end of May 2021.

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As the cases of post-covid-19 mucormycosis rose sharply, several Indian states had marked it as an epidemic [3]. *Rhizopus*, *Mucor* and *Lichtheimia* are the causative fungal species behind mucormycosis [4]. *Rhizopus* spp members like *Rhizopus oryzae*, *Rhizopus microsporus* contributed to over 70% of the reported cases of mucormycosis. Several clinical reports have identified *Rhizopus microsporus* as the c HIS113ausative organism of post-covid-19 mucormycosis events [5, 6]. *Rhizopus* spp belonging to the order *Mucorales*, are reported to be resistant to the established anti-fungal agents.

Thus, mucormycosis has a high mortality rate of over 90% and often involves surgical removal of the infected tissue [7, 8]. Mannans are a component of the fungal cell wall facilitating the cross-linking of hemicelluloses and lignin [9]. Mannans are associated with the fungal glycoproteins on the outer cell wall of fungi.

Endo β-1,4-Mannanase (Mannanase) (EC 3.2.1.78) is an enzyme from *Rhizopus microsporus* involved in the hydrolysis of the mannan backbone of the fungal cell wall [10].

Mannanase enzymes are involved in regulating cell growth and division by coordinating the deconstruction of the cell wall [11]. Mannanase enzyme is involved in mediating the stress responses of fungal cells [12].

Interruptions in the mannan metabolism have proven fatal for fungal species like *Neurospora crassa* [13]. We envisaged that finding an efficient inhibitor of mannanase could help manage the current crisis of post-covid-19 mucormycosis. Although amphotericin B is the current gold standard in treating fungal infections cases, it has several toxic side effects [14].

In our search for a minimal side effect cure for mucormycosis, we shifted to flavonoids to look for safer alternatives [14]. We selected a few flavonoids for checking their ability to inhibit mannanase. We also checked the possibility of the same candidate flavonoid as a potential drug candidate for SARS-CoV-2 infection and the subsequent mucormycosis issue.

Our results showed the flavonoid, 2',4'-dihydroxychalcone was able to dock with mannanase protein and the SARS-CoV-2 main protease and RNA dependent RNA polymerase (RdRP). Thus we present a strong case for further *in-vitro* testing of 2',4'-dihydroxychalcone in the treatment of mucormycosis and SARS-CoV-2 infection.

METHODS AND MATERIALS:

Selection of Flavonoids: To select the best possible flavonoids for docking an extensive literature survey using PubMed, pubtator and carrot 2 webservers were carried out [15].

Preparation of Ligand and Protein for Docking: The 3D structures of the flavonoids under study were downloaded from the PubChem server in sdf format [16]. The Open Babel software was used to convert the sdf files into pdb format [17]. The Autodock application of MGL tools was then utilized to convert the ligands into the final .pdbqt file format by making the necessary changes. Similarly, the pdb file for the proteins was converted to pdbqt files using the Autodock function [18].

Docking using Autodock 4.0: For carrying out the docking investigation, the amino acids involved in protein functioning were incorporated within the grid box set at 60 x 60 x 60 Å (x, y and z) and the grid spacing was kept at 0.5 Å. Autodock analysis was carried out by opting for the Genetic algorithm parameter and for output the Lamarckian output was selected [18]. Discovery software was used to obtain 2D images of the flavonoid-protein docking [19].

Molecular Dynamics Simulation: The mannanase-2', 4'-dihydroxychalcone complex generated by docking was subjected to molecular dynamics simulation. GROMACS was used for the simulation studies. The topology files were created using the CHARMm36 force field [20].

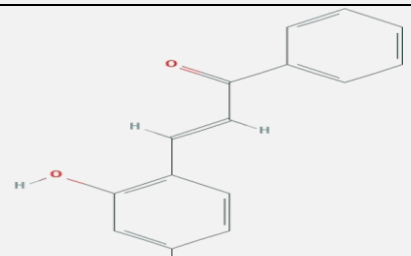
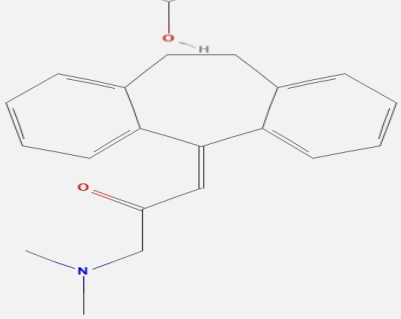
The systems were solvated in a cubic water box of size 10 Å x 10 Å. Briefly, the receptor was prepared under Charmm 27 force field and TIP3P using the Gromacs utility, while the Swiss PARAM server was used to produce the ligand topology [21]. For neutralizing the system, NaCl was added to the system.

The steepest descent approach (1,000 ps) and conjugated descent method (100 ps) were employed for energy minimization (nsteps = 50,000) of each protein-ligand complex in the prepared simulation system. Finally, a 25 ns MD simulation was performed for each docked complex. The root mean square deviation (RMSD) values obtained after the simulation was plotted using Xmgrace software [22].

Drug Likeliness Analysis: The flavonoids were checked for their drug-likeness ability using the Swiss ADME webserver [23].

RESULTS AND DISCUSSION: Our literature search revealed that, amongst flavonoids, it was the chalcones which had the maximum reported anti-fungal activity [24]. We selected the compounds 2',4'-dihydroxychalcone and 2',4'-dihydroxy-3'-methoxychalcone as they had shown strong anti-fungal actions *in-vitro* [25]. The structure of the selected compounds are provided in **Table 1.**

TABLE 1: STRUCTURE OF CHALCONES UNDER STUDY AND THEIR REPORTED INHIBITORY CONCENTRATIONS TO ACHIEVE COMPLETE GROWTH INHIBITION AGAINST FUNGUS

Chalcones	Structure
2',4'-dihydroxychalcone	
2',4'-dihydroxy-3'-methoxychalcone	

The docking results **Table 2, Fig. 1** demonstrated that both the chalcones bound favourably with the fungal mannanase protein. We observed that 2',4'-dihydroxychalcone showed the lowest binding energy of -7.72 kcal/mol and the lowest inhibitory concentration of 2.18 µM. 2',4'-dihydroxy-3'-methoxychalcone had binding energy of -7.42 kcal/mol and inhibition constant of 3.78 µM. The high values obtained with both the chalcones against the Rhizopus protein tally with the previous reports of chalcones inhibiting the growth of the Rhizopus spp of fungi in *in-vitro* experiments [26].

TABLE 2: DOCKING DETAILS OF FLAVONOIDS WITH MANNANASE PROTEIN FROM *RHIZOPUS MICROSPORUS*

Flavonoids	Binding energy (kcal/mol)	Inhibition constant	Amino acids bound to
2',4'-dihydroxy-3'-methoxychalcone	-7.40	3.78 uM	GLU 34, LYS 48, ASN 54, LYS 59, ASN 61, HIS 113, ARG 114, GLY 116
2',4'-dihydroxychalcone	-7.72	2.18 uM	GLU 34, LYS 48, ASN 54, LYS 59, ASN 61, HIS 113, ARG 114

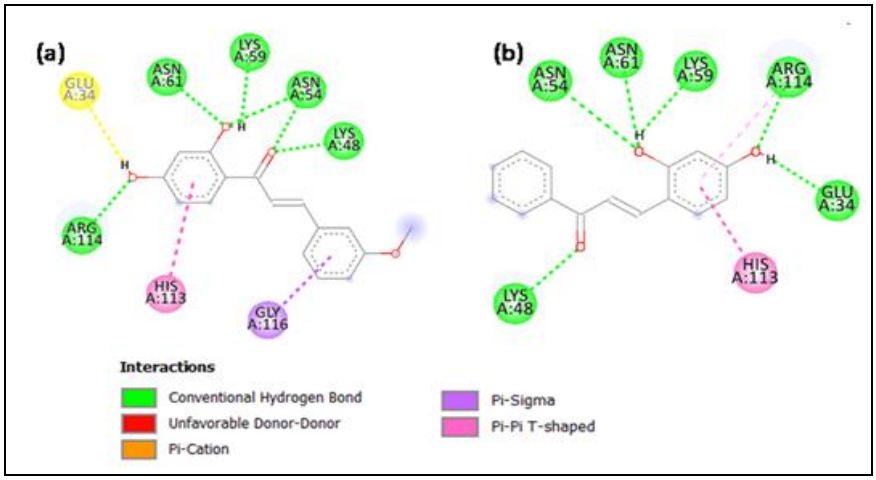


FIG. 1: 2D REPRESENTATION OF THE DOCKING OF *RHIZOPUS MICROSPORUS* ENZYME MANNANASE WITH – (A) 2',4'-DIHYDROXY-3'-METHOXYCHALCONE, (B) 2',4'-DIHYDROXYCHALCONE

The docking of these flavonoids involved a large part of the amino acids residues 44 to 72. These amino acid residues make up the major loop of the mannanase protein. That holds significance as these residues are involved in substrate binding. The binding of the flavonoids to the highly conserved

active site residues of LYS 59 and HIS 113 can affect the activities of the mannanase enzyme [10]. Besides these two residues, the flavonoids also docked with other critical conserved residues like ASP 46, LYS 48, ASN 54, LYS 59 and ARG 114. These amino acid residues were all involved in direct H bonding with the oxygen atoms of mannose molecules. Several of the above amino acid residues interact with the substrate indirectly via water molecules [10]. Although GLU 34 was not involved in substrate binding, its reported flexible nature had an important part in facilitating the attachment of HIS 113 with the oxygen of the substrate. This was evident by the lack of proper attachment and positioning of the substrate when the GLU 34 residue underwent a mutation [10]. LYS 48 is involved in providing specificity to the enzyme and is critical in sterically excluding galactose from the enzyme-substrate binding site [10]. Thus the binding of all of the tested flavonoids to the mannanase protein can potentially hamper the substrate binding, flexibility, specificity and catalytic function of the mannanase protein. We obtained the best docking results with 2',4'-dihydroxychalcone. Hence we proceeded for the molecular simulation study of the complex of 2',4'-dihydroxychalcone and mannanase protein **Fig. 2**.

Using the ligand as the reference alignment molecule, the complex showed RMSD values in the range of 5 nm to 7.5 nm. When the simulation was performed using the protein as the reference alignment molecule, the RMSD values showed a steady binding within the range of 0.05 nm to 0.1 nm for the entire range of the simulation. From the fixed range of RMSD values obtained during the period of simulation we can conclude that the binding of 2',4'-dihydroxychalcone to the mannanase protein would be stable under *in-vitro* conditions [27].

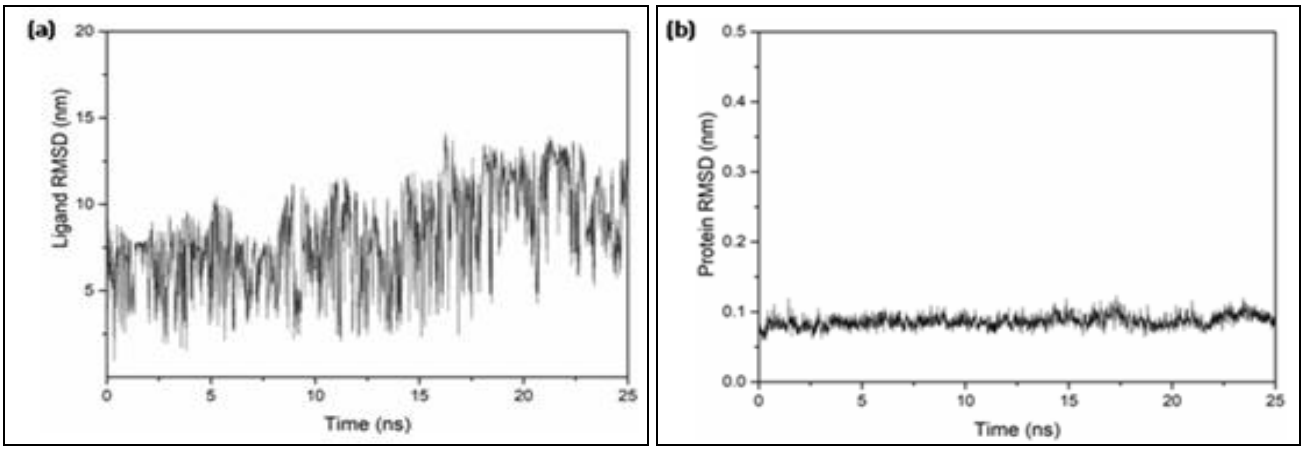


FIG. 2: SHOWS THE PATTERN OF ROOT MEAN SQUARE DEVIATION FOR MANNANASE AND 2,4-DIHYDROXYCHALCONE. (A) RMSD OF 2,4-DIHYDROXYCHALCONE WHERE LIGAND WAS USED AS REFERENCE ALIGNMENT MOLECULE, (B) RMSD OF Cα OF MANNANASE

To further advocate the cause of 2',4'-dihydroxychalcone, we investigated the docking ability of the flavonoid with the SARS-CoV-2 main protease and the RdRP proteins. 2',4'-dihydroxychalcone showed favourable docking properties with both of these two SARS-CoV-2 proteins **Table 3, Fig. 3**.

2',4'-dihydroxychalcone had binding energy of -6.43 kcal /mol, which was better than viomycin (-3 kcal/mol). It is to be noted that viomycin happens to be a prime candidate as a potential SARS-CoV-2 main protease inhibitor [28]. 2',4'-dihydroxychalcone docked with amino acid residues in the substrate-binding region of the protease like MET 165, GLU 166 and the conserved catalytic residue HIS 41, thus highlighting its potential influence in inhibiting the enzyme function [29]. Another factor to keep in mind is the lower binding energy of 2',4'-dihydroxychalcone with RdRP (-6 kcal/mol) in comparison to the -4.14 kcal/mol binding energy of the natural substrate ATP for RdRP [30].

2',4'-dihydroxychalcone docked with the amino acid residues of the palm domain of the RdRP, which included the highly conserved catalytic residue ASP 761 [31]. Thus keeping these factors in mind 2',4'-dihydroxychalcone should be investigated as a curative agent in the fight against SARS-CoV-2. And lastly, we analysed the

pharmacological properties of the flavonoids, out of which 2',4'-dihydroxy-3'-methoxychalcone and 2',4'-dihydroxychalcone passed all the five filters of drug-likeness **Table 4**, thus emphasizing their ability to act as potential drugs.

TABLE 3: DOCKING OF 2',4'-DIHYDROXYCHALCONE WITH SARS-COV-2 PROTEINS

SAR-CoV-2 protein	Binding energy (kcal/mol)	Inhibition constant	Amino acids bound to
Covid Main Protease	-6.43 kcal/mol	19.38 uM	HIS 41, MET 49, MET 165, GLU 166, PRO 168, ARG 188, THR 190
SARS-CoV-2 RdRP	-6.00 kcal/mol	39.9 uM	ASP 761, ALA 797, LYS 798, TRP 800, HIS 810, GLU 811

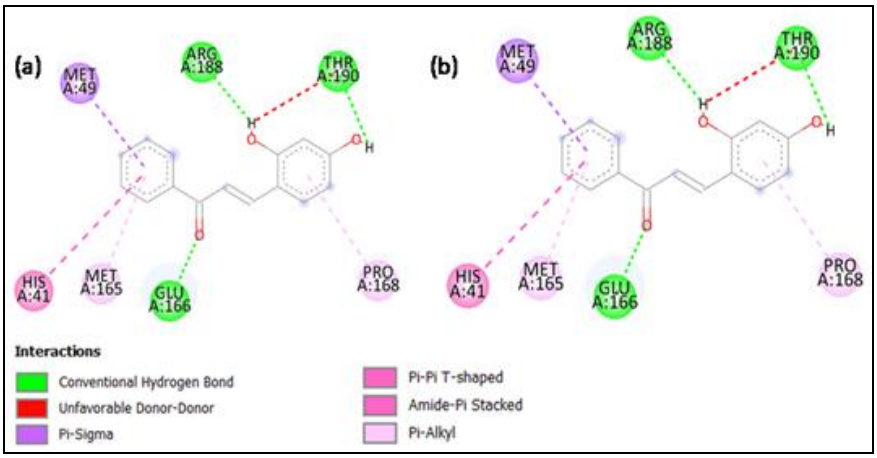


FIG. 3: 2D REPRESENTATION OF THE DOCKING OF 2',4'-DIHYDROXYCHALCONE WITH – (A) COVID MAIN PROTEASE, (B) SARS-COV-2 RDRP

TABLE 4: DRUG LIKELINESS ANALYSIS OF THE CHALCONES UNDER STUDY

Flavonoid	Lipinski filter	Ghose filter	Veber filter	Egan filter	Muegge filter
2',4'-dihydroxy-3'-methoxychalcone	+	+	+	+	+
2',4'-dihydroxychalcone	+	+	+	+	+

CONCLUSION: In this study, we investigated two flavonoids as a curative against post-covid-19 mucormycosis. Out of the two tested flavonoids, 2',4'-dihydroxychalcone showed to be the most promising agent to inhibit the activity of the *Rhizopus* mannanase protein. 2',4'-dihydroxychalcone showed its ability to dock with amino acid residues involved in the functioning of the mannanase protein. Molecular dynamics simulation of the 2',4'-dihydroxychalcone-mannanase complex also showed steady RMSD values. Inhibition of this protein can be fatal to the fungus and thus will allow the control of mucormycosis. As such the flavonoid 2',4'-dihydroxychalcone should be subjected to further study as an antifungal agent. The ability of 2',4'-dihydroxychalcone to bind with the SARS-CoV-2 main protease and RdRP also hold potential for the flavonoid to be looked at as an option for SARS-CoV-2 treatment. Thus, keeping in mind that 2',4'-dihydroxychalcone passed all filters of drug likeliness, it deserves to be investigated as an agent to prevent SARS-CoV-2 associated mucormycosis and as an anti-SARS-CoV-2 agent.

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CONFLICTS OF INTEREST: None to declare.

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