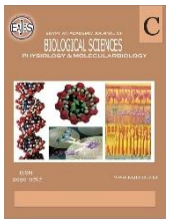
.

Citation: *Egypt.Acad.J.Biolog.Sci. (C.Physiology and Molecular biology) Vol. 17(1) pp1-18 (2025)*
DOI: 10.21608/EAJBSC.2025.403105

Egypt. Acad. J. Biolog. Sci., 17(1):1-18 (2025)

Egyptian Academic Journal of Biological Sciences
C. Physiology & Molecular Biology
ISSN 2090-0767
www.eajbsc.journals.ekb.eg



Structure-Based Discovery of Epigallocatechin Gallate and Withaferin A as Inhibitors of Clostridium difficile Toxin Pathogenesis

Saeedah Aljadani
Department of Basic Sciences, Faculty of Applied Medical Sciences, Al-Baha University, Al-Baha, Saudi Arabia
***E-mail:** saljadaani@bu.edu.sa

ARTICLE INFO
Article History
Received:1/12/2024
Accepted:6/1/2025
Available:10/1/2025

Keywords:
Clostridium difficile; TCDA and TCDB toxins; Epigallocatechin Gallate; Withaferin A; Molecular docking; Molecular dynamics simulations; ADMET analysis; Post docking analysis.

ABSTRACT

Clostridium difficile is a Gram-positive bacterium and a major global health concern due to its role in severe infectious diseases affecting humans and animals. Its prevalence in healthcare settings places a significant burden on healthcare systems worldwide. The primary virulence factors of *C. difficile* are the toxins TCDA and TCDB, which disrupt host cellular machinery and impair normal organ function. Despite ongoing efforts to develop effective therapeutic strategies, no universally successful treatment or preventive measure is currently available.

This study focuses on identifying potential compounds to inhibit the production of TCDA and TCDB toxins, aiming to mitigate *C. difficile* infections. Using a combination of ADMET analysis, molecular docking, post-docking analysis, and molecular dynamics simulations, five candidate compounds were evaluated. Among them, Epigallocatechin Gallate (CID: 65064) and Withaferin A (CID: 265237) demonstrated the highest binding affinities, with docking scores of -8.9 kcal/mol and -9.2 kcal/mol, respectively. Post-docking MMGBSA analysis further validated their binding free energies as -64 kcal/mol and -54 kcal/mol. The interaction analysis revealed that Epigallocatechin Gallate binds with multiple key residues, including TYR283, ASP285, and ARG272, while Withaferin A interacts with residues such as VAL455, ARG462, and SER517. Both compounds exhibited favorable ADMET properties, confirming their safety profiles. Molecular dynamics simulations further assessed their stability under real-time conditions through analyses such as RMSD, RMSF, SASA, H-bonds, MMGBSA, and PCA. The findings suggest that Epigallocatechin Gallate and Withaferin A hold promise as potential inhibitors of *C. difficile* toxin production, paving the way for future therapeutic development.

INTRODUCTION

Clostridium difficile is a gram positive bacteria was firstly introduced in mid-1930s (Voth and Ballard., 2005). It causes serious health issues in many countries. Human beings and animals specifically cattle effected by this infection causing bacteria (Carter., *et al* 2015). C.difficile cause variety of health problems from mild to severe. A broad range of gastrointestinal diseases including diarrhea to severe pseudomembranous colitis caused by clostridium difficile has significantly increased in Europe and America (Sun *et al*., 2010). Clostridium difficile is an anaerobic bacteria, produce toxins which cause infections, major two types are eagerly studied those are main virulence factors named Clostridium difficile toxin TcdA and TcdB (Kuehne., *et al*., 2010) (Voth and Ballard., 2005).

Antibody and cytotoxicity assessment analysis of patient stools showed TcdA and TcdB in it, as TcdA and TcdB are the important biomarkers for diagnosis of C.difficile causing infections (Voth and Ballard., 2005).

The chromosome of C.difficile comprise the encoding part of TcdA and TcdB, by the response of various environmental stimuli these toxins commonly expressed in the late log or stationary phases of growth (Voth and Ballard., 2005) . Asthe bacteria grow, the glycosylation of them regulates variety of physiological events in cellular machinery of host which cause disease. TcdA and TcdB represent homologous sequence and functionality and has 47% structure similarity (Chumbler *et al*., 2016), so it has been proposed that both these proteins are the results of gene duplication (Voth and Ballard., 2005). These two toxins are relatively similar with the other toxins of clostridium difficile. These toxins cause variety of severe infections.

The infection caused by Clostridium difficile develop in body by the ingestion of vegetative organisms which cause acidity in gastric system and develop in colon (Sun *et al*., 2010)). The mechanism of TcdA and TcdB is well defined as it modulates physiology of cell and made alterations in host cellular machinery. LDLR (low density lipoprotein receptor) does not serve as major receptor but facilitate the entry in the cell (Tao *et al*., 2019). The major target of TcdA and TcdB are Ras superfamily of small GTPases (Pruitt *et al*., 2012). Irreversible modification by the process of glycosylation of Rho family members (Carter., *et al* 2015) (Rac1, Cdc42, RhoA, RhoB, Rap2A, and RhoC) leads to the inactivation of small proteins in cell which cause prostration signaling pathways and also cause cell death (Voth and Ballard., 2005). Toxins of clostridium difficile cause inflammation in the colon and tissue damage which causing fluid loss in intestinal lumen and pave the way of diarrhea (Voth and Ballard., 2005). When the spores formed by clostridium difficile are not eliminated from the body of host, the patient facing the illness again and again which is the cause of serious complications and if left untreated cause death of patient (Voth and Ballard., 2005). The infection carriage also reported in infants as they have high number of toxins of Clostridium difficile in their stools (Voth and Ballard., 2005).

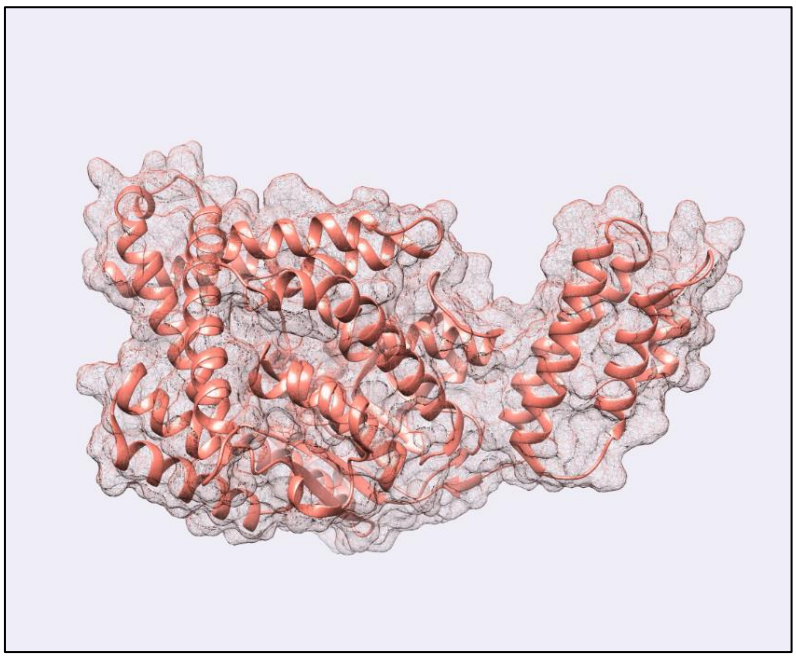


Fig. 1: Glycosyltransferase domain of toxin A (TcdA**).**

Clostridium difficile producing toxin TcdA has four functional domains, GT44 is the N-terminal domain glycosyltransferase also called GTD Figure 2, the protein of TcdA comprise many cofactor binding sites (Popoff., 2018). The toxin TcdA glycosyltransferase domain bind with UDP Mn2+ aid in the activity of glycosyltransferase (D'Urzo *et al*., 2012) (Alvin and Lacy., 2017). The N-terminal domain initiates the glycosylation activity of small host GTPases. The catalyzing of autoprocessing lead to release the N-terminal domain GT44 in the cytoplasm of host cell. The glycosyltransferase TcdA is a four helical structure that leads to the binding with phospholipids (Varela Chavez *et al*., 2015). In the post-translational modification of TcdA, the releasing of N-terminal caused by the autocatalytic cleavage which comprise the active part of toxin in cytoplasm (Reineke *et al*., 2007) (Pruitt *et al*., 2009).

In this study, our goal is to screen the potential compounds that could inhibit the production of toxins TcdA and TcdB in Clostridium Difficile to control the infectious diseases caused by this bacterium. The *in silico* approaches will utilized to identify leading compounds. Epigallocatechin Gallate (EGCG) and Withaferin A were chosen for their potential as inhibitors of Clostridium difficile toxin pathogenesis due to their established bioactive properties. EGCG, a polyphenolic compound found in green tea, exhibits strong antioxidant and anti-inflammatory effects, making it a promising candidate for modulating toxin-mediated inflammation and cellular damage. Withaferin A, a steroidal lactone derived from Withania somnifera (Ashwagandha), has been demonstrated to interfere with various signaling pathways and inhibit the activity of critical enzymes. Both compounds possess the ability to target the glycosyltransferase domains of TcdA and TcdB, thereby preventing the glycosylation of host GTPases. Additionally, their favorable pharmacokinetic profiles and low toxicity make them suitable for further exploration as therapeutic agents against C. difficile infections. Recent progress in phytotherapy has highlighted the promise of natural compounds as valuable supplements to conventional cancer treatments. These compounds not only enhance therapeutic outcomes but also help mitigate the adverse effects commonly associated with standard therapies. Compared to synthetic chemotherapeutic drugs, which often result in severe toxicity and widespread side effects, natural compounds are generally safer and more cost-effective. They demonstrate selective action, targeting cancer cells while preserving healthy tissues, thereby minimizing the physical and psychological toll on patients. Moreover, the production of plant-derived compounds is substantially more economical than the synthesis of traditional pharmaceuticals. This affordability is especially beneficial for low- and middle-income nations, where access to expensive cancer therapies can be challenging. For example, bioactive substances such as Berberine and Curcumin, sourced from abundant natural materials, offer a cost-efficient and sustainable alternative to synthetic drug development, meeting the rising demand for affordable and effective treatments globally.

MATERIALS AND METHODS

Data Retrieval and Preparation*:*

The target protein structure of Clostridium Difficile toxin TcdA was retrieved from RCSB Protein Data Bank (https://www.rcsb.org/search) containing PDB ID: 3SS1. The structure of 3SS1 determined through X-ray diffraction method having resolution 2.20 Å. Clostridium difficile toxin A (TcdA) glucosyltransferase domain having PDB ID: 3SS1contain only chain A comprise five hundred fifty-five amino acids. For further processing, the target proteins were prepared by using PyMol software which is used to visualize, process and animate biomolecules (Yuan *et al*., 2016)**.** PyMOL, a cross-platform molecular graphics application, is extensively used for 3D visualization of proteins, nucleic acids, tiny molecules, electron concentrations, surfaces,

and trajectory (Yuan *et al*., 2017). PyMol was used for removing water molecules from target protein. The default parameters of Swiss PDB viewer utilized for the energy minimization of the receptor (Rangisetty *et al*., 2023). The 3D structures of six natural compounds in SDF format was retrieved from PubChem database (https://pubchem.ncbi.nlm.nih.gov/)**.** The natural compounds were prioritized for their natural properties**.**

Pharmacokinetics Properties Prediction:

Pharmacokinetics properties prediction reveal the information about the intestinal absorption, distribution, blood brain barrier, CaCo2 permeability, toxicity and physicochemical properties of compounds (Siddiquee *et al*., 2024). Modern methods of evaluating drug characteristics are costly but the *in silico* approaches can overcome this problem by minimizing the large numbers of compounds on the basis of their pharmacokinetics and drug likeness properties (Kharchenko *et al*., 2022). PkCSM (https://biosig.lab.uq.edu.au/pkcsm) is publically available server for evaluating such properties, was utilized for this purpose. After analyzing their properties those compounds which did not pass the ADMET criteria were eliminated.

Toxicity Prediction*:*

Evaluating the toxicity of compounds is the most crucial step in the process of drug development (Siddiquee *et al*., 2024). It is necessary to predict the toxicity, tumorigenic, mutagenic and reproductive effects to choose the lead compounds those have lowest risk of failure during drug trials. Data warrior (OSIRIS) was used to predict the toxicity (Srivastava., 2021) and druglikeness of compounds (Parthasarathy *et al*., 2017)**.**

Molecular Docking:

The main purpose of molecular docking is to check the binding affinity and binding pose of ligand within receptor binding site (Fan *et al*., 2019). This process is crucial for knowing that how biomolecules interact with each other and aid in finding the novel compounds to solve queries. PyRx is a virtual screening tool (Dallakyan and Olson., 2015) was utilized to dock five natural compounds against receptor. During docking process Lamarckian Genetic algorithm was used with its default parameters. The energy minimization of all the ligands done. The charge was applied on ligands and receptor to convert them into pdbqt form before running the molecular docking. Docking simulations were carried out using AutoDock Vina, with a grid spacing of 0.375 Å to ensure high-resolution accuracy. The grid box dimensions were optimized to fully encompass the active sites of the target proteins. For BCL-2, the grid size was set to 60 × 60 × 60 points, centered at coordinates (X: -12.5, Y: 14.3, Z: 6.7), with a box size of 15 × 15 × 15 Å. Similarly, for PDL-1, the grid size was 50 × 50 × 50 points, centered at (X: 5.8, Y: -3.2, Z: 18.9), with a box size of 14 × 14 × 14 Å. The grid for CDK4/6 was set to 55 × 55 × 55 points, centered at (X: 18.1, Y: -9.6, Z: 25.7), with a box size of 16 × 16 × 16 Å. Finally, for FGFR, the grid size was 65 × 65 × 65 points, with a box size of 18 × 18 × 18 Å, centered at (X: 10.2, Y: 12.7, Z: 8.4).

The stability of the protein-ligand complexes was assessed by calculating binding free energy (kcal/mol) from the docking results. Conformations with the lowest binding energies were selected for further analysis. Post-docking evaluations were conducted using BIOVIA Discovery Studio, which enabled visualization of critical chemical interactions such as π-π stacking, hydrophobic contacts, and hydrogen bonding. Detailed 2D interaction diagrams generated with LigPlot+ provided insight into the specific residues involved in binding, further clarifying the stability and specificity of the complexes.

The docking protocol's validity was confirmed by redocking co-crystallized ligands into their respective binding sites. The root mean square deviation (RMSD) values of the redocked poses were ≤2 Å, demonstrating the reliability and reproducibility of the protocol. These validated docking results offered a comprehensive analysis of the molecular interactions and binding efficacy of

the selected phytochemicals with their target proteins. Advanced visualization tools, including BIOVIA Discovery Studio and LigPlot+, facilitated a detailed understanding of these interactions, paving the way for subsequent molecular dynamics simulations and therapeutic evaluations.The binding energy calculation between ligand and macromolecule was performed and interaction between the ligand and protein was analyzed by using UCSF chimera software.

Molecular Dynamics Simulation:

A molecular dynamics (MD) simulation was carried out in order to investigate the structural stability of the 3ss1-withaferin A and 3ss1-Epigallocatechin gallate complexes under certain particular physiological settings. A Molecular Dynamics simulation that lasted for one hundred nanoseconds was carried out with the help of the Desmond package, which can be found at Schrodinger suit (Ullah *et al*., 2023). The orthorhombic often boundary box has been selected for each complicated TPI3P water model in order to guarantee that the signifies of the systems will stay unchanged. Na+ and Cl- ions were chosen at random and distributed inside the solvated system in order to keep the salt concentration at 0.15 M (Padhi *et al*., 2022). This was done in order to maintain the salt concentration. Applying the OPLS_2005 force field at a temperature of 300 kelvin and a pressure of 1 atmosphere contributed to the reduction and relaxation of the system (Filipe and Loura., 2022). The first step was to establish a state of release for every complex system.

Recording intervals of one hundred ps were then used in order to carry out the final output.

RESULTS

Data Retrieval:

The structure of target protein was retrieved and prepared by removing water molecules and got energy minimized. The ligands three-dimensional structure was retrieved from PubChem database in SDF format Figure 2. The natural products or remedies become highly accepted to cure several inflammatory, renal, and cardiac diseases as they provide multiple benefits against disorders (Ullah *et al.*, 2024) . The natural compounds play various roles in biotic system (Ullah *et al.*, 2024).

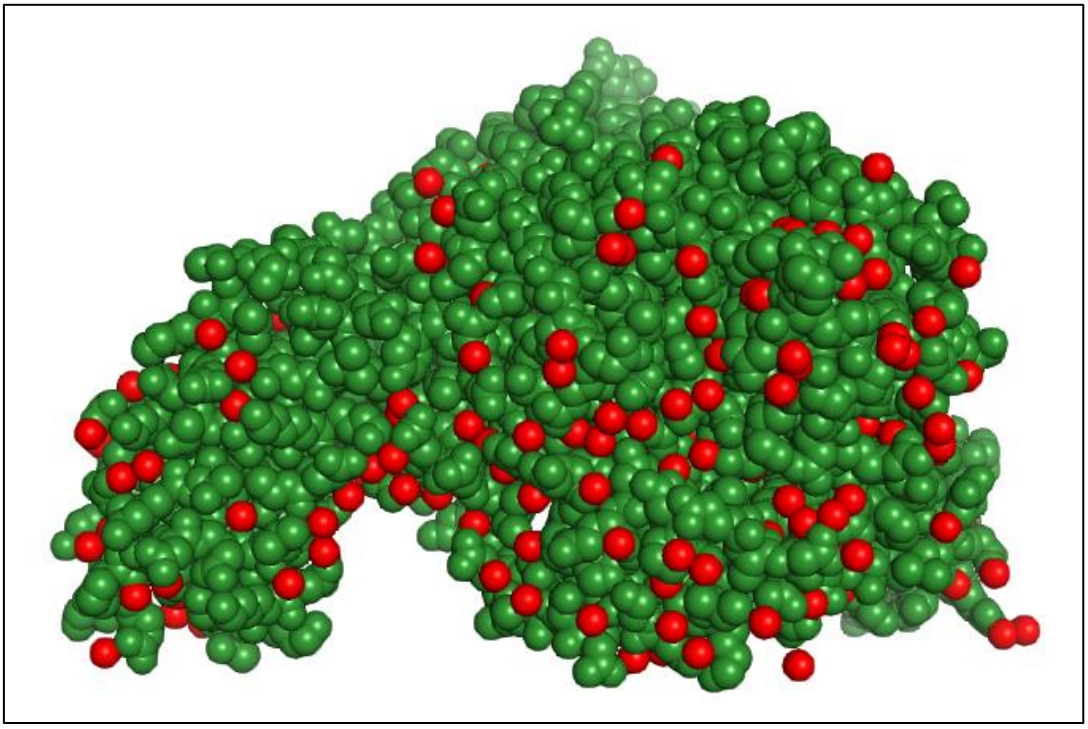


Fig. 2: Structure of Clostridium difficile toxin A glucosyltransferase domain (PDB ID: 3SS1) with water molecules.

Pharmacokinetics Properties Analysis:

The failure of drug development process mainly caused by the failure in pharmacokinetics properties, it is necessary to evaluate those properties in early stages of drug development. Early detection or diagnostics of pharmacokinetics properties aid in discarding the inappropriate compounds (Xiong *et al.*, 2021). Under this study the properties under consideration were blood-brain barrier, water solubility, intestinal absorption, Lipinski rule of five, CaCo2 permeability. Table 1, provides detailed analysis of the ADMET properties of the selected compounds.

Table 1: ADMET Analysis of compounds.

Features	**CID: 2353 Berberine**	**CID: 445154 Resveratrol**	**CID: 5280343 Quercetin**	**CID: 65064 Epigallocatechin Gallate**	**CID: 265237 Withaferin A**
MOL_WEIGHT	336.367	228.247	302.238	458.375	470.606
ROTATABLE_BONDS	2	2	1	3	3
H-Bond ACCEPTORS	4	3	7	11	6
H-Bond Donors	0	3	5	8	2
Intestinal absorption (human)	97.147	90.935	77.207	47.395	85.345
Blood Brain Barrier permeability	0.198	-0.048	-1.098	-2.184	-0.03
LOGP	3.0963	2.9738	1.988	2.2332	3.3529
cLogS	-4.669	-2.864	-2.491	-2.16	-4.469
Caco2 permeability	1.734	1.17	-0.229	-1.521	0.829
Skin Sensitisation	NO	NO	NO	NO	NO

Toxicity Analysis:

Toxicity analysis is the most important step to check the hazardous effect of compounds on living organisms. It is important for human health because certain compounds those are involve in the treatment of disease can affect the other organs in body which might be harmful for health. The compounds which are considered as potential candidate for curing disease can be tumorigenic, mutagenic, irritant, toxic, and effect badly on reproductive system. So, those properties including drug likeness must be under consideration while developing the drug. Table 2, summarizes the toxicity profiles of selected bioactive compounds based on key toxicity parameters.

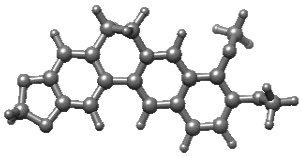
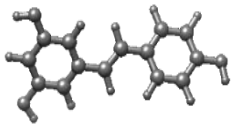
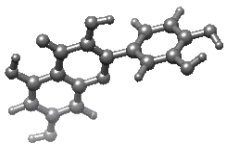
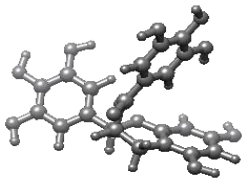
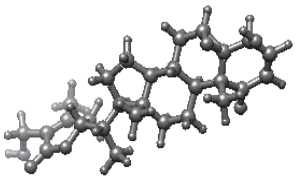
Table 2: Toxicity analysis.

Features	**CID_2353 Berberine**	**CID_445154 Resveratrol**	**CID_ 5280343 Quercetin**	**CID_65064 Epigallocatechin Gallate**	**CID_265237 Withaferin A**
Hepatotoxicity	Yes	No	No	No	No
Ames toxicity	Yes	Yes	No	No	No
Oral Rat Acute Toxicity	2.571	2.529	2.471	2.522	2.779
Oral Rat Chronic Toxicity	1.89	1.533	2.612	3.065	0.918
Druglikeness	-2.2467	-1.6732	-0.082832	-0.32874	1.6889
Mutagenic	None	High	High	None	None
Tumorigenic	None	None	High	None	None
Reproductive Effective	None	High	None	None	Low
Irritant	None	None	None	None	None

Molecular Docking Analysis:

Molecular docking was done to find out the interaction, binding affinity and binding pose between protein and ligand (Ballester and Mitchell., 2010). The results of five compounds show that the Withaferin A and Epigallocatechin Gallate show strong binding affinity. Withaferin A having CID: 265237 and Epigallocatechin Gallate having CID: 65064 show binding affinity -9.2 kcal/mol and -8.9 kcal/mol respectively. Table 3, showing Docking Scores for Selected Compounds and Figure 3, illustrates the molecular interactions between the Clostridium difficile toxin A glucosyltransferase and the two bioactive compounds, Withaferin A and Epigallocatechin Gallate.

Table 3: Representation of docking score with respect to the mentioned compounds names.

PubChem CID	Compound name	3D structure	Docking score (Kcal/mol)
CID_2353	Berberine		-7.4
CID_445154	Resveratrol		-7.2
CID_5280343	Quercetin		-8.4
CID_ 65064	Epigallocatechin Gallate		-8.9
CID_265237	Withaferin A		-9.2

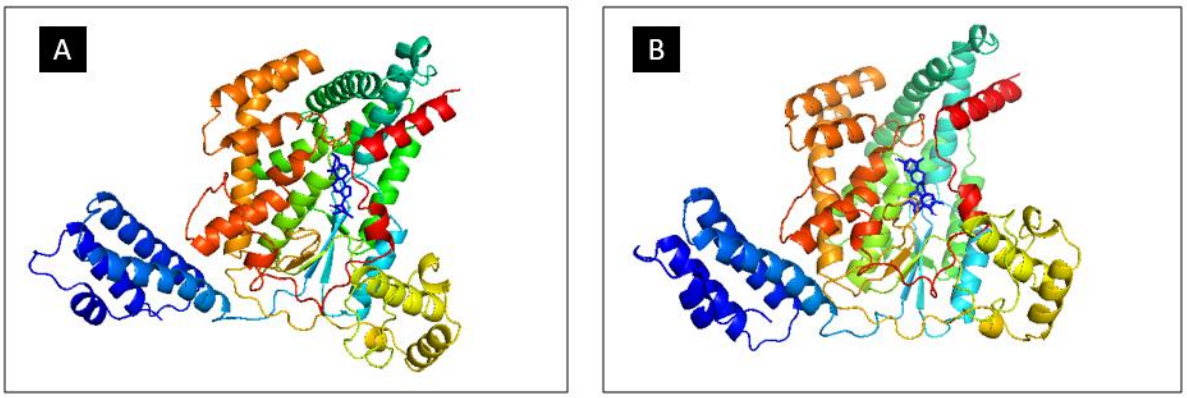


Fig. 3: (A) Structure of Clostridium difficile toxin A glucosyltransferase complexed with Withaferin A. (B) Structure of Clostridium difficile toxin A glucosyltransferase complexed with Epigallocatechin Gallate.

Protein ligand Interaction:

The UCSF chimera software was utilized to visualize the protein residues interaction with specific ligand. The two compounds which passes the ADMET analysis and show best binding affinity in the process of virtual screening were visualize in the UCSF chimera software to know about the interacted residues. The interactive residues of Withaferin A (CID_ 6506) compound with receptor are VAL455, GLY456, ARG462, SER268, ARG272, PHR521, TYR283, ASP285, SER517, and VAL286. The interactive residues of compound Epigallocatechin Gallate (CID_65064) with receptor are TYR283, ASP285, ARG272, ASP287, ASP269, GLN384, ASN383, GLU514, SER517, ILE382, THR464, LEU518, VAL544, ILE465, SER463, PHE521, and ARG462. Figures 4 and 5, illustrate the molecular docking results and interactions of Clostridium difficile toxin A with the bioactive compounds Withaferin A and Epigallocatechin Gallate, respectively.

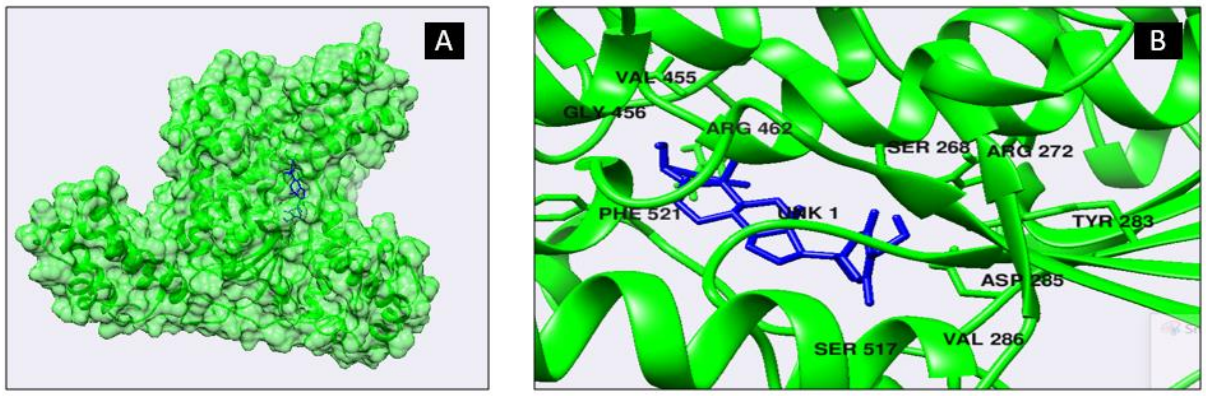


Fig. 4: (A) Clostridium difficile toxin A protein complexed with Withaferin A compound. (B) Shows the interactive residues of protein with ligand.

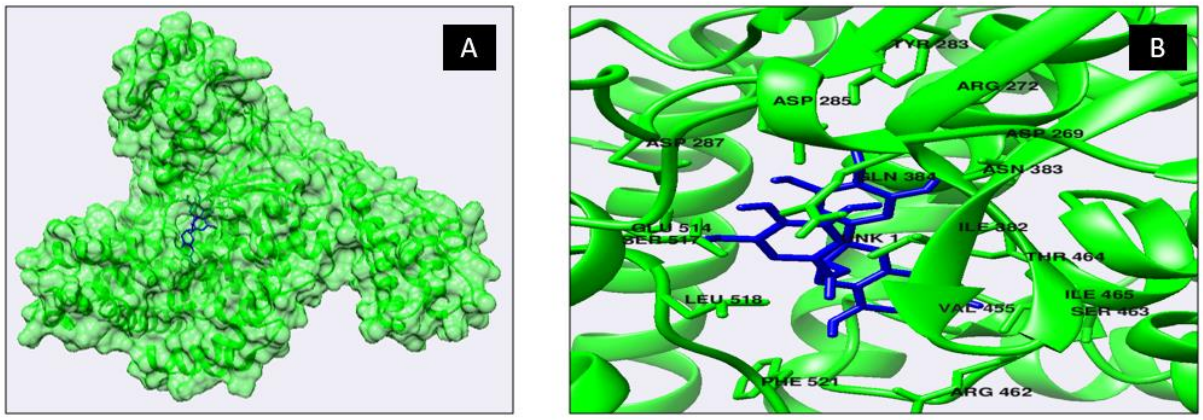


Fig. 5: (A) Clostridium difficile toxin A protein complexed with Epigallocatechin Gallate compound. (B) Shows the interactive residues of protein with ligand.

Molecular Dynamics Simulation:

During the target time, the MD simulation is helpful in gaining an understanding of the interactions that occur between molecules as well as the stability of the 3ss1-withaferin A and 3ss1-Epigallocatechin gallate complexes. Furthermore, it is able to provide an estimation of the structural development that would occur in a complicated system if it were established in an artificial environment (Benson and Daggett., 2012).

RMSD Analysis:

For the purpose of analyzing the conformational change of the target 3ss1 protein in the complex of the desired molecule, which consists of 3ss1-withaferin and 3ss1-Epigallocatechin gallate, a 100 ns MD simulation was carried out, and, as a result, a somewhat comparable RMSD value was found Figure 6 (A). For the purpose of analyzing the conformational change of the target 3ss1 protein in the complex of the desired molecule, which consists of 3ss1-withaferin and 3ss1-Epigallocatechin gallate, a 100 ns MD simulation was carried out, and, as a result, a somewhat comparable RMSD value was found (Dar *et al.*,2018). The protein and ligand in 3ss1-withaferin remained stable during simulation. The ligand withaferin stabilized after 10ns till at the end. The 3ss1-Epigallocatechin gallate remained stable during the running, the ligand Epigallocatechin gallate little bit fluctuation at 10ns and 30ns, after that showed stabilization Figure 6 (B) (Jakhar *et al.*,2020).

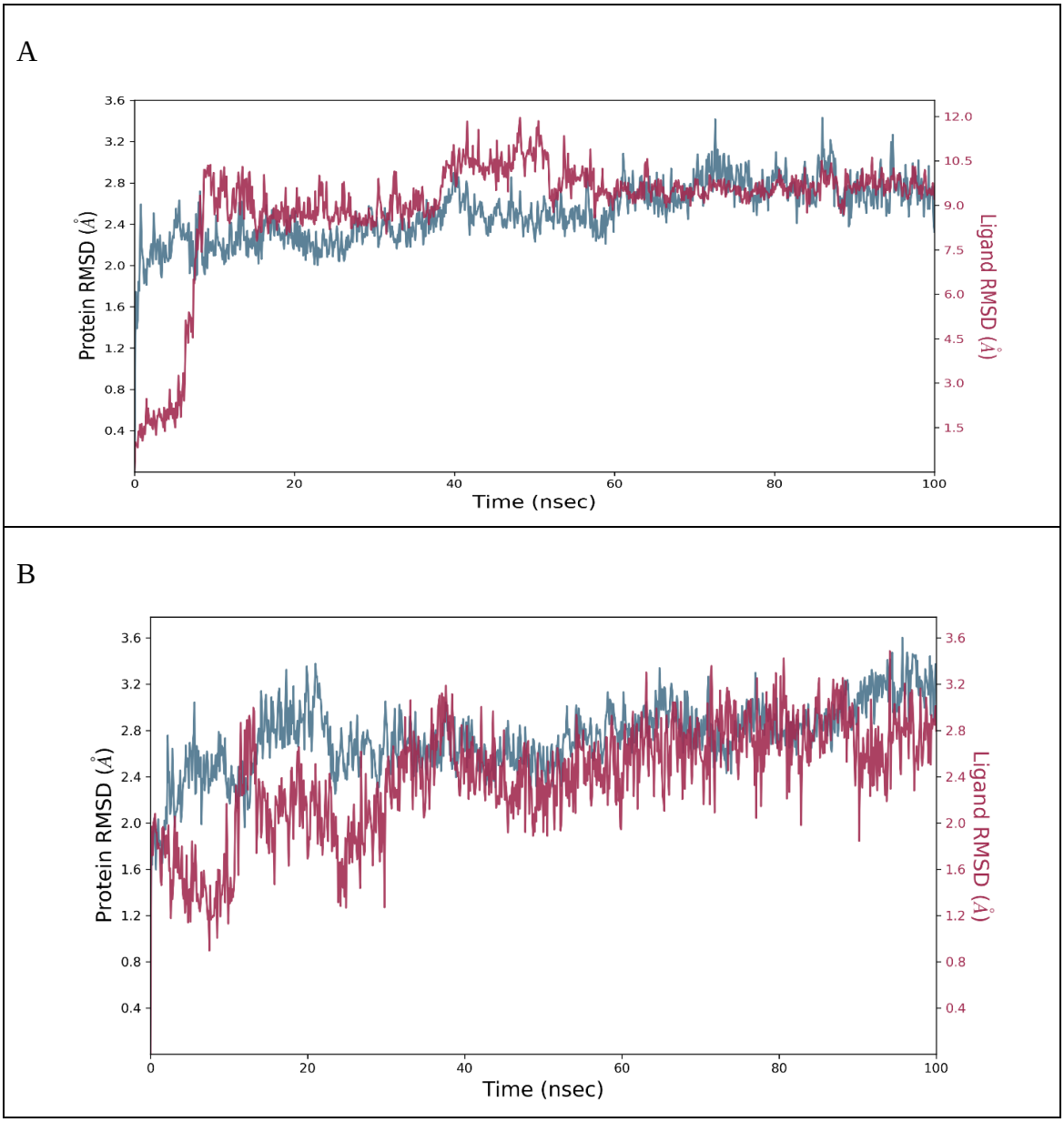


Fig. 6: The RMSD analysis of top two complexes (A) 3ss1-withaferin and (B) 3ss1-Epigallocatechin

RMSF Analysis:

The RMSF values of the molecules withaferin and epigallocatechin gallate complexes with the 3ss1 protein were calculated in order to monitor the alteration in protein structural flexibility that occurred during the attachment of specific molecules to a certain residual site (Opo, *et al.*,2021). This is shown in Figure 7. In Figure 7, each of the selected compounds had a peak area of the 3ss1 protein at residual regions that exhibited the maximum volatility during the course of the simulation.

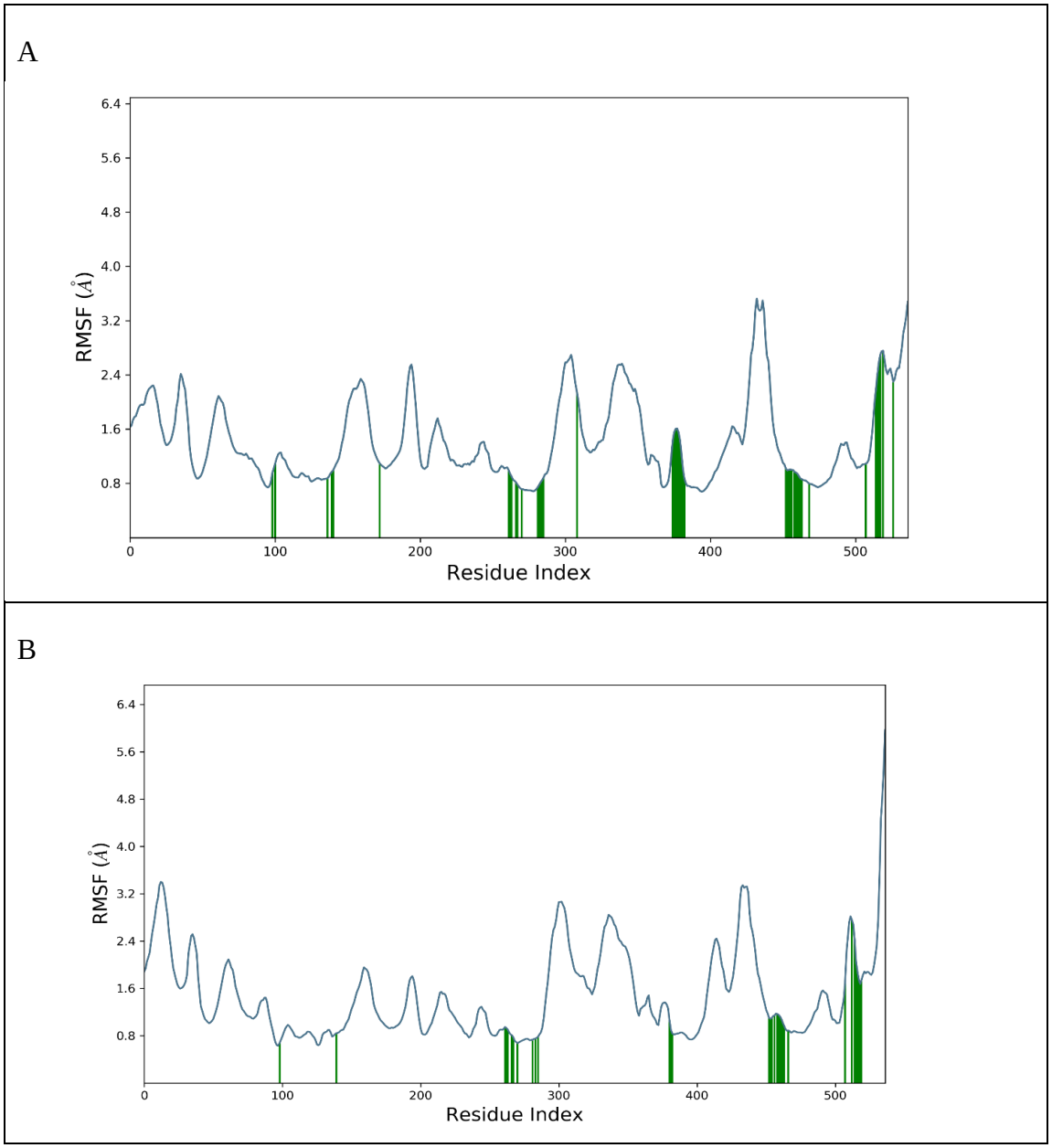


Fig. 7: The RMSF analysis of top two complexes (A) 3ss1-withaferin and (B) 3ss1-Epigallocatechin

Secondary Structure Elements:

A description of the secondary structural elements (SSE) that were present in the 3ss1 protein throughout the simulation can be found in Figure 8. It gives a visual depiction of the distribution of beta strands and alpha helices throughout the 3ss1 protein structure, displaying the distribution in connection to the residue index (Arnittali *et al.*, 2019). These components, when taken as a whole, are responsible for roughly 51.12 and 49.66 percent of the secondary structure of the 3ss1 protein in complexes 3ss1-withaferin and 3ss1-Epigallocatechin gallate, respectively. To be more specific, we may say that roughly 5.61 and 5.33 percent of the secondary structural components are made up of beta-strands, while approximately 45.51 and 44.66 percent are made up of alpha-helices, respectively (Oluwafemi, *et al.*, 2024).

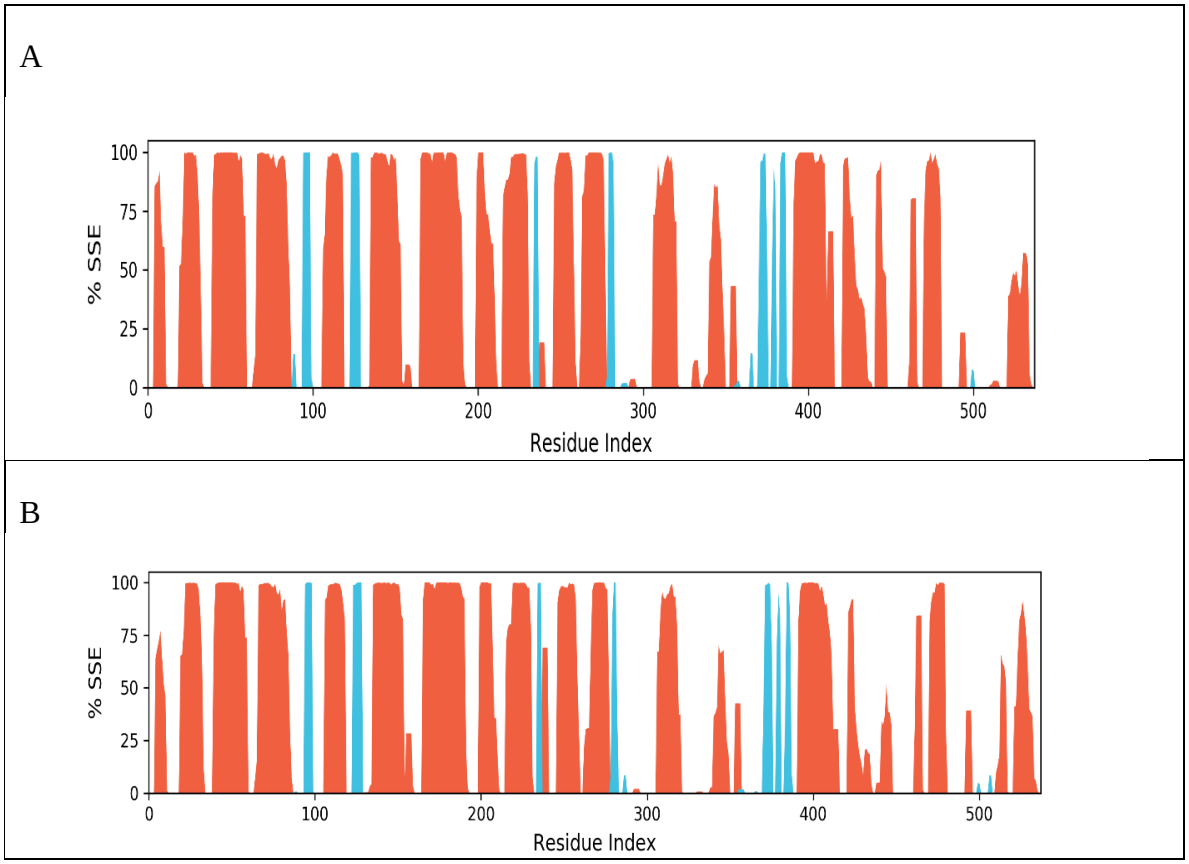


Fig. 8: The SSE plots of top two complexes (A) 3ss1-withaferin and (B) 3ss1-Epigallocatechin

Protein-Ligand Contacts:

An analysis of the interaction between the 3ss1 protein and the chemicals withaferin and epigallocatechin gallate has been performed, and the results are shown in Figure 9 (A). The analysis was based on a number of parameters, including hydrogen bond connections (Santos Silva, *et al.*,2018). The unique interaction is maintained for the whole of the simulation as a result of the recurrent interactions of the ligands, as seen by the illustration in Figure 9. Multiple contacts were created between the compound withaferin and the residues Ala377, Leu378, Gly456, Met458, Glu460, Ala461, and Trp519, which were sustained in line with the simulation duration (Fig. 8A). Multiple contacts were created by the compound Epigallocatechin gallate at the residues Asp269, Arg272, Asp285, Ala377, Ala461, Glu514, Leu518 and Trp519, which were sustained in line with the simulation duration (Fig. 9B).

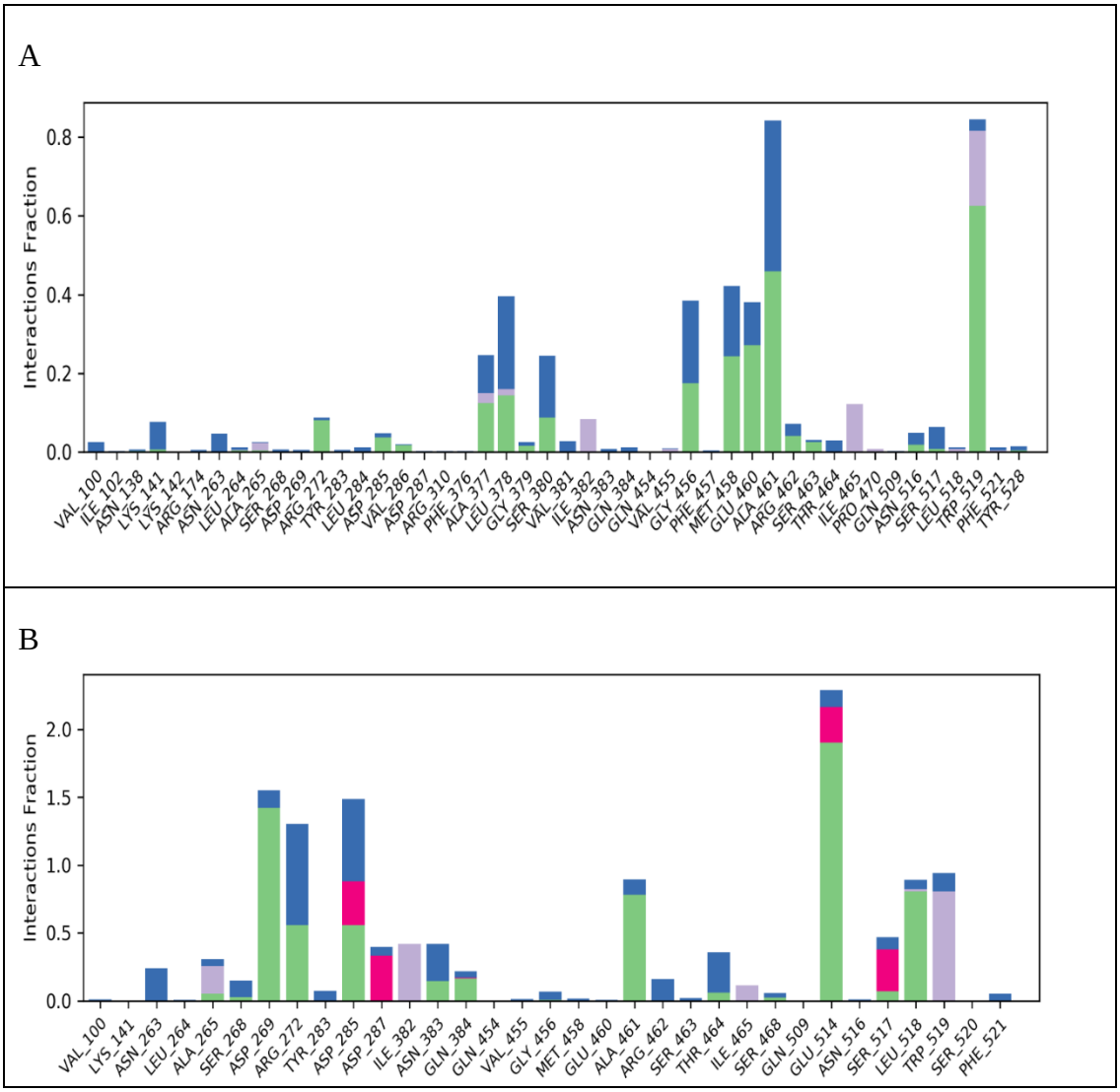


Fig. 9: The interactions analysis of top two complexes (A) 3ss1-withaferin and (B) 3ss1-Epigallocatechin

DISCUSSION

Clostridium difficile is a serious concern in healthcare facilities around the world. C. difficile produces two major toxins, TcdA and TcdB, which are the main virulence factors in the disease (Chumbler Nicole *et al.*,2016). The most common cause of infections involving bacteria linked to healthcare in the US is Clostridium difficile, which can cause fatal illnesses. Bacterial toxins TcdA and TcdB are important factors in the pathophysiology of disease and make appealing therapeutic targets**.** One effective treatment strategy for C difficile infection (CDI) is to neutralize the actions of TcdA and TcdB. Those toxins of C. difficile, an emerging pathogenic agent with antibiotic resistance, are the subject of this study's computational study and molecular docking (Scotti *et al.*, 2017). We examined five structures of natural products, and identified two potential therapeutic candidates to block toxin independently.

The structure of Clostridium difficile toxin A (TcdA) glucosyltransferase domain having PDB ID: 3SS1 retrieved from PDB database. The structure of ligands was retrieved PubChem database in SDF format. Pharmacokinetics properties prediction of Berberine (CID: 2353), Resveratrol (CID: 445154), Quercetin (CID: 5280343), Epigallocatechin Gallate (CID: 65064), Withaferin A (CID: 265237) have been analyzed. A few natural substances' receptor-

binding properties, ADME toxicity, and drug-likeness were examined in more detail. ADME characteristics decide how a drug moves throughout the body (Siddiquee *et al*., 2024. These properties significantly affect a compound's capability to move through the bodies of humans and animal. In the process of developing new drugs, it is essential to maximize the pharmacokinetic properties to fulfil the demands established by clinical trials, therefore determining the possible effectiveness of the medication candidate (Siddiquee *et al*., 2024). A drug molecule with Molecular Weight<500g/mol, ClogP <5, hydrogen-bond-accepting atoms <10, and hydrogen-bond-donating atoms <5 is considered an acceptable pharmaceutical agent in terms of oral administration, according to the Lipinski rule for drug-likeness Kharchenko *et al*., 2022). The absorption rate of molecule which is considered to be a potential lead compound is determined by a LogP value. The log P value and the body's absorption of drug molecules are inversely correlated. The possible compound's solubility is demonstrated by the LogS value Kharchenko *et al*., 2022). pkCSM tool is used to predict the physicochemical and ADMET properties (Muslikh *et al*., 2023). So the Epigallocatechin Gallate (CID: 65064), Withaferin A (CID: 265237) successfully passed the ADME and toxicity criteria.

The target protein and ligands structures got prepared and molecular docking was run to analyze the interaction and binding affinity and binding pose of ligand to protein (Yuriev and Ramsland., 2010). This is an advanced technique of screening libraries to find the best novel compounds. This study aims to find the best therapeutic agent which inhibit the production of toxin in clostridium difficile. The molecular docking was run on five natural compounds then two of them were selected named Epigallocatechin Gallate (CID: 65064), Withaferin A (CID: 265237) having binding affinity -8.9 kcal/mol and -9.2 kcal/mol respectively were selected.

Within a 3ss1 protein or other molecular structure, a molecular dynamics (MD) simulation makes it possible to make predictions about the movement of atoms and the interactions between atoms over a predetermined amount of time. For the purpose of determining the behavior of biomolecules at the atomic level, MD simulations may be conducted. They serve as markers of the ideal sustainability of the compounds, and the RMSD measurements for the complexes Epigallocatechin Gallate (CID: 65064) and Withaferin A (CID: 265237) are used to determine this. Both the chemical Epigallocatechin Gallate (CID: 65064) and the compound Withaferin A (CID: 265237) have average RMSD values of 2.75 Å and 2.54 Å, respectively of their respective compounds (Sargsyan, K., C. Grauffel, and C. Lim., 2017). The rigidity of the protein structure is shown by the fact that the variation of the residues is relatively modest in comparison to the native structural components that are present in the complex frame. Due to the presence of the α-helix, β-sheet, N-terminal, and C-terminal domains, the levels of fluctuation are reported to be at their highest at the beginning and end of the 3ss1 protein (Padhi *et al*., 2022).

To show the effect of a pharmaceutical substance, which causes conformational changes in the protein, it is also possible to employ it within the context of a protein structure (Wilkinson., 2001). According to the findings of a number of studies, Epigallocatechin Gallate (CID: 65064) and Withaferin A (CID: 265237) have the potential to be used as a therapeutic agent in the treatment of Clostridium difficile infection.

Conclusion

This study identified and screened natural compounds with potential inhibitory activity against disease-causing toxins of *Clostridium difficile*. Despite the availability of therapeutic approaches, managing *C. difficile* infections remains challenging. Our findings highlight Epigallocatechin Gallate (CID: 65064) and Withaferin A (CID: 265237) as promising candidates with drug-like physicochemical properties. These

compounds demonstrated potential for further development as therapeutic agents. Future research should focus on detailed preclinical evaluations, including in vitro and in vivo studies, to validate their efficacy and safety, paving the way for novel treatment strategies against *C. difficile*-associated infections.

Declarations:

Ethical Approval: Not applicable.

Conflict of interests: The author has no relevant financial or non-financial interests to disclose

Authors Contributions: Main Author.

Funding: The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.

Availability of Data and Materials: All data supporting the described findings of the study can be obtained from the corresponding authors upon request.

Acknowledgements: The authors acknowledge the kind help of the College members of Faculty of applied sciences /University of Al baha; Dr Saba beigh and Dr Hind Alzahrani.

REFERENCES

Alvin, J.W. and Lacy, D.B., 2017. Clostridium difficile toxin glucosyltransferase domains in complex with a non-hydrolyzable UDP-glucose analogue. *Journal of structural biology*, *198*(3), pp.203-209. https://doi.org/10.1016/j.jsb.2017.04.006

Arnittali, M., Rissanou, A.N. and Harmandaris, V., 2019. Structure of biomolecules through molecular dynamics simulations. *Procedia Computer Science*, *156*, pp.69-78. https://doi.org/10.1016/j.procs.2019.08.181

Ballester, P.J. and Mitchell, J.B., 2010. A machine learning approach to predicting protein–ligand binding affinity with applications to molecular docking .*Bioinformatics* , *26*(9), pp.1169-1175. https://doi.org/ 10.1093/bioinformatics/btq112

Benson, N.C. and Daggett, V., 2012. A comparison of multiscale methods for the analysis of molecular dynamics simulations. *The Journal of Physical Chemistry B*, *116*(29), pp.8722-8731. https://doi.org/10.1021/jp302103t

Carter, G.P., Chakravorty, A., Pham Nguyen, T.A., Mileto, S., Schreiber, F., Li, L., Howarth, P., Clare, S., Cunningham, B., Sambol, S.P. and Cheknis, A., 2015. Defining the roles of TcdA and TcdB in localized gastrointestinal disease, systemic organ damage, and the host response during Clostridium difficile infections. *MBio*, *6*(3), pp.10-1128. https://doi.org/10.1128/mbio.00551-15

Chumbler, N.M., Farrow, M.A., Lapierre, L.A., Franklin, J.L. and Lacy, D.B., 2016. Clostridium difficile toxins TcdA and TcdB cause colonic tissue damage by distinct mechanisms. *Infection and immunity*, *84*(10), pp.2871-2877. https://doi.org/10.1128/iai.00583-16

Chumbler, N.M., et al., 2016.Crystal structure of Clostridium difficile toxin A. *Natur Microbiolology*, 11:1:15002. https://doi: 10.1038/nmicrobiol.2015.2.

D'Urzo, N., Malito, E., Biancucci, M., Bottomley, M.J., Maione, D., Scarselli, M. and Martinelli, M., 2012. The structure of Clostridium difficile toxin A glucosyltransferase domain bound to Mn2+ and UDP provides insights into glucosyltransferase activity and product release. *The FEBS journal*, *279*(17), pp.3085-3097. https://doi.org/10.1111/j.1742-4658.2012.08688.x

Dallakyan, S. and Olson, A.J., 2015. Small-molecule library screening by docking with PyRx. *Chemical biology: methods and protocols*, pp.243-250. https://doi.org/10.1007/978-1-4939-2269-7_19

Dar, K.B., Bhat, A.H., Amin, S., Hamid, R., Anees, S., Anjum, S., Reshi, B.A.,

Zargar, M.A., Masood, A. and Ganie, S.A., 2018. Modern computational strategies for designing drugs to curb human diseases: a prospect. *Current topics in medicinal chemistry*, *18*(31), pp.2702-2719.Fan, J., Fu, A. and Zhang, L., 2019. Progress in molecular docking. *Quantitative Biology*, *7*, pp.83-89. https://doi.org/10.2174/1568026619666190119150741

Filipe, H.A. and Loura, L.M., 2022. Molecular dynamics simulations: advances and applications. *Molecules*, *27*(7), p.2105. https://doi.org/10.3390/molecules27072105

Jakhar, R., Dangi, M., Khichi, A. and Chhillar, A.K., 2020. Relevance of molecular docking studies in drug designing. *Current Bioinformatics*, *15*(4), pp.270-278. https://doi.org/10.2174/1574893615666191219094216

Kuehne, S.A., Cartman, S.T., Heap, J.T., Kelly, M.L., Cockayne, A. and Minton, N.P., 2010. The role of toxin A and toxin B in Clostridium difficile infection. *Nature. 467* (7316) , pp.711-713. https://doi:10.1038/nature09397

Kumar, T.A., Kabilan, S. and Parthasarathy, V., 2017. Screening and toxicity risk assessment of selected compounds to target cancer using QSAR and pharmacophore modelling. *International Journal Of Pharmtech Research*, 10(4), pp.219-224. http://dx.doi.org/10.20902/IJPTR.2017.10428

Muslikh, F.A., Kurniawati, E., Ma'arif, B., Zenmas, S.Z., Salmasfattah, N., Dhafin, A.A. and Prasetyawan, F., 2023. ADMET Prediction of the Dominant Compound from Mangosteen (Garcinia mangostana L.) using pkCSM: A Computational Approach. *International Journal of Contemporary Sciences (IJCS)* ,*1*(1), pp.33-38. https://doi.org/10.55927/ijcs.v1i1.7271

Oluwafemi, K.A., Jimoh, R.B., Omoboyowa, D.A., Olonisakin, A., Adeforiti, A.F. and Iqbal, N., 2024. Investigating the effect of 1, 2-Dibenzoylhydrazine on Staphylococcus aureus using integrated computational approaches . *In Silico Pharmacology*, *12*(2), pp.1-15. https://doi.org/10.1007/s40203-024-00278-1

Opo, F.A.D.M., Rahman, M.M., Ahammad, F., Ahmed, I., Bhuiyan, M.A. and Asiri, A.M., 2021. Structure based pharmacophore modeling, virtual screening, molecular docking and ADMET approaches for identification of natural anti-cancer agents targeting XIAP protein. *Scientific reports*, *11*(1), p.4049. https://doi.org/10.1038/s41598-021-97945-6

Padhi, A.K., Janežič, M. and Zhang, K.Y., 2022. Molecular dynamics simulations: principles, methods, and applications in protein conformational dynamics In *Advances.in protein molecular and structural biology methods* (pp. 439-454). Academic Press. https://doi.org/10.1016/B978-0-323-90264-9.00026-X

Popoff, M.R., 2018. Clostridium difficile and Clostridium sordellii toxins, proinflammatory versus anti-inflammatory response. *Toxicon*, *149*, pp.54-64. https://doi.org/10.1016/j.toxicon.2017.11.003

Pruitt, R.N., Chagot, B., Cover, M., Chazin, W.J., Spiller, B. and Lacy, D.B., 2009. Structure-function analysis of inositol hexakisphosphate-induced autoprocessing in Clostridium difficile toxin A. *Journal of Biological Chemistry*, *284*(33), pp.21934-21940. https://doi: 10.1074/jbc.M109.018929

Pruitt, R.N., Chumbler, N.M., Rutherford, S.A., Farrow, M.A., Friedman, D.B., Spiller, B. and Lacy, D.B., 2012.

Structural determinants of Clostridium difficile toxin A glucosyltransferase activity. *Journal of Biological Chemistry*, *287*(11), pp.8013-8020. https://doi 10.1074/ jbc.M111.298414

Rangisetty, P.T., Kilaparthi, A., Akula, S., Bhardwaj, M. and Singh, S., 2023. RSAD2: An exclusive target protein for Zika virus comparative modeling, characterization, energy minimization and stabilization. *International Journal of Health Sciences*, *17*(1), p.12.

Reineke, J., Tenzer, S., Rupnik, M., Koschinski, A., Hasselmayer, O., Schrattenholz, A., Schild, H. and von Eichel-Streiber, C., 2007. Autocatalytic cleavage of Clostridium difficile toxin B. *Nature*, *446*(7134), pp.415-419. https://doi:10.1038/nature05622

Santos Silva, H., Alfarra, A., Vallverdu, G., Bégué, D., Bouyssière, B. and Baraille, I., 2018. Impact of H-bonds and porphyrins on asphaltene aggregation as revealed by molecular dynamics simulations. *Energy & Fuels*, *32*(11), pp.11153-11164. https://doi: 10.1021/acs. energy fuels.8b01901

Sargsyan, K., Grauffel, C. and Lim, C., 2017. How molecular size impacts RMSD applications in molecular dynamics simulations. *Journal of chemical theory and computation*, *13*(4), pp.1518-1524. https://doi. org/ 10. 1021/acs.jctc.7b00028

Scotti, L., JB Mendonca Junior, F., M Ishiki, H., F Ribeiro, F., K Singla, R., M Barbosa Filho, J., S DaSilva, M. and T Scotti, M., 2017. Docking studies for multi-target drugs. *Current drug targets*, *18*(5), pp.592-604. 10. 2174/ 1389450116666150825111818

Siddiquee, N.H., Hossain, M.I., Talukder, M.E.K., Nirob, S.A.A., Shourav, M., Jahan, I., Tamanna, U.H.A., Das, P., Akter, R., Hasan, M. and Abdullah-Al-Mamun, M., 2024. In-silico identification of novel natural drug leads against the Ebola virus VP40 protein: A promising approach for developing new antiviral therapeutics. *Informatics in Medicine Unlocked*, *45*, p.101458. https://doi.org/10.1016/j.imu.2024.101458

Siddiquee, N.H., Malek, S., Tanni, A.A., Mitu, I.J., Arpa, S.H., Hasan, M.R., Shammi, S.E.J., Chakma, C., Mahinur, M., Wajed, S. and Hossain, M.I., 2024. Unveiling the antiviral activity of 2′, 3, 5, 7-Tetrahydroxyflavanone as potential inhibitor of chikungunya virus envelope glycoprotein. *Informatics in Medicine Unlocked*, *47*, p. 101486. https://doi.org/10.1016/j. imu.2024.101486

Srivastava, R., 2021. Theoretical studies on the molecular properties, toxicity, and biological efficacy of 21 new chemical entities. *ACS omega*, *6*(38), ,pp.24891-24901. https://doi. org/ 10.1021/acsomega.1c03736

Sun, X., Savidge, T. and Feng, H., 2010. The enterotoxicity of Clostridium difficile toxins. *Toxins*, *2*(7), pp. 1848-1880. https://doi.org/10.3390/ toxins2071848

Tao, L., Tian, S., Zhang, J., Liu, Z., Robinson-McCarthy, L., Miyashita, S.I., Breault, D.T., Gerhard, R., Oottamasathien, S., Whelan, S.P. and Dong, M., 2019. Sulfated glycosaminoglycans and low-density lipoprotein receptor contribute to Clostridium difficile toxin A entry into cells. *Nature microbiology*, *4*(10), pp.1760-1769. doi: 10.1038/s41564-019-0464-z.

Ullah, S., Zheng, Z., Rahman, W., Ullah, F., Ullah, A., Iqbal, M.N., Iqbal, N. and Gao, T., 2023. A computational approach to fighting type 1 diabetes by targeting 2C Coxsackie B virus protein with flavonoids. *Plos one*, *18*(8), p.e0290576. https://doi. org/10.1371/journal.pone.0290576

Ullah, S., Rahman, W., Ullah, F., Ullah, A., Jehan, R., Iqbal, M.N., Ali, I. and Tianshun, G., 2024. Identification of lead compound screened from the natural products atlas to treat renal inflammasomes using molecular docking and dynamics simulation. *Journal of Biomolecular Structure and Dynamics*, *42*(9), pp.4851-4861. https://doi.org/10.1080/07391102.2023.2254397

Varela Chavez, C., Hoos, S., Haustant, G.M., Chenal, A., England, P., Blondel, A., Pauillac, S., Lacy, D.B. and Popoff, M.R., 2015. The catalytic domains of C lostridium sordellii lethal toxin and related large clostridial glucosylating toxins specifically recognize the negatively charged phospholipids phosphatidylserine and phosphatidic acid. *Cellular microbiology*, *17*(10), pp.1477-1493 https://doi.org/10.1111/cmi.12449

Voth, D.E. and Ballard, J.D., 2005. Clostridium difficile toxins: mechanism of action and role in disease. *Clinical microbiology reviews*, *18*(2), pp.247-263. https://doi.org/10.1128/cmr.18.2.247-263. 2005.

Wilkinson, R., 2001. Pharmakokinetics: The dynamics of drug absorption, distribution, and elimination. . *Goodman & Gilman's the pharmacological basis of therapeutics*, pp.3-29.

Xiong, G., Wu, Z., Yi, J., Fu, L., Yang, Z., Hsieh, C., Yin, M., Zeng, X., Wu, C., Lu, A. and Chen, X., 2021. ADMETlab 2.0: an integrated online platform for accurate and comprehensive predictions of ADMET properties. *Nucleic acids research*, *49*(W1), pp.W5-W14. https://doi.org/10.1093/nar/gkab255

Yuan, S., Chan, H.S., Filipek, S. and Vogel, H., 2016. PyMOL and Inkscape bridge the data and the data visualization. *Structure*, *24*(12), pp. 2041-2042. http://dx.doi.org/10.1016/ j.str.2016.11.012

Yuan, S., Chan, H.S. and Hu, Z., 2017. Using PyMOL as a platform for computational drug design. *Wiley Interdisciplinary Reviews: Computational Molecular Science*, *7*(2), p.e1298. https://doi.org/10.1002/wcms.1298

Yuriev, E. and Ramsland, P.A., 2013. Latest developments in molecular docking: 2010–2011 in review. *Journal of Molecular Recognition*, *26*(5), pp. 215-239. https://doi.org/10.1002/jmr. 2266

Zadorozhnii, P.V., Kiselev, V.V. and Kharchenko, A.V., 2022. In silico ADME profiling of salubrinal and its analogues. *Future Pharmacology*, *2*(2), pp.160-197. https://doi.org/10.3390/futurepharmacol2020013