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In silico Approaches for Effective and Potential Biocontrol Agents against Targeting Chitin Synthase Protein of Pathogenic Fungus *Alternaria alternata* (Fr.) Keissl. of Onion

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Key words: *Alternaria alternata*, Chitin synthase, Phytocompounds, Molecular docking, Molecular simulation, Drug-likeness

Abstract

This study utilized an *in silico* drug discovery approach, combining molecular docking and molecular dynamics (MD) simulations, to identify novel inhibitors of the fungal virulence factor, Chitin Synthase, an enzyme essential for *A. alternata*'s survival and hyphal growth. The leaf blight-associated fungus from onion was identified as *A. alternata* based on its woolly greyish-brown mycelia, pale cylindrical conidia, and shorter conidiophores. Molecular identification using 18S ITS1 and ITS2 sequencing (566 bp) confirmed 100% similarity with *A. alternata* in GenBank. The sequence was submitted to NCBI under accession number PP894981.1 (JUF0089). A library of 105 phytocompounds was virtually screened, yielding 26 candidates with binding affinities superior to those of the reference inhibitor. Among these, 10 complied with Lipinski's rule of five, indicating favorable drug-likeness. The two most promising candidates, luteolin and azadiradione, were selected as lead compounds for further validation. A 200 ns MD simulation was performed, and subsequent analysis of Root Mean Square Deviation (RMSD), Root Mean Square Fluctuation (RMSF), and hydrogen bond interactions confirmed stable protein-ligand complexes throughout the trajectory, with binding free energies supporting strong inhibitory potential. These results strongly suggest that luteolin and azadiradione act as effective inhibitors by specifically targeting the active site of the Chitin Synthase protein. This research validates these natural compounds as potent starting points for the rational design and development of new antifungal drugs against *A. alternata.* To our knowledge, this is the first *in silico* investigation of phytocompound-based control strategies against *A. alternata* conducted in Bangladesh.

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Introduction

Alternaria alternata is a ubiquitous, opportunistic fungal pathogen known to cause a wide range of destructive diseases in numerous economically important crops, including onion and garlic (Khatun et al. 2023, Rahman et al. 2025). In onions (*Allium cepa*), it is responsible for the development of Alternaria blight disease, characterized by necrotic lesions on leaves and bulbs that lead to significant yield and quality losses during both cultivation and storage (Kumar et al. 2012). The fungus produces various phytotoxic metabolites that interfere with host cell functions and accelerate tissue necrosis. Beyond its agricultural impact, *A. alternata* also poses serious risks to human health, as it can act as an allergen and opportunistic pathogen, triggering respiratory disorders such as asthma, rhinitis, and hypersensitivity pneumonitis (Kustrzeba-Wójcicka et al. 2014). Moreover, some strains produce mycotoxins like alternariol and tenuazonic acid, which are considered potential health hazards due to their cytotoxic and mutagenic effects (Logrieco et al. 2009). Therefore, *A. alternata* represents a critical concern for both sustainable crop production and public health worldwide (Ahmmed et al. 2020, Alam et al. 2023).

Various fungicides are used in agricultural fields to control infections caused by *A. alternata*. However, drug-resistant strains pose a significant public health risk, as resistance may transfer from farms to humans through plant-human interactions (Bengyella et al. 2017). Conventional breeding is often slow in developing resistance due to the rapid evolution of the pathogen (Srivastava et al. 2017, Alam and Rahman 2020). Excess use of fungicides is making fungicide resistant fungus by modifying the target site and activating efflux pump. So, various biocontrol approaches and techniques are becoming more popular for controlling fungal plant disease (Sultana et al. 2020). Natural phyto-compounds are successful in controlling plant diseases, and they may be a safe substitute for chemical fungicides. Certain phytocompound based formulations have been generated and marketed for commercial use (Shuvo et al. 2024). Current research focuses on identifying compounds that disrupt chitin synthesis-a vital component of fungal cell walls mediated by chitin synthase (CHS). Targeting CHS proteins offers a promising approach for developing fungicides that suppress both fungal growth and virulence (Qin et al. 2022).

Molecular docking and inhibitor studies continue to provide valuable insights for designing targeted antifungal treatments. Fungal signal transduction relies heavily on mitogen-activated protein kinases (MAPKs) (Halder et al. 2022). When plants interact with phytopathogenic fungi, fungal MAPKs aid in the mechanical or enzymatic penetration of the host plants, while plant MAPKs are crucial for the activation of plant immunity (Halder et al. 2024). The present study focuses on *in silico* analysis, employing computational methods such as molecular docking and molecular dynamics (MD) simulations to investigate protein-ligand interactions. The objective is to understand the efficacy of antifungal metabolites against *A. alternata* by examining their effects on target

enzymes that may serve as potential and effective biocontrol targets. This research aims to highlight recently discovered natural products with inhibitory properties against the blight fungus *A. alternata*, clarifying their mechanisms of action and assessing their viability as candidate fungicides. *In silico* molecular docking studies were conducted on proteins and enzymes critical for the infection of plants by the blight fungus.

Materials and Methods

Infected onion leaves were collected from commercial fields in Manikganj, Dhaka, Bangladesh (23.8611°N, 90.0004°E) to isolate *A. alternata*. Pathogenic fungus was isolated using tissue planting methods (Sikder et al. 2019). Colony morphology and microscopic features were examined for preliminary identification.

Genomic DNA was extracted using the Promega Wizard kit. The ITS region was amplified with universal primers ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS5 (5′-GGAAGTAAAAGTCGTAACAAGC-3′) (Alam et al. 2010). PCR products were purified using the QIAquick kit (Rahman et al. 2025) and sequenced by Sanger sequencing. Sequence identity was confirmed by BLAST analysis showing 99% similarity.

In silico drug design refers to computer-aided molecular design, involving the systematic creation or identification of pharmaceutical agents through computational techniques. The advancement of *in silico* methods has greatly enhanced hypothesis testing and accelerated progress in pharmacological research.

The target protein, Chitin Synthase (CHS), was selected based on its amino acid composition and its role in the pathogen's survival and pathogenicity (Singh et al. 2006). The FASTA sequence of CHS (Entry: A0A177DPK9) was retrieved from the UniProt database (UniProt Consortium 2019) and modeled *ab initio* using the GalaxyWEB server (Zhang et al. 2008). Model refinement was performed with Galaxy Refine (Ko et al. 2012), and validation was conducted via the SAVES server using the Ramachandran plot (Carrascoza et al. 2014). Models with over 90% of residues in favored regions were considered high quality. Additional validation was performed using ERRAT and Verify3D. Secondary structural elements, including α-helices, β-turns, and random coils, were predicted with 80% accuracy using the SOPMA server (Geourjon and Deléage 1995).

A total of 105 phytochemicals with reported antifungal properties from various Bangladeshi medicinal plants were randomly selected. Active components of nine commercial fungicides were included as reference compounds. Three-dimensional structures of all compounds were retrieved in SDF format from the PubChem database (Cheng et al. 2014).

A ligand is bound to receptor binding sites as part of the molecular docking process, which estimates the binding affinity. Autodock-vina was used to forecast the bound conformation and binding affinity (Seeliger and de Groot 2010). Protein-ligand interactions rely on hydrogen bonds, hydrophobic interactions, and salt bridges. Ligand binding in the protein's active site terminates its functional activity. The CASTp server

predicted the active site (Tian et al. 2018). The grid center for docking was selected as X= 3.0173, Y= 3.7022, and Z= -85.8686, and the size of the grid box was X= 60, Y= 60, Z= 60 Å with a spacing 0.375 Å between the grid points by using MGL tools. All docking was performed using AutoDock Vina which predicts a binding score for each ligand employing the Lamarckian genetic algorithm (Trott and Olson 2010).

The pharmacokinetic properties of the best promising compounds were checked using SWISS ADME, an online server that evaluates drug-likeness (Daina et al. 2017). For drug-like ligands, the molecular weight should not exceed 500 Da (≤500), the cLogP value should remain below 5 (≤5), the number of hydrogen bond donors should be fewer than 5 (≤5), and the number of hydrogen bond acceptors should be less than 10 (≤10).

Docked compounds were examined and visualized with Pymol software. The docked complexes were generated using Pymol, and their two-dimensional representations were created with Discovery Studio 2021 to identify the interactions among amino acids between the protein and the ligand. This analysis exposed the presence of hydrogen bonds and hydrophobic interactions between the drug-like compounds and the protein (DeLano et al. 2002).

MD simulations were conducted on Desmond software on the Linux operating system developed by Schrodinger (Bowers et al. 2006). They were subjected to default parameters such as OPLS3e force-filed, solvated with an orthorhombic box of SPC (single point charge) water molecules, and box wall distance was 10 Å, neutralized by adding the required number of ions and a salt concentration of 0.15 M. Then the energy minimization was performed under 100ps. Each complex was subjected to a final 200 ns molecular run. After the end of the simulation, the trajectories were analyzed using the RMSD, RMSF, and protein-ligand contacts (Khan et al. 2022).

Results and Discussion

Onion plants showing typical leaf blight symptoms are presented in Fig. 1A. The associated fungus was identified as *A. alternata* based on its woolly, greyish-brown submerged mycelia and pale to light brown (Fig. 1B), profusely branched and septate mycelium (Fig. 1C), cylindrical conidia and conidiophores appeared shorter and more vibrant than the conidial body seen under microscopic examination (Fig. 1). Molecular identification using 18S rDNA ITS1 and ITS2 primers confirmed the species (566 bp), showing 100% sequence similarity with *A. alternata* in the GenBank database. The sequence was deposited in NCBI under accession number PP894981.1 (*Alternaria alternata* isolate JUF0089 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence).

A. alternata causes widespread leaf blight in major crops such as onion and medicinal plants like *Centella asiatica*, leading to severe yield losses and economic impact (Alam et al. 2023, Reyes-Tena et al. 2023). Although chemical fungicides offer limited protection,

their use is constrained by resistance development and environmental concerns. Thus, identifying natural compounds with novel anti-fungal mechanisms is essential. Mansouri et al. (2023) reviewed bioactive natural compounds effective against *A. alternata*, emphasizing the need for further validation.

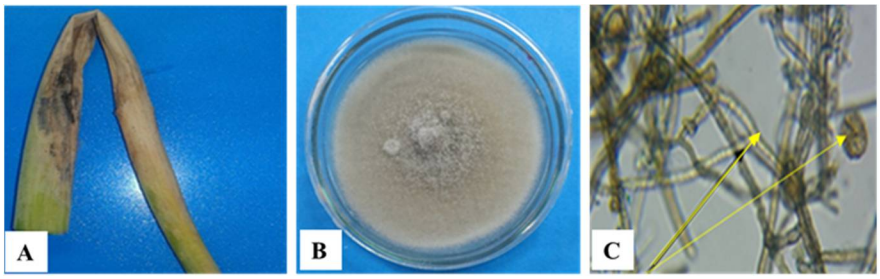


Fig. 1. Morphological characterization of *A. alternata* causing leaf blight disease of onion: (A) leaf blight symptom of onion, (B) mycelial growth, and (C) mycelium and conidia of *A. alternata*.

In silico methodologies provide an efficient approach to understanding and managing *Alternaria alternata* by identifying potential molecular targets and bioactive antifungal compounds through computational analysis. This study consisted of three stages: molecular docking for virtual screening of proteins and ligands, pharmacokinetic evaluation of drug-like compounds, and molecular dynamics simulation of top-performing complexes. Docking results identified azadiradione from *Azadirachta indica* and luteolin from *Lawsonia inermis* as strong binders to chitin synthase (CHS), indicating their potential as fungicide candidates. CHS, a key enzyme in fungal cell wall formation, provides structural rigidity and contributes to pathogenicity (Madrid et al. 2003). A total of 105 compounds from medicinal plants and commercial fungicides (as standards) were screened. The results revealed promising candidates capable of inhibiting key virulence-related enzymes, indicating potential suppression of fungal growth and toxin production. These findings highlight the effectiveness of *in silico* tools as a rapid, cost-effective, and sustainable strategy for developing novel antifungal agents to control *A. alternata* infection in onion and other crops.

In *ab initio* modeling, the GalaxyWEB server generated five models of the target protein, from which Model 1 was selected based on the server's recommendation. The selected structure was further refined using the GalaxyRefine server and subsequently validated through PROCHECK, VERIFY3D, and ERRAT. All quality assessments showed percentile scores above 80%, a quality factor exceeding 90%, and over 90% of residues in favored regions of the Ramachandran plot.

Presents the tertiary structure of the protein, including α-helices, β-strands, and random coils. Secondary structure prediction by the SOPMA server estimated 41.42% α-helices, 3.72% β-strands, and 35.04% random coils (Fig. 2). Additional plots were generated to visualize prediction accuracy and scoring curves based on window width and state parameters.

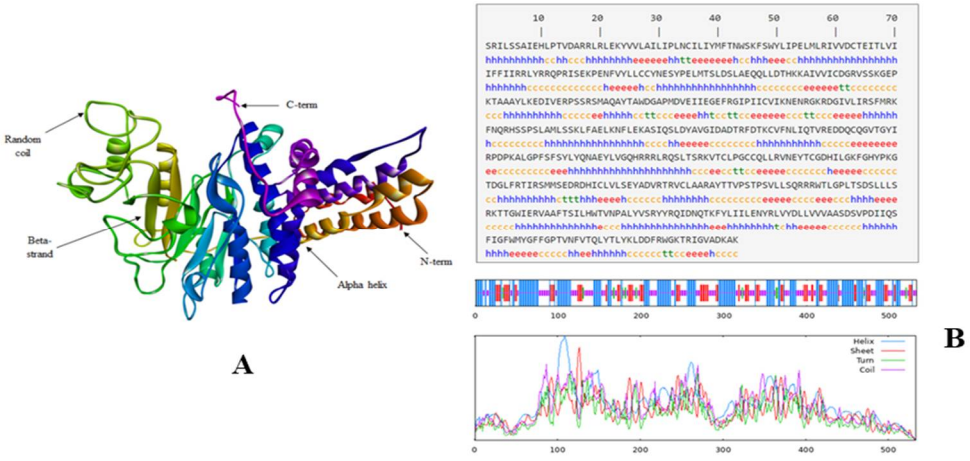


Fig. 2. (A) The 3D structure of the designed protein. The a-helix, b-strand, random coil are indicated by arrows in different colors. (B) Secondary structure analysis of target protein (CHS). The random coil is represented by magenta, the β-turn by green, the expanded strand by red, and the α-helices by blue lines. The X-axis shows the position of the amino acid, and the Y-axis shows the scores for each predicted condition.

Designing effective chemicals against plant pathogenic fungi relies on how certain phyto-compounds or fungicides interact with the binding site of CHS. Difenoconazole, with the highest binding affinity score of -8.1, was chosen as the standard. To identify potential leads, 105 phyto-compounds were docked with CHS. Among these, 26 showed higher binding affinity than the control. The drug-likeness properties of 26 compounds were evaluated via the SwissADME online. 10 phyto-compounds displayed no signs of breaking the laws governing medicine likeness and therefore could be administered as drugs program.

Ligands were evaluated for drug-likeness using Lipinski's rule of five, considering molecular weight (<500 Da), logP (<5), hydrogen bond donors (≤5), and acceptors (≤10). These parameters influence fungicidal activity, absorption, and solubility (Gleeson et al. 2011, Alex et al. 2011). Among the tested compounds, ten phytochemicals satisfied all drug-likeness criteria.

The drug-likeness properties and docking binding affinity of top-ranked potential drug compounds are shown in Table 1 and 2. Azadiradione exhibited the highest binding affinity and hydrogen bonding with chitin synthase (CHS) of *A. alternata*, followed by luteolin. These two compound-protein complexes were selected for further analysis, as shown in Fig. 3. Azadiradione had a binding energy of -9.5 kcal/mol, forming H-bonds with amino acids at positions 525, 356, 220, 219, and 221 of CHS. Luteolin had a binding energy of -9.4 kcal/mol, forming H-bonds with amino acids at positions 301, 321, 435, and 497. The control had a binding affinity of -8.3 kcal/mol and formed two H-bonds at position 402. Higher binding affinity indicates greater effectiveness at the cellular or organismal level. Molecular docking indicated that higher binding affinities correlated

with increased hydrogen bonding (Shamsi et al. 2022). Azadiradione has previously shown inhibitory effects against *Drechslera oryzae*, *Fusarium oxysporum*, and *Alternaria tenuis* (Wasim et al. 2023), while luteolin is also known for its antifungal activity (Alves et al. 2014). These findings identify both compounds as promising bio fungicidal leads.

Table 1. Drug likeness properties of the top-ranked potential inhibitor compounds of CHS protein of *A. alternata*.

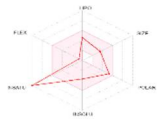
Phyto-compounds	ADME properties		Molecular Formula	Rader Diagram
Azadiradione	Molecular weight	450.57	C28H34O5	
	LogP	4.34		
	H-bond acceptor	5		
	H-bond donor	0		
	Violation	0		
Luteolin	Molecular weight	286.24	C15H10O6	
	LogP	1.73		
	H-bond acceptor	6		
	H-bond donor	4		
	Violation	0		

Table 2. Potential medicinal drugs' molecular docking binding affinities with *A. alternata*'s target protein CHS.

Compound name	PubChem ID	Sources	Binding affinity (Kcal/mol)	Hydrogen bond formation	Amino acid residues
Azadiradione	12308714	*A. indica*	-9.5	5	ARG525, ARG 356, LEU220, SER219, ALA221
Luteolin	5280445	*L. enermis*	-9.4	4	TYR301, YR497, CYS321, SER435
Difenoconazole (Control)	86173	Amister Top	-8.3	2	ARG406, SER402

The structural stability of the top two CHS complexes-azadiradione_CHS and luteolin_CHS-was evaluated through 200 ns molecular dynamics (MD) simulations, along with the difenoconazole_CHS complex as a control (Fig. 4). In the azadiradione_CHS complex, the protein RMSD stabilized around 4.8 Å after an initial rise, indicating overall structural stability, while the ligand RMSD fluctuated between 2-14 Å, suggesting partial ligand instability, possibly due to side-chain flexibility. In the luteolin_CHS complex, the protein RMSD gradually increased and stabilized near 5.6 Å, with the ligand RMSD ranging from 1.6-6.4 Å, reflecting greater stability than azadiradione. For the difenoconazole_CHS control, the protein RMSD reached approximately 6.4 Å, and the ligand RMSD stabilized around 15 Å, indicating significant deviation and potential ligand detachment. Overall, azadiradione displayed the least protein deviation but moderate ligand instability, whereas luteolin maintained balanced protein-ligand stability. In contrast, difenoconazole showed high ligand instability, implying weaker binding affinity.

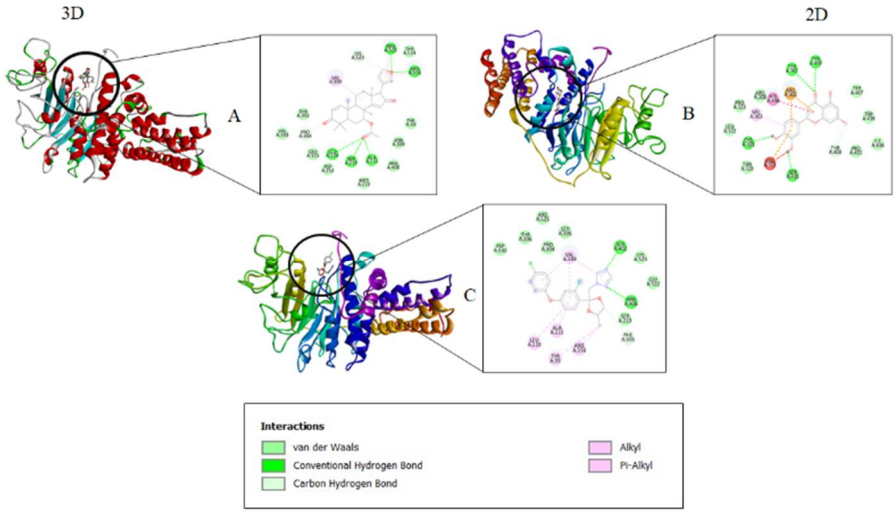


Fig. 3. Binding of (a) azadiradione, (b) luteolin, and (c) difenoconazole (control) with CHS. The CHS protein is represented in two and three dimensions (Ribbon) and displays different interactions and complex docking fits.

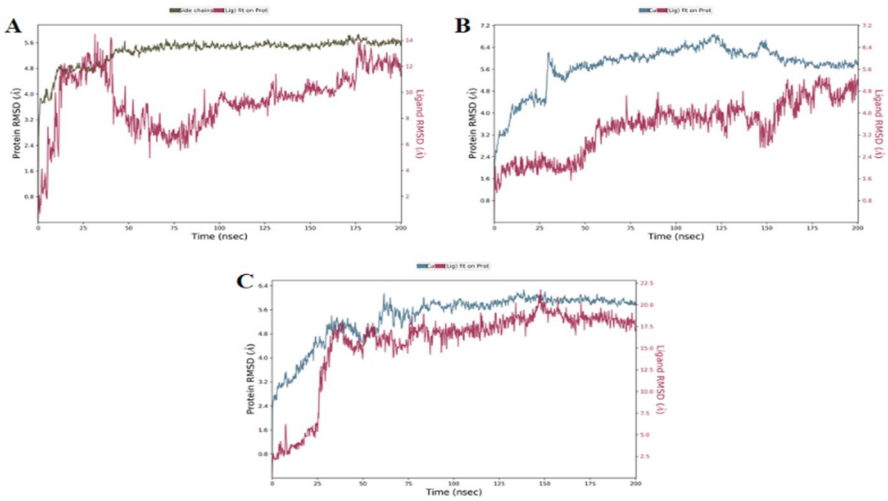


Fig. 4. Simulation graph of RMSD (root mean square deviation) values of the protein-ligand: (A) CHS_azadiradione, (B) CHS_luteolin, and (C) CHS_difenoconazole.

In addition to overall dynamics, RMSF analysis was performed to evaluate residue-level flexibility of the CHS protein (Fig. 5). The binding site residues of the CHS_azadiradione complex (ARG525, ARG356, LEU220, SER219, ALA221) exhibited a few erratic but elevated fluctuations (Fig. 5A), whereas those of the CHS_luteolin complex (TYR301, TYR497, CYS321, SER435) showed reduced fluctuations (Fig. 5B), indicating greater stability compared to the control complex (Fig. 5C).

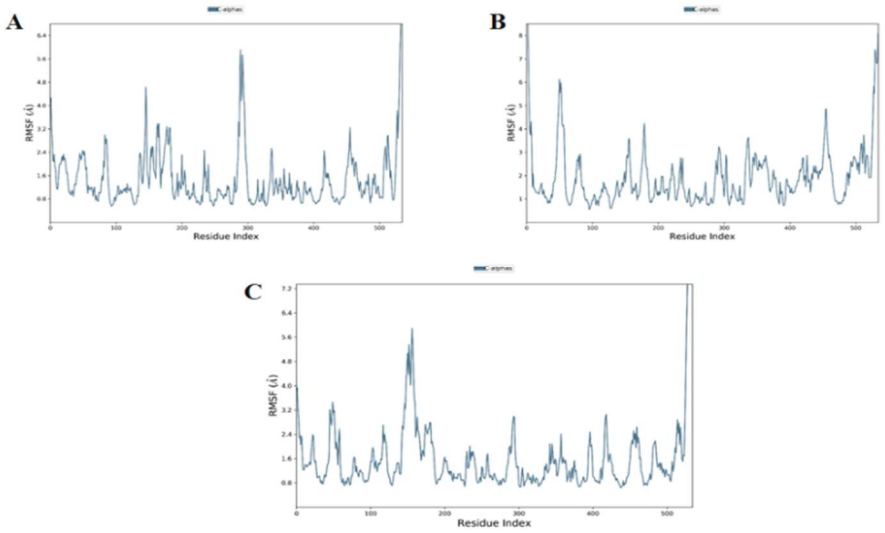


Fig. 5. Simulation graph of root-mean-square fluctuation (RMSF) values of the protein-ligand. (A) CHS_ azadiradione (B) CHS_luteolin (C) CHS_difenoconazole.

Interaction stability was further assessed by monitoring hydrogen bond formation between the receptor and ligand throughout the simulation. In the azadiradione complex, hydrogen bonds, water bridges, and ionic interactions were formed with ARG525, ARG356, LEU220, SER219, and ALA221 residues (Fig. 6A). The luteolin complex established hydrogen bonds and water bridges with TYR301, TYR497, CYS321, and SER435 residues (Fig. 6B), while the control (difenoconazole) formed fewer interactions, primarily with ARG406 and SER402 (Fig. 6C).

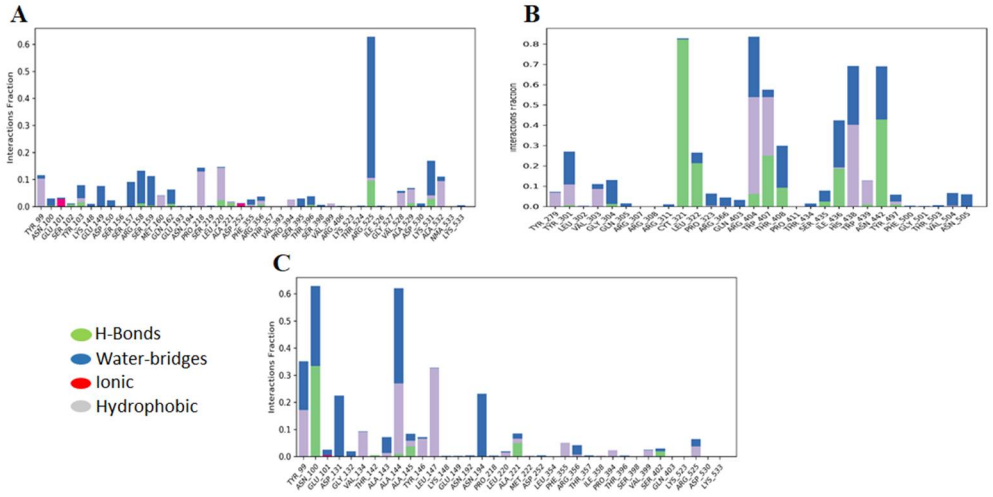


Fig. 6. Contact maps of: (A) CHS_azadiradione, (B) CHS_luteolin, and (C) CHS_difenoconazole complexes.

Molecular dynamics (MD) simulations provided insight into complex stability and protein-ligand interactions (Zanger and Schwab 2013). Analyses of H-bonding, protein Cα RMSD, and RMSF (Liu et al. 2018) showed that the CHS-luteolin complex maintained stability comparable to the CHS-difenoconazole reference. RMSF results indicated minimal fluctuations in catalytic and non-catalytic regions, suggesting stable ligand binding. Both luteolin and azadiradione remained stable during simulation, confirming their potential as high-affinity CHS inhibitors.

Despite the wide use of synthetic fungicides, *A. alternata* blight remains difficult to control due to emerging resistance. This work is the first to explore the antifungal mechanisms of these compounds against *A. alternata*. Luteolin exhibited the strongest binding, surpassing the reference fungicide difenoconazole. Further *in vitro* and *in vivo* studies on pure phytocompounds are needed to validate their efficacy. These compounds hold promise as biorational fungicides or scaffolds for developing novel antifungal agents, contributing to sustainable disease management strategies.

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