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IDENTIFICATION OF POTENTIAL BIOACTIVE DSBA-INHIBITORS AS AN ANTIMICROBIAL RESTORATION STRATEGY: AN *IN-SILICO* SCREENING AND ADMET STUDY

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Keywords:

AMR, DsbA-inhibitor, Molecular docking, SAR, Molecular dynamics simulation and ADMET

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ABSTRACT: Antimicrobial resistance continues to cause antibiotics treatment failure and millions of deaths globally. Inhibition of a double-stranded binding protein-A enzyme (DsbA), machinery in bacteria resistance and pathogenesis mechanisms make the bacteria vulnerable to existing antibiotics, allowing restoration of antimicrobial efficacy. However, there is a gap in identifying bioactive and safe DsbA-inhibitors as a therapeutic restoration strategy. In this *in-silico* screening study, a co-crystallized *E. coli*-DsbA enzyme protein was used as the target. Seven bioactive ligands: linoleic acid, aloesin, azadirachtin, nimbin, palmitic acid, stearic acid, and aloeresin-A, showed maximum docking scores of low binding affinity (kcal/mol): -6.8345, - 5.9434, - 7.2810, -6.2394, -6.5958, -6.7170 and -6.9554 respectively, lower than the standard (9AG): -4. 6919. The ligands obeyed Lipinski's rule of 5. The Root Mean Square Deviation (RMSD) of E. coli-DsbA complexes with azadirachtin, and aloeresin-A displayed remarkable stability throughout the simulations. Molecular dynamics simulation on azadirachtin and aloeresin-A showed great docking scores and modes over 10000ps and revealed lowest binding affinity scores, remain stable in the active site. Additionally, astructural-activity relationship study was done on azadirachtin as the top-ranking ligand in which 2-oxopropanal moiety revealed lowest binding energy of -7.4190(kcal/mol). Finally, the test ligands were subjected to *in-silico* ADMET prediction analysis and showed good pharmacokinetics drug likeness properties and safety. The results obtained are a great step in discovering safe DsbA-inhibitors (antivirulence agent) for *in-vitro* and *in-vivo* validation studies in the antibiotics restoration strategy against resistant bacteria.

INTRODUCTION:

Background of Study: Antimicrobial resistance (AMR) occurs when microbes no longer respond to medicines that were formally used to inhibit or kill them, making infection treatment and control very difficult [1, 2].

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Antimicrobial overuse and misuse in humans, animals and plants are still the core causes of AMR [3], The World Health Organization has declared AMR among the top ten global threats [1, 3, 4, 5]. The global annual mortality and economic burden of AMR is projected to be 10 million deaths and decrease in global GDP by between 1.1% and

3.8% (USD 100 trillion) respectively, if no effective action is taken, posing a substantial disease and economic burden by 2050 [6, 7]. There were a projected 1.27million, and 4.95 million global annual deaths, directly linked and associated with bacterial AMR respectively in 2019 [1]. Low- and middle-income countries in Africa account for a significant portion of the global burden of AMR [8, 9]. In Nigeria for instance, the complex nature of AMR challenges revolves around a large population of over 200 million people, poor health infrastructure, and chaotic supply chains [10].

The bulk of AMR burden depicts its multifaceted challenges in agriculture and human health, coupled with long years of antimicrobial development pathways and few antibiotics FDA approval rates in the last three decades [11, 12]. The AMR wave has reached every antimicrobial class; antibiotics, antiviral, antifungal, and antimalarials [1, 13, 14, 15]. This impact has led to the survey which revealed the urgent call to rename AMR to communicate the seriousness of the impacts to everyone [16].

A recent study at Imperial College London and the University of Texas at Austin has discovered a new approach in the fight against antibiotic resistance by targeting a bacterial protein called Double Stranded Binding Protein-A (DsbA), which is a thiol-disulfide oxidoreductase that pathogens use as machinery in transferring disulfide bonds to create folding patterns leading to an inability for a drug to inhibit or kill the bacteria [17]. The DsbA enzyme is a periplasmic protein in microbes that catalyses the formation of disulfide bonds in newly synthesized substrate proteins, which is essential for their stability and pathogenesis [17, 18, 19]. DsbA plays a critical role in both Gram-negative and Gram-positive bacteria by facilitating disulfide bond formation essential for protein folding and stability which leads to resistance principle [20]. DsbA enzymes are found in various classes of pathogens, including proteobacteria, chlamydiales, actinobacteria, and bacilli [20]. The DsbA inhibitors works by inhibiting this DsbA enzyme preventing the formation of disulfide bonds which the bacteria use for resistance and pathogenesis, thereby making the microbes vulnerable to existing antibiotics by regaining their efficacy to kill or inhibit bacteria pathogens [17]. The revolutionary benefits of DsbA-inhibitor lie in its antivirulence activities as they target the virulence factors of a pathogen, reducing its harmful effects on the host [21]. It differs from traditional antibiotics by not killing the pathogen but rather disarming its virulence mechanisms, potentially reducing the development of resistance [21, 22, 23]. Antibiotics such as β-lactams (piperacillin-tazobactam and ceftazidime) are among the antimicrobials that can develop resistance *via* the DsbA mechanism [24].

Recent studies have identified various inhibitors such ashalicin, phenylthiophene and phenoxy-phenyl derivatives targeting DsbA enzymes in bacteria, showcasing their potential as antivirulence agents in pathogens like *E. coli* and *Salmonella enterica*, disrupting virulence factor assembly [20, 21]. In addition, the rate at which these microbes develop resistance even to new antibiotics, and the vulnerability of the pathogens to antibiotics created by DsbA-inhibitors have made restoration strategy unavoidable scientific tasks, banking on the dual inhibitory and antivirulence benefits.

The current advancement has shown that the contributions of computational tools/artificial intelligence like *in-silico* molecular docking and ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) screening tools in drug development have been of great value [25, 26]. Molecular docking is a valuable tool in drug development that aid in lead drug candidate optimization, virtual screening, polypharmacology, and drug repositioning, as well as the study of structure-activity relationships, and nutraceuticals development [25, 26, 27]. The ADMET analysis plays a crucial role in drug development by evaluating the pharmacokinetic and safety profiles of potential drug candidates [28, 29]. The invaluable ADMET analysis in getting the lead drug candidate helps in the early exclusion of undesirable compounds, reduction in adverse effects, accelerated development, and improved safety and efficacy, which contribute to the overall efficiency, cost-effectiveness, and success rate of drug development [29]. There are notable drugs that have been discovered, developed, and approved by the United States, Food and Drug Agency (FDA) through the aid of molecular docking for human use. Notable few examples are raltegravir, saquinavir, indinavir, ritonavir, oseltamivir, captopril, dorzolamide,

zanamivir [30]. Records for decades have shown many potent and safe drugs developed from plant sources [31]. It is the premises that ignited the choice of using compounds of plants that have been used by man for centuries as potential test ligands for this research. The seven best-recorded docking test ligands out of the thirty-three were randomly selected from the following plants with long history of usage by man in addition to their established antibacterial characteristics: the edible seed of *Gundelia (Gundelia tournefortii.* L: linoleic acid, stearic acid, and palmitic acid) [32], neem leaves (*Azadirachta*: numbi and azadirachtin) [33], aloe vera leaves (*Aloe barbadensis miller*: aloesin and aloeresin A) [34], before retrieving them from PubChem [35]. The palm oil from palm kernel seed (*Elaeis guineensis*) also contains stearic acid, palmitic acid, and linoleic acid [36].

One of the key areas of WHO Global AMR research agenda topics on human health, which proposes the need for new antimicrobial development [37], in addition to the scientific community's race in developing DsbA-inhibitors that are potent and safe in humans, formed the core objective of this study. It is important to note, current study in bacteria has shown that inhibition of DsbA enzymes to not just prevent resistance development but also attenuate growth and virulence in the pathogen [21]. This study aimed to identify potent and safe DsbA-inhibitors for antibiotics' efficacy restoration against resistant bacteria. Bioactive compounds were evaluated to determine *E. coli*-DsbA inhibitory activities and safety using *in-silico* screening: molecular docking, molecular dynamics stimulation, structure-activity relationship, ADME Swiss, and toxicity database evaluations.

Our study will improve theknowledge and understanding of DsbA-inhibitor and could a lead new antimicrobial therapeutic strategy when further *in-vivo* and *in-vitro* studies have been performed.

MATERIALS AND METHODS:

Preparation of the Target Proteins for Modelling Purpose: In this target preparation, X-ray crystal structures of *E. coli*-DsbA (*Ec*DsbA) which was co-crystallized along 9AG (a phenoxy phenyl) having PDB IDs 6BQX [38, 39] was retrieved from the Protein Data Bank [40].

The remaining chains with the enzyme were deleted from the PDB files to enable the enzyme protein work as a monomer [41]. Study has shown that, docking to monomeric forms yielded binding energies comparable to dimeric forms, suggesting reliable predictions regardless of the structures that can effectively interact with potential inhibitors, facilitating drug discovery efforts targeting this critical enzyme [42, 43].

Further removal of water and non-essential small molecules co-crystallized with the protein-ligand complexes were done. Notably, their low energies were generated using the CHARMM27 force field [44, 45]. After polar hydrogen atoms were added in Molecular Operating Environment software [46]. Furthermore, each *E. coli*-DsbA and its co-crystallized ligands were separated and saved as separate files. Finally, the co-crystallized ligands were used as the standard references in this study.

Preparation of the Ligands for Modelling Purpose: As a traditional screening step, all the test ligand molecules' (nimbin, azadirachtin, palmitic acid, stearic acid, aloesin, aloeresin-A and linoleic acid) 3-dimenional structures were retrieved from the PubChem database [35] and prepared by the MOE version 2015 software. The MOE Protonate 3D tool and CHARMM27 force field were respectively used to protonate and generate low energy structures to a gradient of 0.001 kcal/mol to relieve any steric clashes or strains. The temperature and pH were set to 300K and 7.4 respectively to mimic standard physiological conditions. Finally, the prepared molecules were saved to a molecular database for the molecular docking study.

Molecular Docking: The cavities occupied by the co-crystalized ligands were considered as the binding sites of the *Ec*DsbA enzyme. The three main stages implemented in MOE Dock Tool were employed in docking the dataset into the active site as follows: firstly, the program performed a stochastic search to generate all combinations of angles for each ligand from its single 3-D conformation. Next, the Triangle Matcher tool placed a collection of poses, generated from the pool of ligand conformations, into the protein target binding site. Finally, the GBVI/WSA dG scoring function computed the binding free energy of the

ligand from a given pose by taking cognizance of plethora of factors, such as the average rotational and translational entropy terms, energy lost because of the flexibility of the ligand, hydrogen bonding, metal contacts and a desolvation term due the volumes of the atoms of the protein and ligand in contact with solvent. The program was set to retain the top 10 poses for each ligand. Note that the docking parameters were validated by using only the 3-D affinity grids which reproduced the experimental poses of the co-crystallized ligands within root mean square deviation (RMSD) of < 2.0 Å. Docking conformations were ranked on the predicted binding affinities and resemblance to the experimental EcDsbA-ligand binding modes seen in the crystal structure. Only these top-ranking conformations were considered further for the structure-activity relationship (SAR) study and Molecular Dynamics (MD) simulations.

Structure-Activity Relationship Study: The top-ranking ligand from the molecular docking study was selected as lead compound for the SAR studies. To probe the SAR landscape surrounding this lead, a set of analogues was rationally designed by systematically replacing specific moieties within the ligand structure that were identified as crucial for ligand-target interactions with a range of isosteric substituents. To achieve this, the fragments database available in MOE was used for the systematic replacement, ensuring that each resulting analogue had molecular mass < 1000 g/mol and a Lipinski violation count below the number observed in the parent compound. These analogues were then docked into the EcDsbA binding site to investigate the impact of these structural modifications, steric and electronic factors on the binding affinity.

Molecular Dynamics (MD) Simulations: In a nutshell, to further investigate the stability of the ligand-protein complexes of the top-ranking test ligands, we performed 10000ps MD simulations of the apo form of EcDsbA and each of its complexes with the top-ranking test ligands. The docked ligands served as starting structures for simulations. Ligand topologies were generated using SwissParam [47, 48]. MD simulations were performed using GROMACS 2020.4 software [49, 50] with the CHARMM27 force field. Protein-ligand complexes were solvated using TIP3P water [51], in a cubic box, with a minimum distance of 1.0 nm between any protein atom to the box edge. The solvated system was neutralized with Na^+ and Cl^- ions. Energy minimization was performed using the steepest-descent gradient method. The systems were restrained using an isothermal-isochoric ensemble (NVT) followed by an isothermal-isobaric ensemble (NPT) for 100 ps. Temperature and pressure were maintained at 300 K and 1.0 bar with a modified Berendsen thermostat [52], and Parrinello-Rahman Barostat [53] respectively. Bond lengths were constrained using the LINCS algorithm [54], and long-range electrostatic forces were calculated using the particle-mesh Ewald scheme (PME) with grid spacing 0.16 nm [55]. The cut-off ratios of 1.2 nm for Coulomb and van der Waals potentials were used for the calculation of short-range nonbonded interactions. The simulations were carried out at a time-step of 2 fs and the simulated trajectories further visualized using VMD 1.9.3 [56]. Finally, the root mean-square deviation (RMSD), radius of gyration (Rg), root mean square fluctuation (RMSF), and number of hydrogen bonding interactions formed were analysed using the tools included in Gromacs 2020 and plotted Python matplotlib [57].

ADMET *In-silico* Pharmacokinetic Prediction: The ADMET study remains a vital tool in lead drug development by evaluating the pharmacokinetic and safety profiles of potential drug candidates using ADME and toxicity tool respectively [29]. This methodology involves retrievingthe molecular structure of each test ligands from PubChem data, prepared, and transferred to the submission to page of Swiss-ADME [58] and toxicity tool [59] respectively. Finally, the results are saved in files after analysis. The potential ADME pharmacokinetics properties are usually predicted using the following parameters: total polar surface area (TPSA), estimated aqueous solubility (ESOL), gastro-intestinal absorption (GIA), CytochromeP450 protein enzymes interaction, blood brain barrier (BBB), and P-glycoprotein (P-gp) [58] while mutagenicity, developmental toxicity and 50% lethal dose (LD50) are used for toxicity analysis [59].

RESULTS AND DISCUSSION:

Docking Test Ligands Compounds: From **Fig. 1** above, hydrogen bonding interactions, of the ligands with the active site residues are depicted in

cyan with cylindrical bars, with cylindrical bars representing the strength of the bond. Most of the hydrogen bonding interactions occur between the His32 residue of the EcDsbA CPHC catalytic motif. Aloeresin-A and azadirachtin occupy the same region of the binding pocket's hydrophobic groove, forming strong hydrogen bonding contacts with the His32 residue. Aloesin and nimbin occupy the same portion of the binding pocket and establish strong hydrogen bonding interactions with the Gln35 residue of the binding pocket. Palmitic acid and stearic acid share the same binding mode, both exhibiting strong hydrogen bonds with the Arg142 residue, resulting in similar binding affinities of approximately 6.7 kcal/mol.

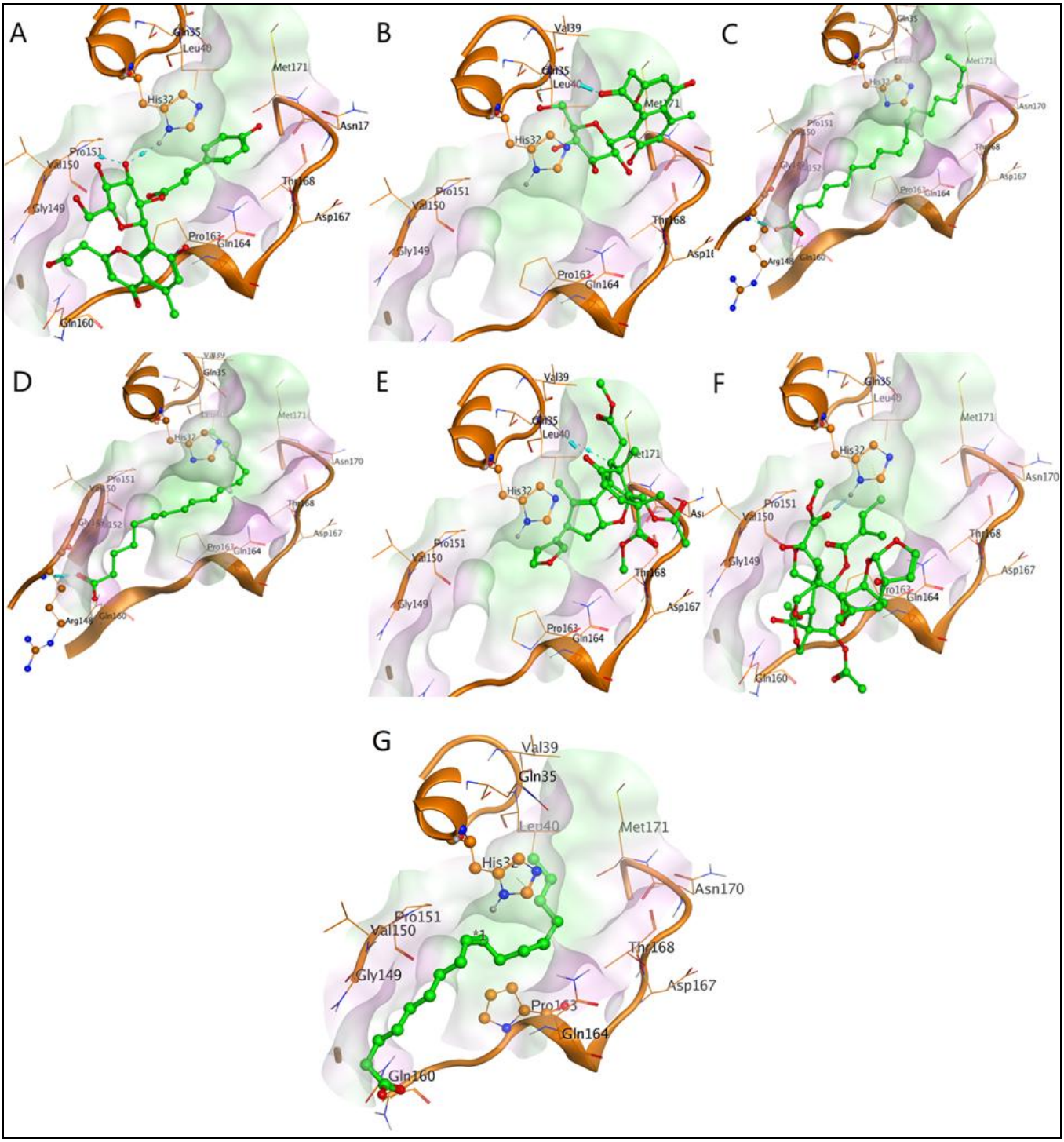
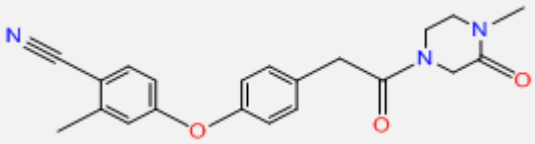
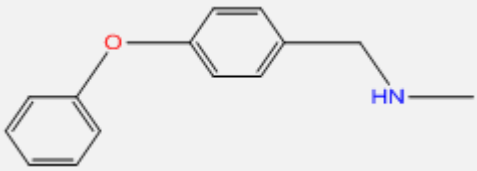
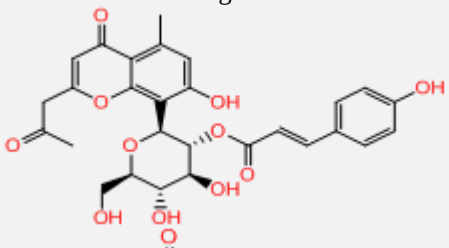
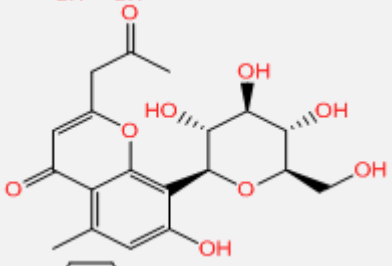
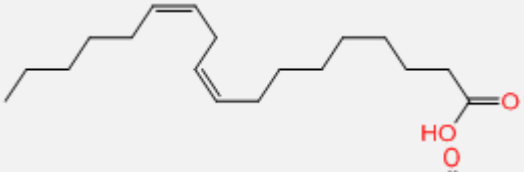
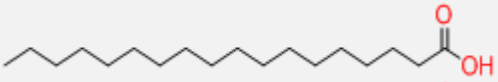
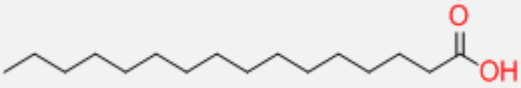
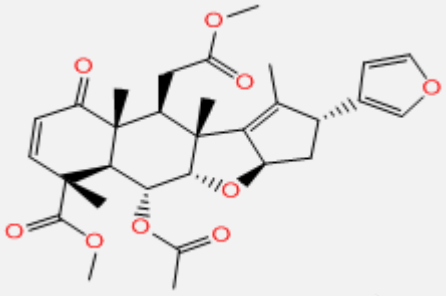
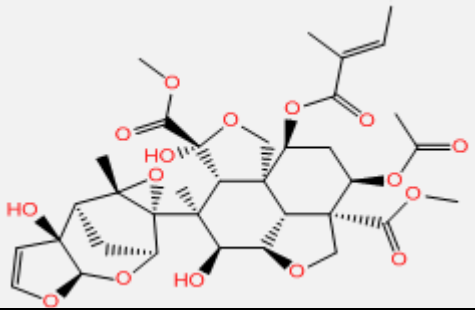


FIG. 1: BINDING MODES AND INTERACTIONS OF THE TOP-RANKING CONFORMATIONS OF THE LIGANDS IN THE BINDING SITE OF ECDSBA. (A) ALOERESIN-A (B) ALOESIN (C) STEARIC ACID (D) PALMITIC ACID (E) NIMBIN (F) AZADIRACHTIN (G) LINOLEIC ACID

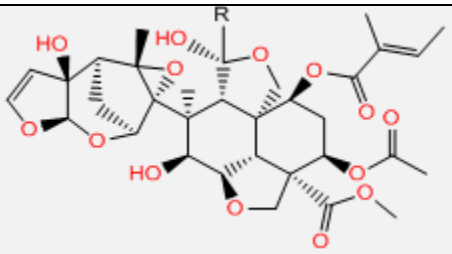
TABLE 1: DOCKING SCORES OF THE BEST POSE OF THE CO-CRYSTALLIZED AND TEST LIGANDS

Ligand	Name/ID	Binding affinity (kcal/mol)	RMSD (Å)	Lipinski violation count
Standard ligands				
	O7P	-8.3799	1.2854	0
	9AG	-4.6919	0.2729	0
Test ligands				
	Aloeresin-A	-6.9554	1.6024	2
	Aloesin	-5.9434	2.0827	0
	Linoleic acid	-6.8345	1.4715	0
	Stearic acid	-6.7170	1.5512	0
	Palmitic acid	-6.5958	1.9078	0
	Nimbin	-6.2394	1.9076	1
	Azadirachtin	-7.2810	1.1523	2

Antibiotics resistance remains a global health threat with urgent need for action in developing molecules to restore the efficacy of the antimicrobials that have developed resistance against microbial pathogens. This silico screening intends to achieve that goal. Binding affinity quantifies drug-target interactions, RMSD measures structural similarity, and Lipinski violation count predicts drug-likeness. These parameters are crucial for rational drug design, virtual screening, and lead optimization.

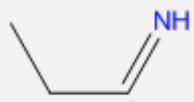
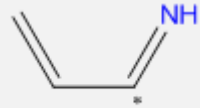
Antibiotics resistance global health threats need urgent action in developing molecules to restore the efficacy of the antimicrobials that have developed resistance against microbial pathogens. This silico screening intends to achieve that goal. Binding affinity quantifies drug-target interactions, RMSD measures structural similarity, and Lipinski violation count predicts drug-likeness. These parameters are crucial for rational drug design, virtual screening, and lead optimization. The test ligands with docking scores better than standard were recorded. From **Table 1**, it showed that the seven test ligands exhibited the lowest binding affinity between -7.2810 to -5.9434(kcal/mol) compared to the co-crystallized standard (9AG) -4.6919 (kcal/mol) indicating their better drug likeness potentials with respect to their binding with drug targets. The whole test ligands obeyed Lipinski's rule of 5 with four of them having zero and the highest recording 2 count predicting their good drug properties. The test ligands, azadirachtin, demonstrate with lowest binding affinity (-7.2810 vs. -4.6919) (kcal/mol), despite a larger structural deviation (1.1523 vs. 0.2729), with standard respectively suggesting potential for improved binding interactions. Additionally, the test ligand's lower Lipinski violation count (2 vs. 0) indicates better adherence to drug-like properties, potentially leading to improved oral bioavailability and reduced toxicity. The test ligand, linolic acid showed a binding affinity of -6.8345 kcal/mol and an RMSD of 1.4715 Å, demonstrating a competitive interaction compared to the standard ligand's -4.6919 kcal/mol and 0.2729 Å. Notably, both ligands have zero Lipinski violations, but the test ligand's stronger binding affinity suggests enhanced potential for efficacy, making it a promising candidate in drug development. All the seven recorded test ligands posed great stability as none of them showed high RMSD values approximately more than 2Å as required with azadirachtin having the lowest of approximately 1A indicating high stability. These great docking scores of the ligands positioned them for potential anti-DsBA inhibitors drugs.

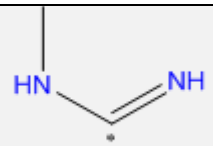
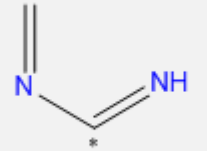
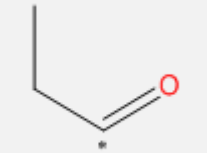
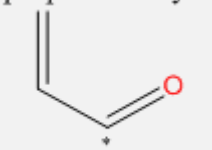
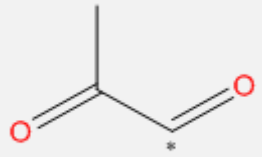
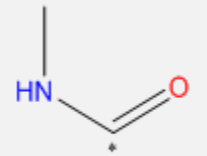
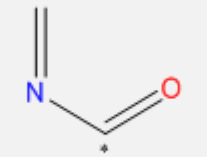
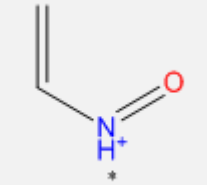
TABLE 2: SAR RESULT

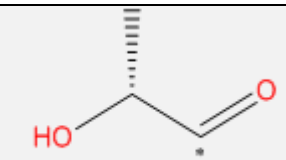


MW: 662.68 g/mol

Good Linkers

R	RMSD (Relative to azadirachtin)	Binding affinity (kcal/mol)	Substituent MW (g/mol)	Lipinski violation count
 propan-1-imine	0.1078	-7.3167	57.09	2
 prop-2-en-1-imine	0.1078	-7.1711	54.07	2

 *N*-methylformimidamide	0.0266	-7.3278	58.08	2
 *N*-methyleneformimidamide	0.0266	-7.2682	56.07	2
 propionaldehyde	0.1037	-7.4156	57.07	2
 acrylaldehyde	0.1037	-7.2718	56.06	2
 2-oxopropanal	0.0688	-7.4190	72.06	2
 *N*-methylformamide	0.0240	-7.3462	59.04	2
 *N*-methyleneformamide	0.0240	-7.2654	56.04	2
	0.1039	-7.2124	60.07	2
 *N*-oxoethenaminium	0.1039	-7.0633	58.06	2

 (*R*)-2-hydroxypropanal	0.1092	-7.0011	73.07	2

Points marked with asterisk **(*)** indicate atoms of attachment to the parent scaffold. MW = Molecular weight

Bioisosteric Replacement and its Impact on Binding Affinity: In the docking studies, the top-scoring test ligand, azadirachtin, was found to engage in a crucial hydrogen bond interaction with the CPHC motif of the EcDsbA receptor through its methyl formate moiety (MW 60.05 g/mol). Guided by azadirachtin's drug-like properties (Lipinski violation count < 3, MW < 720.71 g/mol), we systematically replaced the methyl formate group with fragments from the MOE database. We ensured that each generated analogue maintained a molecular weight below 1000 g/mol and a Lipinski violation count below 3. This rule is essential for maintaining drug-like physicochemical properties in the resulting compounds. Candidate drugs adhering to exhibiting lower attrition rates in clinical trials, increasing their chances of reaching the market [58].

The SAR docking scores showed RMSD (relative to azadirachtin) of the 12 substituent linkers valueswere less than 1Å which is great stability criteria. N-methylformamide and N-Methyl-eneformamide both exhibited the best relative RMSD of 0.240, suggesting a favourable binding conformation compared to the original compound, azadirachtin, with an RMSD of 1.1523. Lower RMSD values indicate greater stability and conformational fidelity within the binding site, enhancing the likelihood of effective interactions and potentially improving the compound's efficacy in SAR docking studies. Interestingly, azadirachtin analogues featuring bioisosteres closely resembling the methyl formate moiety in terms of hydrogen bond donors/acceptors, molecular weight, and spatial orientation displayed binding affinities like the parent compound **Table 2**. This aligns with the nature of bioisosteres, chemical substitutes offering comparable physical and chemical properties within a molecule. In drug design, bioisosterism empowers us to fine-tune desired biological or physical characteristics while preserving a consistent chemical framework.

This strategy can minimize toxicity, modulate bioavailability, and influence the activity and metabolism of lead compounds [60]. Remarkably, analogues incorporating propan-1-imine (57.09 g/mol), N-methylformimidamide (58.08 g/mol), propionaldehyde (57.07 g/mol), 2-oxopropanal (72.06 g/mol), and N-methyl-formamide (59.04 g/mol) as replacements exhibited binding affinities between -7.30 and -7.42 kcal/mol, surpassing that of azadirachtin itself. This suggests an optimal substituent molecular weight range of 57.0 to 72.0 g/mol. Notably, all **Table 2** analogues retained the same hydrogen bonding interactions observed in azadirachtin between the methyl formate moiety and His32 of EcDsbA, further bolstering the notion that bioisosteric replacements can yield analogues with highly similar biological activity. **Fig. 3** showcases the 3D hydrogen bonding interactions of these five promising azadirachtin analogues.

In contrast, replacing the methyl formate group with bulky moieties exceeding 100 g/mol drastically reduced binding affinity, resulting in positive binding energy values. This detrimental effect can be attributed to unfavourable steric and electronic factors introduced by these larger groups. **Fig. 4** provides a clear visual representation of how substituent molecular weight impacts the binding energy of each resulting analogue. It readily demonstrates that lighter substituents (below 100 g/mol) displayed strong binding (approximately -7.0 kcal/mol), while heavier ones (above 100 g/mol) showed positive binding energy, indicating no interaction with the EcDsbA receptor. Importantly, azadirachtin analogues containing methyl formatebioisosteres with masses between 53.0 and 100.0 g/mol exhibited comparable binding affinity to EcDsbA, showcasing their potential as valuable lead compounds for further development. **Fig. 5** displays the collection of the bulky moieties hindering EcDsbA binding of the azadirachtin analogues.

Replacing the methyl formate group with these moieties leads to positive binding energies, suggesting hindered interactions with the EcDsbA receptor

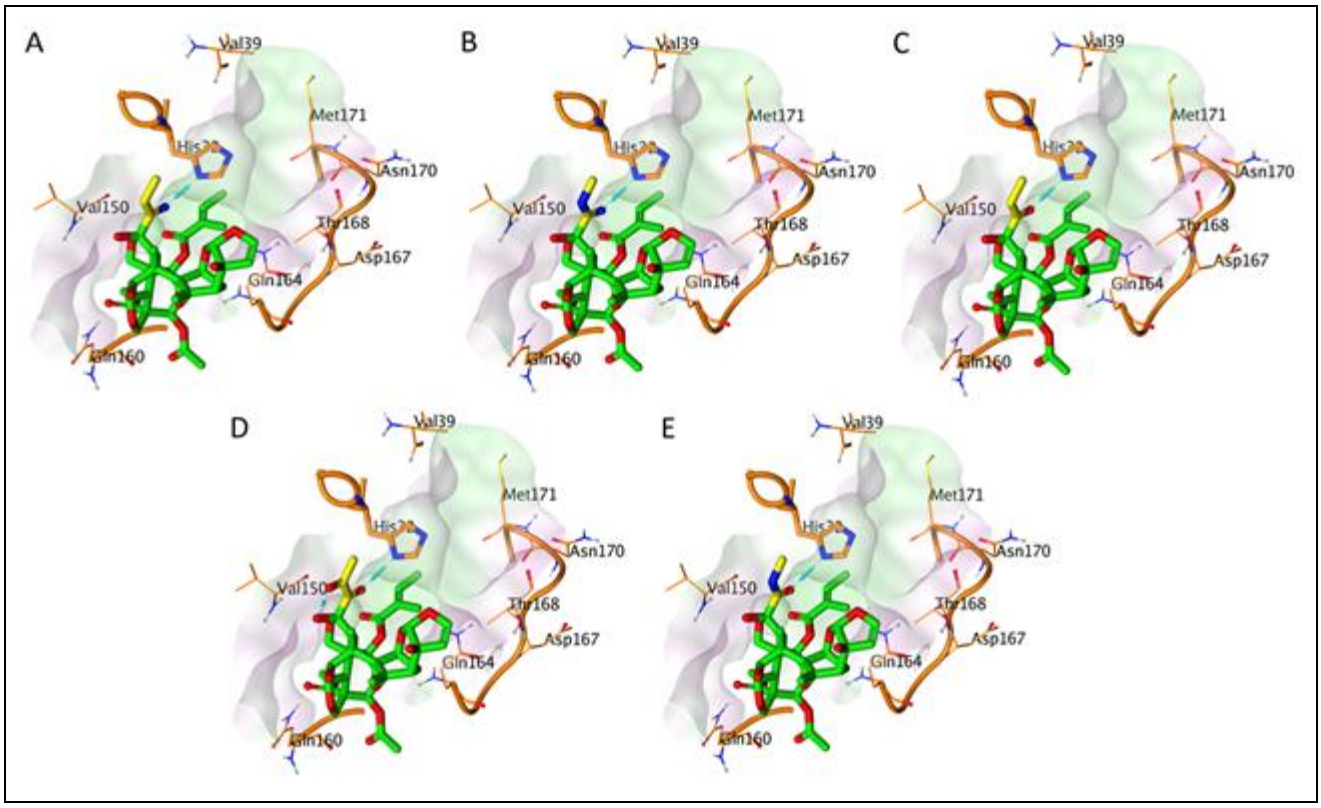


FIG. 2" INTERACTION DIAGRAMS OF THE TOP-RANKING ANALOGUES OF AZADIRACHTIN, WITH THE METHYL FORMATE MOIETY REPLACED WITH VARIOUS BIOISOSTERES

(A) propan-1-imine analogue. (B) N-methyl-formimidamide analogue. (C) propionaldehyde analogue. (D) 2-oxopropanal analogue. (E) N-methylformamide analogue. Strikingly, despite employing diverse bioisosteres in place of methyl formate moiety, these top-ranking azadirachtin analogues all retain the crucial hydrogen bonding pattern observed in the parent compound's interaction with the EcDsbA receptor, as revealed by their interaction diagrams. The carbon atoms of the substituting moiety are coloured yellow for emphasis and the hydrogen bonding interactions are shown in cyan. For clarity, non-interacting amino acid residues interfering with the ligand interactions with the target were deleted and the His32 residue is made bold.

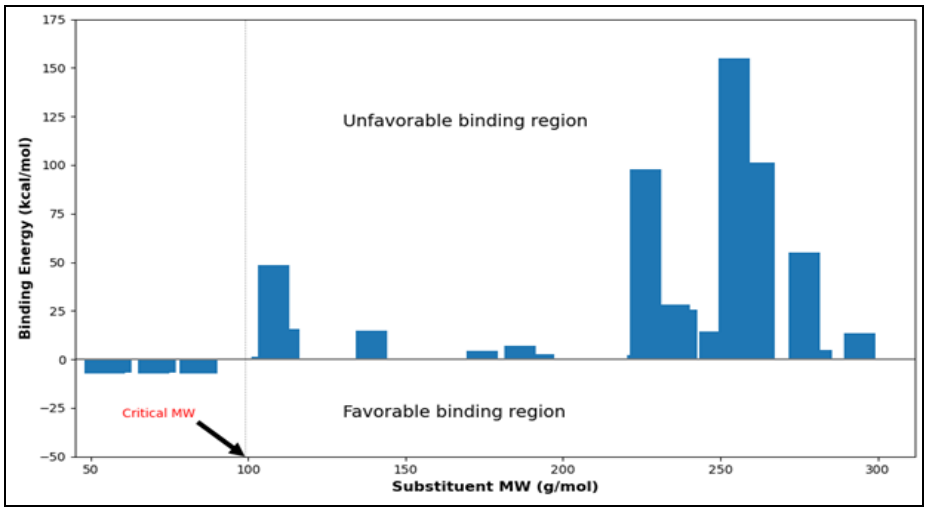


FIG. 3: DISTRIBUTION OF THE BINDING AFFINITIES OF THE AZADIRACHTIN ANALOGUES AS A FUNCTION OF THE SUBSTITUENT'S MOLECULAR WEIGHT

This revealed a key relationship between substituent molecular weight and binding affinity. Lighter substituents (below 100 g/mol) displayed strong binding (approximately -7.0 kcal/mol), while heavier ones (above 100 g/mol) showed positive binding energy, indicating no interaction with the EcDsbA receptor. 100 g/mol appears to be the "critical mass" for optimal binding of the bioisosteres to the EcDsbA receptor. Notably, azadirachtin analogues containing methyl format-ebioisosteres with masses between 53.0 and 100.0 g/mol exhibited comparable binding affinity to EcDsbA, highlighting their potential as lead compounds for further development.

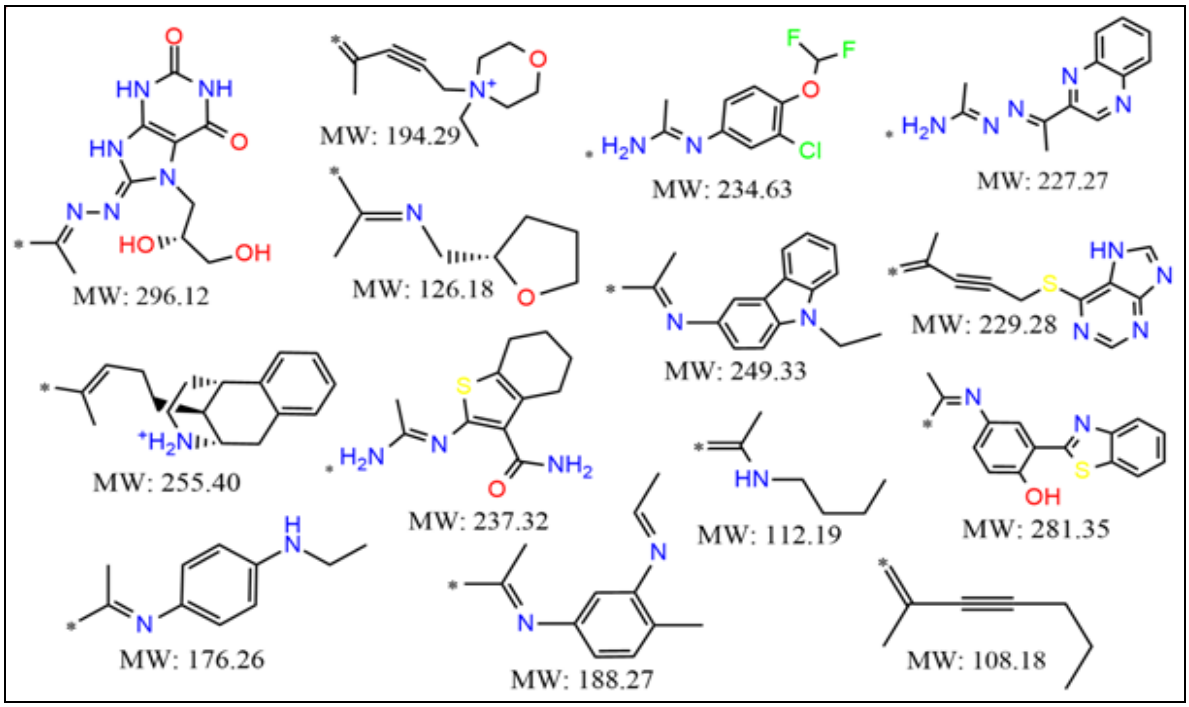


FIG. 4: BULKY MOIETIES HINDERING ECDSBA BINDING AZADIRACHTIN ANALOGUES. Points marked with an asterisk (*) indicate atoms of attachment to the parent scaffold. MW = Molecular weight.

This collection displays bulky moieties that disrupt binding in azadirachtin analogues. Replacing the methyl formate group with these groups leads to positive binding energies, suggesting impeded interactions with the EcDsbA receptor. Steric hindrance, where the bulky moieties collide with the receptor and potentially unfavourable electronic factors are likely culprits. The least of them recording MW; 108.18 and the highest with 296.12. Bulky moieties in Azadirachtin analogues can hinder protein binding due to steric hindrance, which disrupts the spatial fit between the ligand and the target protein. Increased molecular mass may exceed optimal size thresholds for effective binding, potentially reducing affinity and efficacy in biological interactions, ultimately impacting therapeutic outcomes.

Molecular Stimulation of Top-Ranking Ligands-Azadirachtin and Aloeresin-A: (A) Root Mean Square Deviation (RMSD): EcDsbA and its complexes with azadirachtin and aloesin A displayed remarkable stability throughout the simulations, with average RMSD values consistently hovering around 0.1 Å. This indicates minimal overall structural deviations upon ligand binding. (B) Root Mean Square Fluctuation (RMSF): Analysis of residue flexibility revealed minimal fluctuations in residue positions, highlighting the inherent stability of EcDsbA. Notably, the CPHC motif residues (29-32), crucial for ligand binding, exhibited exceptional stability. (C) Radius of Gyration (Rg): Unbound *E. coli*-DsbA showed slightly more dynamic behaviour, exhibiting average Rg values of 1.71 nm. Upon ligand binding, both the azadirachtin-EcDsbA and aloesin A-EcDsbA complexes displayed minimal changes in Rg, averaging 1.72 nm and 1.70 nm, respectively, suggesting ligand binding doesn't significantly alter the overall protein size. (D) Hydrogen Bonding Interactions: Azadirachtin maintained a stable average of 2 hydrogen bonds with EcDsbA throughout the simulation, with

transient increases to 4 around 3.5 and 9.5 ns. Aloeresin A, on the other hand, formed an average of 3 hydrogen bonds, showcasing a higher dynamic range, reaching a peak of 6 interactions around 9 ns.

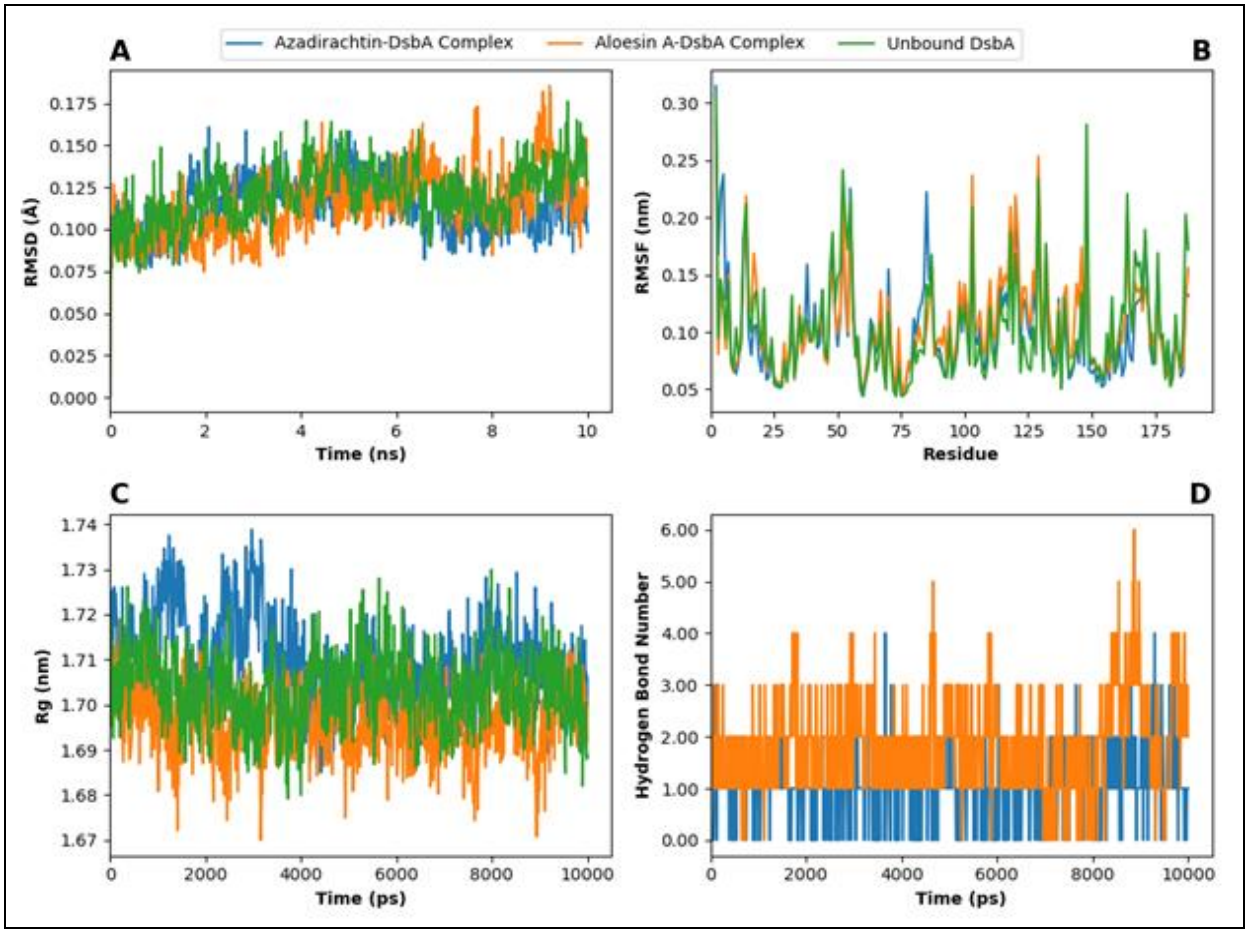


FIG. 5: DEPICTION OF THE DYNAMIC BEHAVIOUR OF ECDSBA AND ITS COMPLEXES WITH TOP-RANKING TEST LIGANDS (AZADIRACHTIN) IDENTIFIED IN MOLECULAR DOCKING STUDIES

TABLE 3: ADME RESULTS

Ligand	TPSA	LogP (o/w)	ESOL LogS	ESOL Solubility (mg/ml)	ESOL Class	GI absorption	BBB permeant	P-gp substrate	CYP1A2 inhibitor	CYP2,3 inhibitor	Bio-availability Score
Aloeresin-A	183.96	1.34	-3.62	1.31E-01	Soluble	Low	No	Yes	No	No	0.17
Aloesin	157.66	-0.45	-1.48	1.31E+01	Very soluble	Low	No	No	No	No	0.55
Stearic acid	40.13	5.67	-5.73	5.32E-04	Moderately soluble	High	Yes	No	Yes	No	0.85
Palmitic acid	40.13	4.94	-5.02	2.46E-03	Moderately soluble	High	Yes	No	Yes	No	0.85
Azadirachtin	215.34	0.88	-4.34	3.33E-02	Moderately soluble	Low	No	Yes	No	No	0.17
Nimbin	118.34	3.17	-4.2	3.45E-02	Moderately soluble	High	No	No	No	No	0.55
Linoleic acid	40.13	3.17	-4.2	3.45E-02	Moderately soluble	High	No	No	No	No	0.55

TPSA = Total Polar Surface Area, ESOL = Estimated Aqueous Solubility, GI = Gastro-Intestinal, BBB = Blood Brain Barrier, P-gp = p-glycoprotein, CYP2= CYP2C19, CYP2C9, CYP2D6. CYP3 = CYP3A4

Total Polar Surface Area (TPSA): This is a measure of the ability for the drug to permeate living cells and if the value is greater than 140 angstroms squared ($Å^2$) tends to be poor at permeating cell membranes, while a TPSA less than 90 $Å^2$ is usually needed for molecules to penetrate the blood-brain barrier and act on receptors in the central nervous system [58]. Stearic acid, palmitic acid, and linoleic acid were predicted to permeate living cells compared to other ligands.

Estimated Aqueous Solubility (ESOL): This Swiss ADME parameter indicates aqueous solubility predictions directly from molecular structure [61]. The results showed that stearic acid, palmitic acid, azadirachtin, numbin, and linoleic acid are moderately soluble, while aloeresin A and aloesin, are soluble and very soluble in aqueous solution respectively.

Gastro-Intestinal Absorption (GIA): Stearic acid, palmitic acid, numbin, and linoleic acid were observed to have high GIA pharmacokinetic properties, while azadirachtin, aloeresin-A, and aloesin were predicted to exhibit low absorption.

Blood Brain Barrier (BBB): Greatly, stearic acid, palmitic acid, and linoleic acid were the ligands predicted to have the ability to pass BBB.

Cytochrome P450 (CYP): This represents a large supergroup of enzymes responsible for drugs and xenobiotics [58]. Inhibition of these enzymes by drugs can lead to potency reduction. All test ligands predicted no inhibition by Cytochrome P450 (CYP2C19, CYP2C9, CYP2D6, CYP3A4) except stearic acid and palmitic acid that showed inhibition against CYP1A2 enzymes.

P-glycoprotein (P-pg): This is a protein membrane transporter that affects drug cellular ADMET and efficacy by interaction which revealed azadirachtin and aloeresin-A as potential substrates.

Bioavailability Score: This vital parameter in ADME Swiss analysis has a value ranging between 0 and 1, where a higher score indicates a greater likelihood of a compound having more than 10% oral bioavailability in rats based on its physicochemical properties and adherence to Lipinski's rule of five. Stearic acid and palmitic acid ligands gave very high score (0.85) close to maximum, while aloesin, numbi and linoleic acid showed high score (0.55), half of the peak maximum value. Finally, azadirachtin and aloeresin-A (0.17) predicted low score which can be improved upon during the action drug dosage formulation processes.

Toxicity: Mutagenicity assessment parameter via in silico toxicity plays a crucial role in identifying genetic hazards early in drug development, guiding compound selection, and improving safety assessments by predicting potential mutagenic effects. The results showed negative for test ligands: numbi, azadirachtin, aloesin, and aloeresin-A, indicating its non-mutagenicity propertyavailable in the toxicity biodata. Developmental toxicity indicator aims to estimate the potential risks to embryonic and foetal development by analysing the structural features and properties of the drug candidate.

Most of the compounds were predicted to be non-toxicants except numbi, which showed toxicity while Azadirachtin is not available yet in the bio-data toxicity tool. Lethal Dose 50 (LD50) is a standard measure used in toxicology to determine the dose of a substance that is lethal to 50% of the test population, especially for acute toxicity [62]. Note, standard measure LD50 value less than 50mg/kg is considered toxic but all test compounds gave above that mark making them safe compounds on LD50 criteria.

Linoleic acid, stearic acid, aloesin, and palmitic acid recorded values above 2000mg/kg which is considered non-toxic in the regulation guideline. Linoleic acid and stearic acid excellently scored above 5000mg/kg which is normally associated with being safe in humans. Stearicacid, linoleic acid and palmitic acid showed best LD50 results with over 4000mg/kg each establishing potential safety characteristics.

TABLE 4: TOXICITY STUDY RESULTS

Ligand Name	Mutagencity	Developmental Toxicity	LD_{50} (mg/kg)
Aloeresin A	Mutagenicity Negative	Developmental NON-toxicant	240.17
Aloesin	Mutagenicity Negative	Developmental NON-toxicant	2473.33
Stearic acid	N/A	Developmental NON-toxicant	5406.7
Palmitic acid	N/A	Developmental NON-toxicant	4871.62
Azadirachtin	Mutagenicity Negative	N/A	54.76
Nimbin	Mutagenicity Negative	Developmental toxicant	671.83
Linoleic acid	N/A	Developmental NON-toxicant	5329.66

Predicted Toxicity of Test Ligands from TEST Software: LD50 & Mutagenicity (Consensus Algorithm), Developmental Toxicity (Hierarchical Clustering).

CONCLUSION: AMR is a top global health threat making infectious disease management more challenging than ever. Restoration of antibiotics efficacy and effectiveness against resistant microbes *via* DsbA inhibition, thougha new approach, holds compelling promises in patients' infectious disease treatment and control.

Remarkably, the molecular docking modes and scores of the seven test ligands, in respect to binding affinity, revealed thatnumbi and azachdirachtin with the least and highest scores respectively, outperformed the standard ligand (9AG). The top-ranking compounds, azachdirachtin, and aloeresin-A, showed optimum dynamic modes after investigation for molecular docking simulation dynamics. Azadirachtin with leading molecular docking stimulation scores, also gave a great performance by the whole moieties when subjected to SAR for further evaluation. The 2-oxopropanal moiety showed the highest energy affinity. All the test ligands complied with Lipinski's rule of 5 with a maximum of 2 rule violations. Notably, most of the compounds performed well in Swiss ADME and toxicity analysis respectively by exhibiting drug-likeness potentials which can be improved upon during formulation. The overall results of this study have showed seven bioactive compounds with high DsbA inhibitory activity, in addition to good drug likeness's properties. There is a need for further wet *in-vivo* and *in-vitro* study, as this research holds a very huge hope for antimicrobial restoration strategy against resistance microbes and infectious diseases management in general.

Data Availability Statement: The data presented in this work can be found in the online websites as referenced in the study.

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