Molecular dynamics simulation of bioactive compounds of *Withania somnifera* leaf extract as DNA gyrase inhibitor

Sartaz Beguma, Daniel M. Shadrackb, Ferister M. Josepha and Valence M. K. Ndensendoa

aSchool of Pharmacy, St. John’s University of Tanzania, Dodoma, Tanzania; bDepartment of Chemistry, Faculty of Natural and Applied Sciences, St. John’s University of Tanzania, Dodoma, Tanzania

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1. Introduction

*Withania somnifera* (L) (Ashwagandha) leaf extract is traditionally used in managing and treating bacterial infections. A combination of experimental and computational methods was used to investigate the related antibacterial mechanism. Leaf extract showed strong antibacterial activity against *S. aureus*. Moreover, molecular docking established that withanolide C, a compound obtained from methanolic leaf extract binded strongly to DNA gyrase enzyme. Molecular dynamics simulation and molecular mechanics Poisson-Boltzmann surface area binding free energy suggested withanolide C to be stable at the active site of DNA gyrase B. The compound binded in a different fashion as compared to chlorobiocin a known DNA gyrase inhibitor. Present finding suggests that the antibacterial activity of *W. somnifera* is due to inhibition of DNA gyrase by withanolide C. This finding serves as the basis for development of novel antimicrobial agents.

CONTACT Daniel M. Shadrack dmshadrack@gmail.com or mshadrack@sjut.ac.tz Department of Chemistry, Faculty of Natural and Applied Sciences, St. John’s University of Tanzania, Dodoma, Tanzania

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base for designing potent antimicrobial agent targeting DNA gyrase.

2. Results and discussion

2.1. In vitro anti-bacterial activity

Antibacterial activity of the *W. somnifera* leaf extract was carried out using standard disc diffusion method against *Staphylococcus aureus*, *Salmonella typhi* and *Escherichia coli*. *E. coli* was isolated from urine while *S. typhi* was isolated from stool and *S. aureus* in pus of the patients admitted at Dodoma Regional Referral Hospital and then cultured and investigated. The zone of inhibition was used to evaluate the effectiveness of the extracts. Figure 2 shows the antibacterial activity of the crude extract at different concentrations and standard drugs against *S. aureus*, *E. coli*, and *S. typhi*. The zone of inhibition observed for *S. aureus* at the concentrations of 0.5, 1.0, 1.5 and 3.0 g/mL was 18, 20, 24 and 28 mm, respectively, whereby the zone of inhibition observed for vancomycin (30 mg), penicillin (10 mg) and gentamycin (10 mg) was 25, 20 and 14 mm, respectively. The crude extract of *W. somnifera* at concentration of 3.0 g/mL exhibited maximum inhibition towards the antimicrobial resistant strain, *S. aureus*. Though the plant is known to cure certain infections and typhoid fevers traditionally in Tanzania, the same extract did not show any significant activity against *E. coli* and *S. typhi* where the zone of inhibition observed was 7 mm for all concentrations. This implies that the extract targets other infectious species not investigated in this study.

2.2. Molecular docking study

Molecular docking was performed to major *W. somnifera* compounds viz; withaferin A, withanoside IV, withanoside V, withanolide A-D and a standard chlorobiocin (Figure 1) against DNA gyrase A and B, to relate the observed in vitro antibacterial activity and establish the mechanism of action. Docking calculations to DNA gyrase-A revealed different binding modes between the reference drugs, chlorobiocin, and the best docked compound withanolides A. The conformation with lowest binding free energy of $-8.8$ kcal/mol corresponding to chlorobiocin, was observed to bind at the two alpha helixes formed by residues ARG491, GLU487, GLN484, GLU480, ALA373 and ARG376 by forming hydrogen bonds with GLN484 and GLU487 (Figure 3a). Interestingly, when withanolide extracts were docked to DNA gyrase-A showed to bind to a different site, for example, withanolide A, which showed a lowest binding free energy of $-9.0$ kcal/mol, was accommodated to a different binding site compared to chlorobiocin (Figure 3b). The observed binding preference and affinity suggests that these class of compounds might work with different mechanisms compared to chlorobiocin. The ability of the extracts to bind and inhibit DNA gyrase-B was also performed (Table 1). Docking results showed that, all the compounds possessed similar binding free energy, with withanolide C being more potent. Docking

Figure 1. Bioactive compounds of *Withania somnifera* leaf extract investigated in this study.

Figure 2. Antibacterial activity of methanolic extract of *Withania somnifera* and standard drugs at different concentration.
calculations performed on flexible residues improved binding free energy compared to rigid protein (Table 1), the predicted inhibition constant \(K_i\) was calculated using \(\Delta G = -RT\ln K_i\), where, \(\Delta G\) is the binding free energy, \(R\) is the universal gas constant and \(T\) is temperature (298.15 K). The observed \(K_i\) values for withanolide C was nearly 9-folds potent than chlorobiocin, a known inhibitor of DNA gyrase, suggesting that withanolide C could be potent inhibitor. The binding modes of chlorobiocin and withanolide C in DNA gyrase-B active site are presented in Figure 3c,d. It is interesting to observe that both chlorobiocin and withanolide C interacted with Ile76, however, the peculiarity in binding and the increased potency of withanolide C is related to its ability to interact with Ile64 and Asn32. Such interactions are not observed in chlorobiocin, instead it interacts with residues from the \(\alpha\)-sheets at the bottom of the pocket and His83 from loop 6 (see Figure 3 and supporting information Figure S1). It seems that interaction with Ile76 is essential in inhibiting DNA gyrase activity and interaction with Asn32 observed for withanolide C suggests its increased potency as an antibacterial agent. The observed difference in binding modes and interaction with residues could be a way for improved activity in withanolide C. Would withanolide C remain stable in the active site interacting with residues observed from docking calculation or otherwise? This was a motivational question that led us to establish the stability of the withanolide C in DNA gyrase-B pocket by classical MD simulation and free energy calculations.

2.3. Classical molecular dynamic (MD) simulation

The methanolic leaf extract of \(W. somnifera\) was previously reported to yield withanolide C (Bessalle & Lavie, 1992). Our docking simulation further suggested that, withanolide C showed potent anti-bacterial activity against DNA gyrase. Since the leaf extracts of \(W. somnifera\) are traditionally used to manage different disease conditions including bacterial infections, MD simulation was carried out to investigate the stability of withanolide C in the active pocket of DNA gyrase. Two independent MD simulation of 100 ns each was run. The root mean square deviation (RMSD), root mean square fluctuations (RMSF), radius of gyration (Rg) and hydrogen bond distances were analysed to establish the related stability. The RMSD provides information on the stability of the complex. The C-\(\alpha\) RMSD (Figure 4) of the protein equilibrated after 37 ns and remained stable with fluctuation ranging from 0.18 to 0.23 nm. The RMSD of the ligand obtained

Table 1. Binding free energy (\(\Delta G\) (kJ/mol)) of withanolide leaf extracts against DNA gyrase A and B subunits.

| Compounds    | \(\Delta G_{rigid}\) | \(\Delta G_{flexible}\) | \(K_i (\mu M)\) | \(\Delta G_{rigid}\) | \(\Delta G_{flexible}\) | \(K_i (\mu M)\) |
|--------------|----------------------|------------------------|----------------|----------------------|------------------------|------------------|
| Chlorobiocin | -8.8                 | -8.3                   | 0.47           | -8.3                 | -8.6                   | 0.47             |
| Withaferin A | -7.7                 | -8.1                   | 0.20           | -7.8                 | -7.3                   | 0.20             |
| Withanoside IV | -8.6              | -7.3                   | 0.93           | -8.2                 | -7.8                   | 0.93             |
| Withanoside V | -8.8                 | -7.8                   | 0.47           | -8.6                 | -7.8                   | 0.47             |
| Withanolide A | -9.0                 | -7.8                   | 0.47           | -8.6                 | -7.8                   | 0.47             |
| Withanolide B | -8.1                 | -7.3                   | 0.28           | -8.9                 | -7.9                   | 0.28             |
| Withanolide C | -8.1                 | -8.7                   | 0.05           | -9.9                 | -9.9                   | 0.05             |
| Withanolide D | -8.4                 | -8.2                   | 0.17           | -9.2                 | -9.2                   | 0.17             |

Figure 3. Binding modes of chlorobiocin in DNA gyrase subunit A (a) and subunit B (c), binding mode of withanolide A in DNA gyrase A (b), binding mode of withanolide C in DNA gyrase B (d).
after least square fit shows few fluctuations during the first 10 ns, and then remained stable until 100 ns time. It is interesting to note that the observed changes in ligand RMSD at the start of the simulation suggested changes in binding pose of the ligand. This motivated us to investigate the changes in binding pose of the ligand. Supporting information Figure S3 suggests that the ligand changed from its initial docking pose and exhibited different orientation within the pocket as suggested by the snapshot taken at 80 ns (supporting information Figure S4). The internal motion and fluctuations of the residues were analysed by calculating the RMSF (Figure 4b). Higher fluctuations were observed to residues forming the loop 5 (Figure 4b) at the region 63 to 73. Other region observed to have large fluctuations is 140 to 160. Despite of the fluctuation observed at loop 5, residue 64 interacted with the ligand, and is part of the residues forming the active site (Figure 5b). It was interesting to observe that, although loop 5 is forming the active site, still shows fluctuations compared to other loops which are forming the active site. The RMSF suggest that the complex was stable with subtle fluctuations in the loop which are expected.

Figure 4. (a) RMSD for complex and ligand (b) pose RMSD of ligand (c-d) hydrogen bond distances between residues Asn32 and ligand oxygen (O), and residue Thr132 and ligand hydroxyl group (OH), (d-e) free energy profiles for the distances between Asn32 and Thr132 and the ligand atoms.
To understand how much withanolide C exhibited changes, hydrogen bond distance (Figure 4c,d), was measured for atoms that formed hydrogen bonds. The distances suggest that withanolide C exhibits orientation within the pocket. The free energy (F) as the function of distance (d) was calculated using

\[
F = -k_B T \ln(P(d)),
\]

where, \(k_B\) is the Boltzmann constant, \(T\) is Temperature and \(P(d)\) is the probability distribution of the distance. The Free energy for Asn32-O(lig) (Figure 4e) shows a flat free energy surface with \(d = 3.5\) to 4.8 nm, suggesting a strong to weak hydrogen bond interaction. However, the distance between Thr132-OH(lig) shows two free energy minima at \(d = 2.3\) and 4.5 nm corresponding to strong and weak hydrogen bonds, respectively. It was observed that, these residues highly interacted with withanolide C suggesting stable complex and hence inhibition of the DNA gyrase activity, which ultimately result to death of the bacteria.

Rg provides information on the compatibility and size of system. The calculated Rg value (supporting information Figure S5) show fluctuations ranging from 1.58 to 1.62 nm. It is observed that, at the beginning of the simulation the system had higher Rg value of 1.62 and decreased to 1.58 nm during the last 100 ns. The decrease Rg value suggest compatibility and small size which reflect that withanolide C was held tightly in the DNA gyrase pocket.

### 2.4. Molecular mechanics Poisson-Boltzmann surface area calculation

Docking calculations are good in predicting the binding and orientation of the ligand, however, poorly estimates the binding affinity and related stability. Free energy methods based on molecular mechanics Poisson-Boltzmann surface area (MM-PBSA) was used to re-score docking results and binding affinity. The binding energy and energy decomposition contribution to free energy are presented in Table 2 and Figure 5, respectively, which suggests strong binding affinity. van der Waal and non-polar energetic favorably contributed to the interaction and binding of withanolide C to DNA gyrase, followed by electrostatic energetic terms. However, polar energy unfavorably contributed to the interaction (Table 2). Decomposition analysis and residues contribution to binding free energy showed that; Ile76 highly contributed with \(-17\) kJ/mol followed by Ala72 and Ile64 (Figure 5a,b). As observed for the hydrogen bond distance analysis (Figure 4c–f), withanolide C remained in the pocket interacting with Asn32, Ile76, Ala73 and Ile64 as observed also for the free energy decomposition analysis.

### 3. Material and methods

#### 3.1. Plant collection

Fresh leaves of W. somnifera were collected in March 2019 from Tukuyu-itete street with GPS location 9° C 15’ 37.512” South and 33° C 38’ 35.124” East, Runge district, Mbeya, Tanzania. The plant was identified by a botanist and authenticated at the St John’s University of Tanzania. The specimen was preserved at the herbarium of the St John’s University of Tanzania.

#### 3.2. Extraction

The under-shade air dried, pulverized samples were soaked twice for 48 h in methanol. The filtered crude extract was concentrated in vacuo using a rotary evaporator while maintaining the water bath temperature below 40°C to avoid thermal decomposition of labile compounds. The
obtained crude extract was refrigerated at $-4^\circ$C until further analysis.

### 3.3. In vitro antimicrobial assay

In vitro antibacterial activity was carried out using a standard disk diffusion method (Adhikari et al., 2017; Tendencia, 2004) at Microbiology laboratory, Dodoma Regional Referral Hospital, Dodoma, Tanzania. Bacterial strains ($S. aureus$, $S. typhi$ and $E. coli$) were isolated, cultured and inoculated into Mueller Hinton agar plates and incubated at $37^\circ$C for 24 h and then identified and stored under appropriate condition for further process using standard methods. A whatman filter paper number 1 (round shape of 5 mm in diameter) was used to prepare the disk because of its high loading capacity and particle retention. The disks were immersed into the $W. somnifera$ leaf extract solution at concentrations of 0.5, 1, 1.5 and 3.0 g/mL for 12 h and then the disks were placed on the agar plates containing bacterial inoculums. Standard antibiotics (vancomycin, penicillin, and gentamycin for $S. aureus$ while chloromphenicol, ciprofloxin and erythromycin for $E. coli$ and chloromphenicol, ciprofloxin and gentamycin for $S. typhi$) were used as reference drugs. The plates were incubated at $37^\circ$C for 24 h prior to determination of results, followed by measuring the zone of inhibition in millimeters by using a ruler. The diameter of the zone of inhibition was related to the susceptibility of the isolate. The zonal diameters of the disk containing the extracts and standard control drugs were interpreted using the criteria published by the Clinical and Laboratory Standards Institute (Cockerill, 2010).

### 3.4. Molecular docking

The chemical structures of the compounds used for docking and MD simulation study was obtained from PubChem database (Kim et al., 2016). The molecules were prepared using Open Babel (O’Boyle et al., 2011), Gasteiger charges and hydrogen were added at pH of 7.4, energy minimized and converted to pdbqt format in Open Babel (O’Boyle et al., 2011). The protein structure for DNA gyrase A (1AB4) (Cabral et al., 1997) and DNA gyrase B (1KZN) (Lafitte et al., 2002) were obtained from RCSB PDB (protein data bank) (Rose et al., 2012). The protein was prepared using Open Babel (O’Boyle et al., 2011) tool where missing hydrogen and Gasteiger charges were added, and converted to pdbqt format. Two blind docking experiments were performed, the first experiment involved rigid protein structure while in the second residues at the active site were allowed to be flexible. The binding site of the protein was detected using the co-crystallized inhibitor of DNA gyrase. Before performing the actual docking the method was validated by re-docking chlorobacin which give an RMSD value of 0.25 Å. The RMSD value of $\leq 3$ Å implies that the tool can reproduce the binding pose. This permitted us to continue with the docking experiments.

### 3.5. MD simulation

Classical MD simulation was performed as previously described (Shadrack et al., 2020). Briefly, the conformation with best binding energy from docking calculation was used as initial structures for MD simulation. Antechamber and general amber force field (GAFF) were used to obtain ligand topology, protein topology was built using amber03 force field. The system was solvated with TIP4P (Abascal & Vega, 2005) water model, Na$^+$ ions were added to make the system electrically neutral. Energy minimization was performed to remove any cruces and constraints from docking calculation. The system was equilibrated at an NVT (constant number of particles, Volume and Temperature) ensemble using Berendsen thermostat at 300 K for 500 ps and at NPT (constant number of particles, Pressure and Temperature) ensemble for 1 ns with pressure maintained at 1 bar using Parrinello-Rahman barostat. Production run was performed in an NPT ensemble for 100 ns, v-rescale (Bussi et al., 2007) thermostat was used for temperature coupling at 300 K and Parrinello-Rahman was used for pressure coupling at 1 bar. Particle mesh Ewald (PME) (Essmann et al., 1995) method was used to treat long-range electrostatic interactions with a cutoff distance on 10 Å while covalent bonds were constrained using LINCS algorithm (Hess et al., 1997), an integration time step of 2 fs was used in all simulations. For control purpose, a second MD simulation run was performed as described above for 100 ns. All energetic terms were monitored until completion of the simulation time. Analysis was performed from the equilibrated MD, VMD and Chimera were used to visualize the results.

### 3.6. MM-PBSA binding free energy calculation

In this study, MM-PBSA was calculated using $\varphi_{\text{mm-pbsa}}$ tool (Kumari et al., 2014). The binding free energy ($\Delta G_{\text{bind}}$) for protein-ligand interaction is expressed in Equation (1)

$$\Delta G_{\text{bind}} = G_{\text{complex}} - (G_{\text{protein}} + G_{\text{ligand}})$$

$$G_X = <E_{MM}> + <E_{Sol}> - T\Delta S$$

$$<E_{MM}>=<E_{bonded}> + <E_{non-bonded}>$$

$$<E_{bonded}> = <E_{vdW}> + <E_{elec}>$$

where, $<E_{MM}>$ is the average molecular mechanics energy terms in vacuum which includes bonded and non-bonded interactions, $<G_{Sol}>$ is the free energy of solvation which includes $G_{\text{polar}}$ and $G_{\text{non-polar}}$, $\gamma$ is a coefficient related to surface tension, SASA is the solvent accessible surface area, $b$ is the fitting parameter and $T\Delta S$ is the entropic contributions to free energy. The binding free energy was calculated using a single trajectory, where a total of 300 snapshots were evenly extracted at a predetermined intervals from the equilibrated MD trajectory. The solvent dielectric constant was 80, solute dielectric constant was 2, $\gamma$ was 0.0226778 kJ/Mol/Å$^2$ and $b$ was 3.84928 kJ/Mol, the PB equation was solved by using the linear PBsolver.
4. Conclusion
Findings reported in this work suggests *W. somnifera* leaf extract to possess antibacterial activity against *S. aureus*. Withanolide C one of the major bioactive compound binds strongly to DNA gyrase B, this could be its mechanism of action against *S. aureus*. MD simulation results suggested that withanolide C remained stable in the active site through out 100 ns simulation time. The present results will provides decisive role for future studies on developing new antimicrobial upon further in vivo experiments.

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No potential conflict of interest was reported by the authors.

ORCID
Daniel M. Shadrick http://orcid.org/0000-0002-4436-1487

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