Identification of potential inhibitors from *Andrographis paniculata* bioactive compounds against extended-spectrum β-lactamases through *in silico and in vitro* approaches

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Abstract

The present study aims to identify potential medicinal plant-based extended-spectrum β-lactamase (ESBL) inhibitors from Andrographis paniculata using both in silico and in vitro approaches. The ESBLs were obtained from the protein data bank. The structures of phytoconstituents were obtained from the PubChem database. The compounds were docked against different ESBLs (targeted proteins) using AutodockTools followed by molecular dynamics simulation. In silico results were further validated using in vitro testing through the disc diffusion method. The molecular docking revealed that most of the phytoconstituents have a good binding affinity. The binding energy and in vitro study of the phytoconstituents of Andrographis paniculata were compared with the standard inhibitor for ESBL i.e. clavulanic acid .14- Acetylandrographolide (AAD) showed good binding with the ESBL proteins, having the best values reported in the docking with OXA-10.

Simulation of the complex of AAD and OXA-10 showed that the complex was relatively steady as evidenced by the lack of sudden fluctuations in the values of root mean square deviations, the radius of gyration and solvent-accessible surface area. Further confirmation of the in silico approach was done by an in vitro study against ESBL-producing organisms which showed inhibitory results. From this study, we can conclude that A.paniculata may have the potential to inhibit ESBLs and may be considered for treating bacterial infections.

Keywords: ESBL, Docking, Molecular Dynamic Simulation, β-lactamase inhibitors, *Andrographis paniculata.*

Introduction

Antibiotics have been playing a significant role in treating bacterial infections for ages now and among them, β-lactam antibiotics are most widely used for the treatment of bacterial infections as they have lesser side effects and more efficacy[40]. However, in recent years, there has been an increasing report of bacterial resistance against β-lactams. This is because of the production of β-lactamases, an enzyme that can hydrolyze the amide bond present in the β-lactam ring[11]. These enzyme-producing bacterial strains are now increasingly reported from different parts of the world and nearly 500 different β-lactamases have been reported worldwide[56]. Based on the Ambler classification, these β-lactamases were classified into four classes: they are A, B, C and D. Classes A, C and D are known as serine β-lactamases and class B is known as metallo-β-lactamases which require zinc for their action[4].

In recent years, there has been a rise in the prevalence of class A and D extended-spectrum β-lactamases (ESBL) that are clogging the treatment process against gram-negative bacteria as they confer resistance to penicillins, cephalosporins and monobactam[8]. The most commonly detected ESBL variant among the coliforms belongs to CTX-M, TEM and SHV[13]. The most problematic and difficult to treat β-lactamase producing Gram-negative bacteria are extended-spectrum β-lactamases (ESBL) producing Enterobacteriaceae such as *Klebsiella pneumoniae, Klebsiella oxytoca, Escherichia coli and* non-lactose fermenting bacteria like *Pseudomonas aeruginosa*, *Serratia marcescens* etc.[8,12,41]

To tackle this problem, a few inhibitors were introduced such as clavulanic acid, sulbactam, tazobactam etc. against the serine β-lactamases[17]. A combination of the β-lactams and β-lactamase inhibitors such as clavulanic acid with amoxicillin, tazobactam with piperacillin, sulbactam with ampicillin etc. was universally used during the treatment process of bacterial infections[9]. The combined treatment can enhance the efficacy of antibiotics against ESBL producing Gram-negative bacteria[50]. These inhibitors form covalent bonds with the β-lactamases that in turn resist the hydrolysis of the β-lactam antibiotics[10].

Unfortunately, these inhibitors were confined to a subset of class A enzymes and have become ineffective; more importantly, they do not include other emerging extended β-lactamases and necessitate better treatment options for β-lactamase producing organisms. Therefore, the search for more widely effective ESBL inhibitors is the need of the

hour[36]. This leads to a reconsideration of medicinal plants for finding new compounds that may directly inhibit cells or enzymes[46]. According to reports from the WHO, around 80% of the global population still depends on medicinal plants for treating various ailments[48]. At least 25% of modern medicines are derived from medicinal plants with a combination of recent advanced technology and traditional wisdom[25].

Since medicinal plants have been proven to contain many phytocompounds that are known to exhibit several biological activities, there is high hope for obtaining novel ESBL inhibitors from them. Thus, in the present study, an attempt was made to explore the medicinal plant *Andrographis paniculata* (local name: Chiorta in Assam) to identify potential and novel ESBL inhibitors since it is believed to have many medicinal aspects which include its usage in the treatment of cancer, diabetes, skin infections, influenza, diarrhoea etc.

The plant is also reported to possess protective activity against various liver disorders, upper respiratory tract infections, fever, herpes, sore throat, hepatitis and various other chronic diseases[5,37,53]. Therefore, a bottom-up approach was carried out to screen and identify potential ESBL inhibitors from well-identified bioactive compounds of *A. paniculata*, using both *in silico* and *in vitro* approaches.

Material and Methods

In silico studies

Preparation of ligand for docking: Phytocompounds previously reported from *A.paniculata* were chosen for the docking study and they were obtained from different sources like PubMed, Google Scholar, PubChem and Springer using related keywords. They are the terpenes such as Andrographolide (AD), Neoandrographolide (NA), Isoandrographolide (IA) andrographiside (AG) andrograpanin (AP), 14-Acetylandrographolide (AAD), 14-Deoxyandrographolide (DAL), 14-Deoxyandrographiside (DAS), 14-Deoxy-11,12-didehydroandrographolide (DDA) and flavones such as 5-Hydroxy-7, 8, 2, 3-tetra-methoxyflavone (TTM), 5-Hydroxy-7,8-dimethoxyflavone (HDM), 5-Hydroxy-7, 8,2,5-tetramethoxyflavone (HTF), 5,7,2,3 tetramethoxyflavone[20,26,44,53]. The 3D structures of these compounds were retrieved from the PubChem database in the .sdf format. The .sdf format was converted into the .pdb format using Open Babel software[39]. Then, using Autodock tools, the .pdb files were converted into.pdbqt files[18].

Preparation of proteins for docking: The 3D structures of the proteins were obtained from the protein data bank (www.https://rscb.org/). The proteins of choice are the ESBL variants such as SHV-1 (PDB ID: 4GDB), OXA-10 (PDB ID: 5MNU), PER-2 (PDB ID: 4D2O), GES-5 (PDB ID: 4GNU), TEM-1(PDB ID: 5HVI), CTX-M-14 (PDB ID: 6MD8). The .pdb files of the protein were then converted into .pdbqt files using the AutoDock function as per established protocols[18].

Molecular docking using AutoDock: The molecular docking of phytoconstituents with the ESBLs was carried out using the AutoDock function of MGL tools. A genetic algorithm was selected for search parameters among the given options[18]. The output option was set to Lamarckian. The Discovery Studio software was used for the analysis of the protein-ligand interactions.

Molecular Dynamic Simulation (MD): The MD simulation was conducted for the complex using GROMACS 2021.3 for a time scale of 100 ns. The AMBER99SB-ILDN force field was used to perform the simulation[31]. The force field topology file of the small molecule was obtained by using ACPYPE and protein topology was generated using the pdb2gmx tool of GROMACS[51]. The protein-ligand system was solvated with the TIP3P explicit solvent-water model. A water box of 1 nm from the center of the protein was created. The system was neutralized using counter-ions (Na^+ ions and/or Cl^- ions). The energy minimization was performed using the steepest descent and conjugate gradient for 50,000 steps. Then, the protein backbone was frozen and solvent molecules with counter-ions were allowed to move for two 500 ps position restrained equilibration MD runs.

The simulation was performed under periodic boundary conditions with NVT followed by an NPT ensemble. During the position restraint MD runs, V-rescale and Parrinello-Rahman algorithms were used to keep the temperature and pressure constant. Finally, 100 ns of production MD runs were performed. The pressure of the protein-ligand system was maintained at 1 bar by isotropic pressure coupling in the X, Y and Z components to a Berendsen with a time constant $\tau = 2.0$ ps and compressibility of $4.5 \times 10{-}5$ bar^{-1} in all three dimensions. The electrostatic interactions were calculated by the PME algorithm with an interpolation order of 4 within a grid spacing of 0.16 nm. The time steps for the simulations were 2 fs. The LINCS algorithm was used to constrain all bond lengths. The analysis was performed using GROMACS 2018.3 package[1,7,22,30,42,57] and the plots were viewed using GRACE.

In vitro Test

Plant Sample Collection and Extraction: *A. paniculata* was collected from the local areas of Diphu, Karbi Anglong and it was identified at Tripura University, Tripura, where a herbarium sample (Accession no. 2064) was deposited. The leaves were cleaned first and air-dried in the shade. The leaves were crushed into powder using a grinder. The extraction was done by the process of maceration where the grounded powder was mixed with the methanol in a ratio of 1:10[33]. Then it was filtered by Whatmann filter no. 1 and the filtrate was dried using a rotary evaporator. The dried crude plant extract was weighed and stored in a screw cap container at 4°C for further study. The crude extract was reconstituted in a DMSO solution for *in vitro* testing[38].

Gas chromatography-mass spectrometry (GC-MS) analysis of the extract: The crude extract of *A. paniculata* was assessed for the presence of bioactive compounds by GC-MS analysis and molecular docking was further performed with the phytoconstituents detected against the target proteins.

Microbial Strains: Previously identified *Klebsiella pneumoniae* strains with the ESBL phenotype were selected for the *in vitro* study.

***In vitro* study:** An *in vitro* study was performed to validate the *in silico* findings and confirm the inhibitory properties of the plant extracts. The study was carried out using the Kirby-Bauer disc diffusion method[6]. The organisms were initially grown in the sterile peptone water and their turbidity was checked by comparing it with 0.5 McFarland's standard. Then lawn culture was prepared on the sterile Mueller Hinton agar (MHA) media with cotton swabs. Antibiotic discs of ceftazidime (30µg) alone and ceftazidime containing crude plant extract at different concentrations (20µg, 40µg and 60µg) were placed on the surface of the media. Ceftazidime in combination with clavulanic acid was used as a control. It was then incubated at 37ºC overnight and observed for the zone of inhibition. An increase in the zone size of ceftazidime in combination with plant extract when compared with ceftazidime alone was considered an ESBL inhibitor.

Results and Discussion

The identification of the inhibitory potential of the reported phytochemicals from *A. paniculata* was based on hydrogen bond formation, their inhibition constant and the interaction between the ligands and active sites of the ESBL variants. Out of the 25 phytoconstituents (Table 1) considered in this study (13 from previously identified phytoactive compounds, 12 identified in the present through GC-MS analysis), a total of 15 phytoconstituents, when compared with their reference standard clavulanic acid were found to have a good binding affinity towards the selected ESBL protein with significant binding scores and binding energy ranging from -5.52kcal/mol to -8.86 kcal/mol.

A total of 15 (60%) ligands were found to have a good binding affinity for OXA-10 and 16 (64%) for SHV-1 and a total of 15 (60%), 12 (48%), 9 (36%) and 5 (20%) numbers of the ligands were also found to have a good binding affinity towards GES-5, TEM-1, PER-2 and CTX-M-14 respectively (Table 1).

Table 1
Docking score of *A.paniculata* bioactive compounds against selected ESBL

S. N.	LIG	Selected extended spectrum β-lactamase (ESBL)																	
		OXA-10 (PDB ID: 5MNU)			SHV-1 (PDB ID: 4GDB)			PER-2 (PDB ID: 4D2O)			TEM-1 (PDB ID: 5HVI)			CTX-M-14 (PDB ID: 6MD8)			GES-5 (PDB ID: 4GNU)		
		BE (kcal/	H	Ki	BE (kcal/mol)	H	Ki	BE (kcal/mol)	H	Ki	BE (kcal/mol)	H	Ki	BE (kcal/mol)	H	Ki	BE (kcal/mol)	H	Ki
1	CA	-6.41	6	19.97 µm	-4.55	3	460.63 µm	-5.21	2	152.91 uM	-4.44	6	558.99 uM	-6.63	5	15.8 uM	-5.49	6	95.22 uM
2.	AD	-7.97	5	1.44 µm	-6.28	2	24.8 µm	-4.77	3	316.3	-7.31	2	4.35 uM	-7.20	5	5.29 uM	-8.86	3	318.86 nM
3.	NA	-7.07	3	6.60 µm	-5.52	6	90.62 uM	-5.36	6	117.73 uM	-1.54	2	74.18 uM	-6.24	3	26.52 uM	-8.03	2	1.33 uM

15	14	13	12	11	10	9	8.	7.	6.	5.	4.
NPD*	TET	HTF	HDM	TTM	DDA	DAS	DAL	AAD	AP	AG	IA
-4.82	-6.30	-6.99	-6.50	-6.99	-6.80	-7.70	-7.27	-7.67	-7.14	-7.25	-7.27
0	4	5	5	7	1	4	3	6	2	6	3
291.35mM	23.91 uM	7.47 uM	17.3 uM	7.48 uM	10.37 uM	2.25 uM	5.35 uM	2.38 uM	5.89 uM	4.84 uM	4.70 µm
-3.46	-6.66	-6.12	-6.69	-6.10	-7.00	-6.29	-6.73	-6.56	-6.39	-6.01	-7.47
0	0	3	4	5	1	3	3	3	5	5	8
2.9 mM	13.06 uM	32.28 uM	12.42 uM	33.62 uM	7.43 uM	24.33 uM	11.66 uM	15.47 uM	20.72 uM	39.6 uM	3.36 uM
-4.21	-4.80	-5.60	-5.04	-5.39	-5.45	-5.36	-5.64	-0.47	-3.51	+0.50	-6.0
0	0	2	0	7	0	5	3	1	0	2	2
814.76 uM	301.22 uM	78.05 uM	204.6 uM	111.86 uM	101.26 uM	117.05 uM	126.97 uM	450.15 uM	2.67 uM	-	39.75 uM
-4.27	-5.50	-4.76	-5.11	+2.14	+10.37	-6.75	-5.48	-6.72	-5.71	+4.14	-6.85
0	1	4	2	2	5	5	4	6	4	5	3
1.04 mM	92.98 uM	322.14 uM	179.37 uM	-	-	11.23 uM	96.64 uM	11.94 uM	65.17 uM	-	9.49 uM
-3.57	-5.60	-6.94	-6.95	-4.36	-6.43	-6.69	-5.38	-5.25	-6.32	-6.35	-5.59
0	3	2	2	5	3	2	4	3	5	6	4
2.41 uM	78.47 uM	8.24 uM	8.06 uM	632.37 uM	19.33 uM	12.43 uM	114.47 uM	142.84 uM	23.46 uM	22.22 uM	79.96 uM
-4.82	-6.49	-6.79	-6.70	-5.38	-7.89	-7.37	-7.13	-7.99	-7.12	-6.52	-6.94
0	2	6	3	10	4	4	5	5	2	6	3
291.64 uM	17.54 uM	10.47 uM	12.31 uM	113.13 uM	1.65 uM	3.95 uM	5.89 uM	1.4 uM	6.08 uM	16.69 uM	8.15 uM

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16	17	18	19	20	21	22	23	24	25	26
PHY*	HDC*	ODD*	PTD*	THD*	OTD*	EIA*	HTC*	AZU*	PMM*	BHM*
-3.91	-4.64	-5.05	-4.93	-4.10	-3.57	-6.99	-7.20	-6.51	-3.38	-4.03
2	3	4	4	2	0	6	1	5	2	2
1.35 mM	397.19 uM	199.56 uM	241.44 uM	986.01 uM	2.42 mM	7.47 uM	5.31 uM	16.81 uM	3.32 mM	1.1 mM
-3.78	-4.25	-4.4	-3.65	-4.20	-3.58	-6.81	-6.44	-6.62	-3.25	-3.45
1	0	3	1	2	0	6	1	4	1	2
1.69 mM	769.48mM	599.43mM	2.1 mM	830.92 uM	2.38 mM	10.2 uM	19.06 uM	14.16 uM	4.13mM	3.05mM
-1.96	-2.84	-2.99	-3.14	-2.90	-4.04	-2.64	-6.84	-6.34	-2.32	-3.28
0	1	2	1	1	0	3	1	4	0	2
36.77 mM	8.31 mM	6.41 mM	5.01 mM	7.48 mM	1.09 mM	11.64 mM	9.7 uM	22.51 uM	19.77mM	3.97 mM
-3.07	-3.15	-4.51	-3.82	-3.37	-3.25	-3.26	-5.95	4.67	-4.24	-3.61
0	2	3	4	0	0	2	0	1	0	3
5.62 mM	4.94 mM	496.17 mM	1.57 mM	3.41 mM	4.17 mM	4.09 mM	43.25 uM	-	436.54mM	2.24 mM
-2.60	-1.95	-4.08	-2.69	-3.41	-3.30	-6.36	-6.79	-4.88	-3.48	-3.51
0	0	2	1	2	0	3	2	3	3	2
12.5 mM	37.17mM	1.02 mM	10.72 mM	3.16 mM	3.82 mM	21.7 uM	10.6 uM	263.03 uM	2.88 mM	2.7 mM
+190.15	-4.52	-4.86	-4.80	-4.37	-3.73	-7.40	-7.20	-6.41	-3.98	-3.93
0	2	4	4	1	0	2	3	6	2	4
-	486.22 uM	275.84 uM	305.72 uM	629.3 uM	1.86 mM	3.78 uM	2.46 uM	19.88 uM	1.2 mM	1.31 mM

LIG= Ligand, BE- Binding energy, H- Hydrogen bond, Ki- Inhibition constant andrographolide (AD), Neoandrographolide (NA), Isoandrographolide (IA) andrographiside (AG) andrograpanin (AP), 14-Acetylandrographolide (AAD), 14-Deoxyandrographolide (DAL),14-Deoxyandrographiside (DAS), 14-Deoxy11,12 didehydroandrographolide (DDA),5 Hydroxy 7, 8,2', 3' tetramethoxyflavone (TTM), 5 Hydroxy 7,8 dimethoxyflavone(HDM), 5-Hydroxy 7, 8 ,2',5'- tetramethoxyflavone (HTF), 5,7,2,3 - tetramethoxyflavone(TET) , Neophytadiene (NPD), Phytol (PHY), Hexadecanoic acid (HDC), 13,16- Octadecadienoic acid (ODD), Pentadecanoic acid (PTD), 3,7,11,15 Tetramethyl-2-hexadecen-1-ol(THD), Octadecyne (OTD), Ethyl isoallocholate (EIA), hexa-1,3,5-trienyl]cyclohex-1-en-1-carboxaldehyde(HTC), 1H-Cyclopropa[3,4]benz[1,2-e]azulene-5,7b,9,9a-tetrol, 1a,1b,4,4a,5,7a,8,9-octahydro-3-(hydroxymethyl)-1,1,6,8-tetramethyl-, 5,(AZU), Pentadecanoic acid, 14-methyl-, methyl ester (PMM), 2-Butanone, 4-hydroxy-3-methyl (BHM) *Compounds identified from *A.paniculata* in the present study through GC-MS

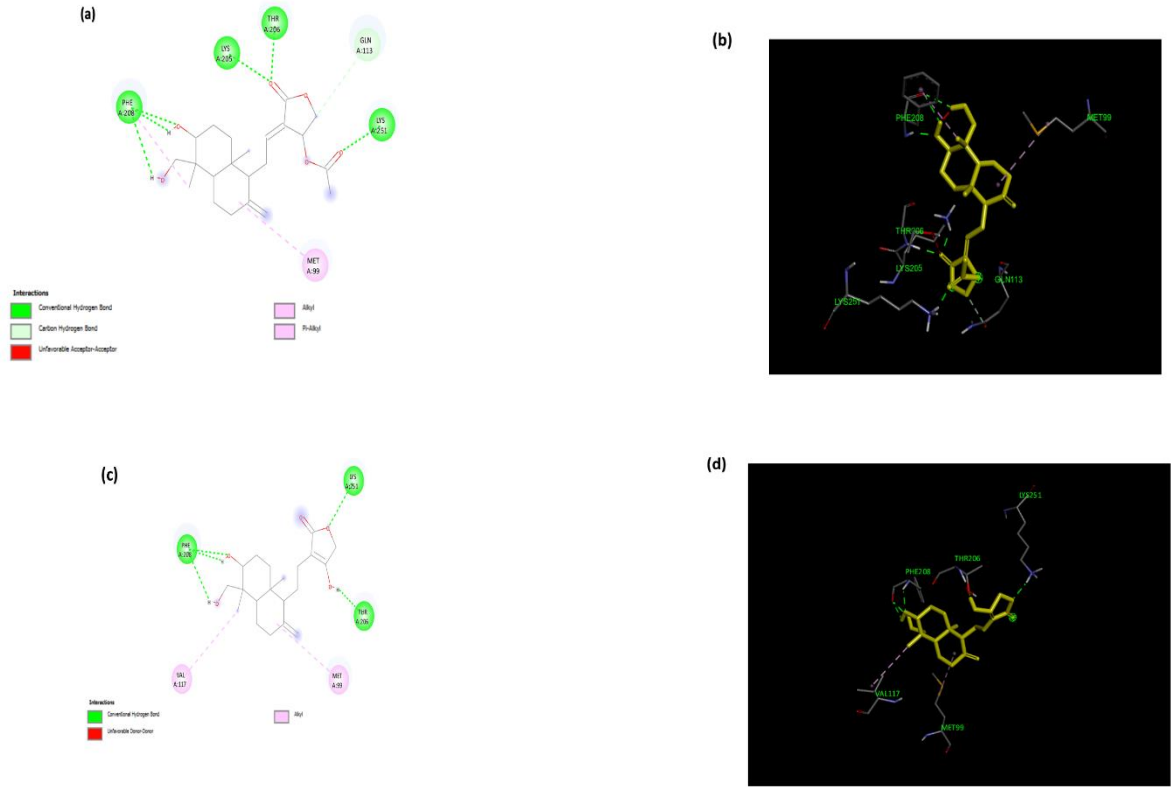

Figure 1: (a) and (b) 2D and 3D representation of the docking of14-acetylandrographolide and OXA-10 (c) and (d) 2D and 3D representation of the docking of andrographolide and OXA-10

However, the analysis of interactions in docked complexes suggested that out of the 15 ligands showing good binding affinity, AAD and AD have the highest binding score with the highest number of hydrogen bond interactions with the proteins. AAD is bound to at least 2 to 3 amino acid residues present in the active site of each target protein and it showed the highest number of 5 hydrogen bonds with the active site of OXA-10, as evidenced from the 2D and 3D images (Figures 1a and 1b).

AD also showed convenient interactions with OXA-10 forming hydrogen bonds with two of the active binding site amino acid residues which include THR 206 and PHE 208 (Figures 1c and 1d). Both AAD and AD have also shown interactions with SHV-1 where maximum of 3 H-bonds were formed by AAD with 2 of the active binding sites (ASP 106 and SER 106) of the target protein. So, based on the docking score and the number of hydrogen bond formations with the target proteins, a representative phytoactive compound, 14-Acetylandrographolide, previously reported from this plant, was selected for molecular dynamic simulation analysis against ESBL the protein (OXA-10).

The analysis revealed that the protein structure and the complex of the protein and ligand obtained their energy minimization gradually, showing no sudden fluctuations in the total energy in both systems (Figures 2a and 2b). The root mean square deviation (RMSD) value of the protein structure alone showed a minimal deviation and the RMSD value stayed constant at approximately 0.1 nm (Figure 3a).

The RMSD value for the protein-ligand complex showed a steady value of around 0.1 nm from the start of the simulation. At 40 ns, there was a minor decrease in the RMSD value of the complex, which gradually increased between 40 ns to 50 ns and remained constant at around 0.2 nm throughout the length of the simulation (Figure 3b).

The RMSD value of the ligand with respect to the protein backbone rose to 4nm till 10 ns, after which there was a small gradual reduction in the RMSD value to 3 nm, approximately to 65 ns. The RMSD value then increased to approximately 4 nm from 70 ns and stayed constant for the remainder of the simulation (Figure 3c).

The RMSF values of the amino acid residues in the protein alone and the protein-ligand complex did not show much variation except in residue 196. Some of the residues in the range of 66 to 85 showed a relatively higher value at 0.25 nm, along with residues 180, 196 and 211. In the case of the protein-ligand complex, the RMSF value for amino acid residue 196 increased to around 0.5 nm with the other residues showing relatively similar values as in the case of the protein alone (Figures 4a and 4b). The radius of gyration (Rg) of the protein alone remained steady during the course of the simulation and the Rg value was maintained at approximately 1.8 nm.

In the case of the complex, there was a marginal increase in the Rg value till 10 ns following which it lowered and the Rg value remained at an average of 1.8 nm during the course of

the simulation (Figures 5a and 5b). The solvent-accessible surface area (SASA) of the protein and the protein-ligand complex remained steady at 125 nm^2 (Figures 6a and 6b). Analysis of the hydrogen bonding between the protein and the ligand showed that 3 hydrogen bonds were formed at the start of the simulation.

The later part of the simulation showed the presence of 1 hydrogen bond between the protein and the ligand (Figure

Further, an *in vitro* study was carried out to validate the *in silico* analysis and it exhibited a good inhibitory effect against ESBL-producing bacterial strains. A remarkable increase in the zone of inhibition was observed when ceftazidime was combined with the plant extracts at different concentrations and showed maximum inhibitory effects when plant extract (40µg) was combined with ceftazidime (Table 2).

Table 2
***In-vitro* testing of *A.paniculata* crude extract against ESBL producing strains**

Test organism	CAZ (mm)	CAC (mm)	CAZ/PE (20µg) (mm)	CAZ/PE (40µg) (mm)	CAZ/PE (60µg) (mm)
K.pneumoniae (S10W)	22	27	27	28	28
K.pneumoniae (S17S1)	18	23	23	24	23
K.pneumoniae (S2S2)	20	26	21	20	23

CAZ-Ceftazidime, CAC- Ceftazidime/Clavulinic acid, CAZ/PE-Ceftazidime/Plant extract

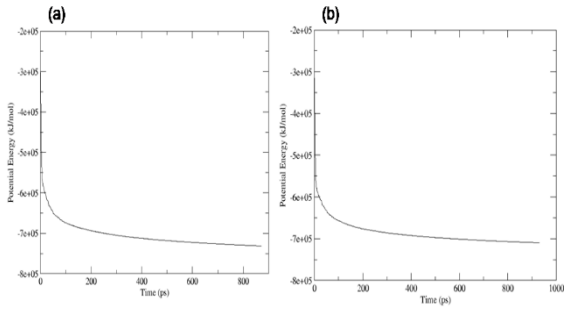

Figure 2: Potential energy minimization of the (a) protein alone and (b) protein-ligand complex; over a period of 100 ns.

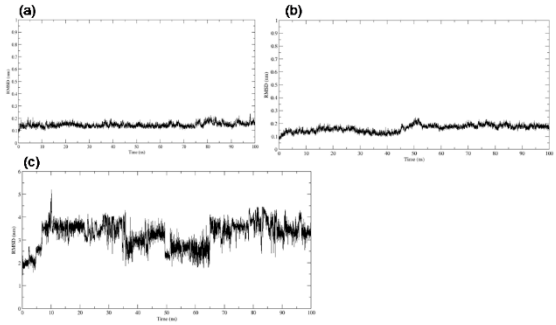

Figure 3: RMSD value of (a) protein alone, (b) protein-ligand complex and (c) ligand alone, during the course of simulation.

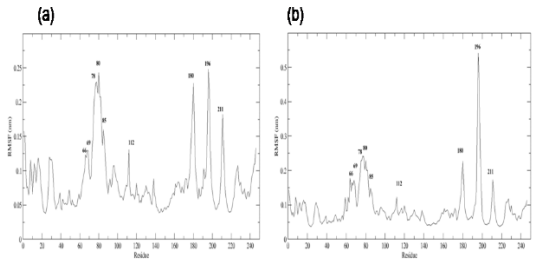

Figure 4: RMSF value of amino acid residues in (a) protein alone and (b) protein-ligand complex.

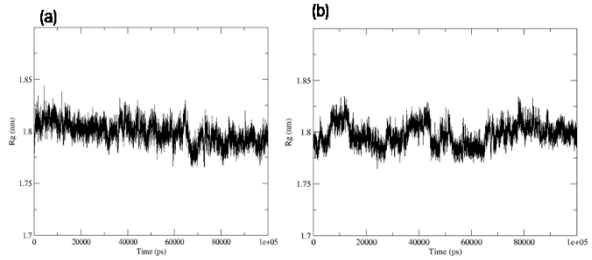

Figure 5: Rg value of (a) protein alone and (b) protein-ligand complex during the course of simulation.

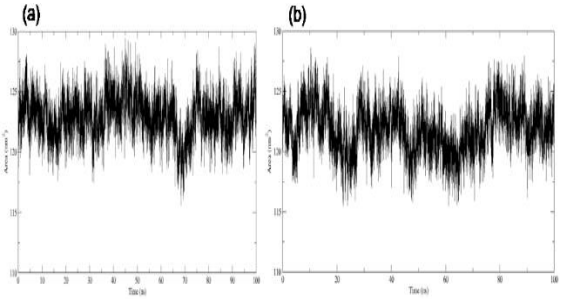

Figure 6: SASA value of (a) protein alone and (b) protein-ligand complex during the course of simulation.

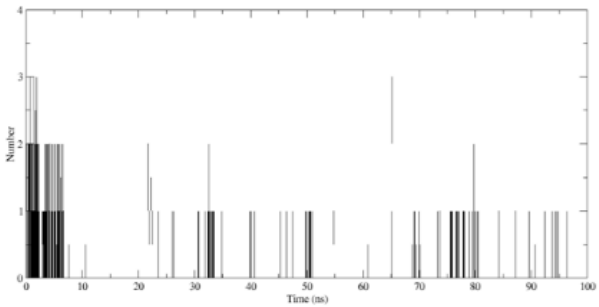

Figure 7: Hydrogen bonding of the ligand with the protein in the protein-ligand complex.

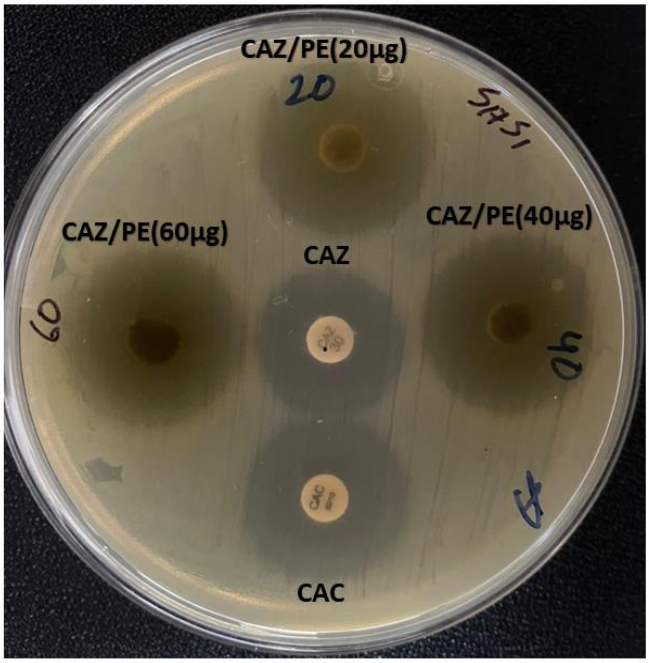

Figure 8: CAZ- Ceftazidime, CAC- Ceftazidime/Clavulanic acid, CAZ/PE-Ceftazidime/Plant extract

β- lactam antibiotics have been playing a pivotal role in treating several bacterial infections[29]. However, due to the increasing use and misuse of these antibiotics, many bacteria have developed resistance to them. The most common mechanism of resistance against these drugs involved the production of β-lactamases by bacteria. These β-lactamases are also continuously evolving with new variants reported every year from different parts of the world[12,16]. So, to overcome this threat, there is a need to discover and develop new inhibitors against the β -lactamase enzymes, which will help in lessening the burden of β-lactam drug resistance and also help in reusing outdated antibiotics[19].

Since various medicinal plants are known to contain a wide range of bioactive compounds, there is a growing interest in these natural products, either as pure compounds or as standardized plant extract, which may provide unlimited opportunities for new drug leads[35]. Hence, in this present study, we have considered a medicinal plant named *A. paniculata* which has a wide range of bioactive phytoconstituents and is known for its ability to treat various ailments. This plant is known to contain a wide variety of phytoconstituents that have been categorized as diterpene lactones, flavonoids and polyphenols[23]. So, there are more than 20 diterpenoids and nearly 10 flavonoids that have been reported from this species over the past three decades[28].

Many studies have also reported that these phytocompounds exhibit antibacterial properties against different types of bacteria[34,59]. So, for examination of its inhibitory property against the ESBL-producing variants, the known phytoconstituents of *A. paniculata* and the phytoconstituents detected in the present study were priorly screened by molecular docking against different variants of ESBL. During the screening process, the active sites of the β-lactamases were targeted to find the lead compound that could interact with them. The ligands showing the lowest binding energy and higher H-bond formation during the ligand-protein interaction were considered to be potent inhibitors of the ESBL. A similar approach to the study was also conducted by researchers who targeted the active sites of NDM-1 to find out its potential inhibitors[45].

In the molecular docking study, it was observed that more than 10 phytoconstituents showed good binding energy; however, comparatively, AAD and AD exhibited a significant binding affinity against ESBLs (OXA-10, SHV-1, TEM-1, CTX-M-14, GES-5) (Table 1). The higher affinity of AAD and AD may be due to H-bond formation between amino acid residues and glycoside moieties[14]. Interestingly, Afroze et al[2] also reported that AAD and NA were found to be the best compounds while screening for antibacterial activity by the *in silico* method. To further validate the docking study, a molecular simulation (MS) study was carried out by selecting one representative phytoconstituent (14-Acetylandrographolide) with good binding energy and higher hydrogen bonding with the target protein.

In the MS study, the gradual minimization of potential energy in both the protein and the protein-ligand complex shows that in both cases, the protein is close to its natural structure. An energy-minimized structure will also portray the size of the active site close to its native state and the active site amino acid residues will be displayed in their natural conformations, yielding a better interpretation of results[43,47]. The RMSD values of the protein-ligand complex and the ligand alone did not show any major fluctuations, indicating the stability of the interaction between the protein and the ligand[55]. The 0.1 nm difference in the RMSD values

of the complex in comparison with the protein structure alone after 60 ns of the simulation indicated that the ligand caused structural changes to the protein as compared to the protein alone[27].

The fluctuations in the RMSD values of the ligand indicated the binding of the ligand in different orientations to the protein binding site[32]. There was an increase in the RMSF value of specific residues in the complex of the protein-ligand as compared to the RMSF values of the protein alone. Increases in RMSF values have been previously linked with lowered catalytic efficiencies of proteins[3,58]. Increased RMSF indicated the flexibility of the amino acids to accommodate the ligands within the binding site of the protein[49]. The steady value of Rg indicated that the protein maintained its structural integrity both when simulated alone and in complex with the ligand.

In the case of the ligand, the marginal increase in the early part of the simulation followed by the lowering of the Rg indicated an increased interaction between the ligand and the protein binding site[54]. The approximately constant SASA value of both the protein and the protein-ligand complex showed that the protein retained its native structure and there was no unfolding of the protein[15]. The occurrence of hydrogen bonding between the protein and the ligand indicates the thermodynamic stability and specificity of the molecular interaction between the ligand and the protein[21,24].

To further validate and confirm the inhibitory property of the phytoconstituents, the crude extract of *A. paniculata* was further assessed by combining it with ceftazidime at different concentrations and comparing it with the zone of inhibition of ceftazidime alone. In the present study, an enlarged zone size of ceftazidime, when combined with the plant extract at 40µg and 60µg, was observed against the test organism in comparison to ceftazidime alone. Also, the zone size of ceftazidime in combination with plant extract shows similarity with the zone size of ceftazidime/clavulanic acid, indicating the inhibitory property of *A.paniculata* against the ESBLs. There has been a lot of research on the antibacterial properties of the above-mentioned medicinal plant, but those studies lack the approach to identify the inhibitory properties of this very plant against the ESBLs.

Our study attempted to find the possibility of the phytoconstituents of *A. paniculata* acting against ESBLs. One of the drawbacks of the study was the non-utilization of a particular phytoactive compound for the *in vitro* study which made it difficult to decide whether the inhibitory effect was the sole responsibility of a single compound or if it was because of the combined effects of the phytoconstituents. Analysis of the inhibitory properties of crude extract against target proteins (ESBL) is lacking as well. So, further studies can be carried out by taking specific compounds to check the inhibition of the target proteins. Nevertheless, this study achieved in delivering a clue that *A.paniculata* can be one of the potential candidates for finding potential β-lactamase inhibitors. A few researchers also claimed that the phytoconstituents of *A. paniculata* are safe for human consumption[2,52]. So, rationalized combination therapy of cephalosporins and the plant extract may be recommended to enhance the efficacy of the β-lactam antibiotics in acting against the ESBL-producing bacteria.

Conclusion

Drug resistance has been a major concern in the treatment process of various infections caused by the β- lactamase producing organisms and finding a good inhibitor is an urgent necessity. Thus, this study reveals a fair and satisfactory inhibitory activity of *A. paniculata* against β-lactamase producing organisms.

Considering its good binding affinity and its stability with active sites of the proteins (ESBL) in molecular docking and molecular simulation studies with a visible inhibition in the disk diffusion method, this plant extract may be taken into account for a combination therapy along with the β- lactam antibiotics that will augment the efficacy of the β-lactam drugs against the ESBL- producing organisms. This approach may be an upturn in treating bacterial infections.

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