In-silico screening and identification of potential inhibitors against 2Cys peroxiredoxin of Candidatus Liberibacter asiaticus

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ABSTRACT
Huanglongbing (HLB) is a worldwide citrus plant disease-related to non-culturable and fastidious α-proteobacteria Candidatus Liberibacter asiaticus (CLas). In CLas, Peroxiredoxin (Prx) plays a major role in the reduction of the level of reactive species such as reactive oxygen species (ROS), free radicals, and peroxides, etc. Here, we have used structure-based drug design approach was used to screen and identify the potent molecules against 2Cys Prx. The virtual screening of fragments library was performed against the three-dimensional validated model of Prx. To evaluate the binding affinity, the top four molecules (N-Boc-2-amino isobutyric acid (B2AI), BOC-L-Valine (BLV), 1-(boc-amino) cyclobutane carboxylic acid (1BAC), and N-Benzoyl-DL-alanine (BDLA)) were docked at the active site of Prx. The molecular docking results revealed that all the identified molecules had a higher binding affinity than Tert butyl hydroperoxide (TBHP), a substrate of Prx. Molecular dynamics analysis such as RMSD, Rg, SASA, hydrogen bonds, and PCA results indicated that Prx-inhibitor(s) complexes had lesser fluctuations and were more stable and compact than Prx-TBHP complex. MMPBSA results confirmed that the identified compounds could bind at the active site of Prx to form a lower energy Prx-inhibitor(s) complex than Prx-TBHP complex. The identified potent molecules may pave the path for the development of antimicrobial agents against CLA.

KEYWORDS
Peroxiredoxin; Candidatus Liberibacter asiaticus; oxidative stress; quantum mechanics; molecular dynamics simulation

1. Introduction

Huanglongbing (HLB) or citrus greening is a worldwide, highly devastating citrus plant disease associated with phloem limited, non-culturable, fastidious α-proteobacteria. Candidatus Liberibacter asiaticus’ (CLas), ‘Ca. L. africanus’ (CLaf), and ‘Ca. L. americanus’ (CLal) are three important species and transmitted through Asian citrus psyllid (Diaphorina citri Kuwayama) (Bové, 2006; Halbert & Manjunath, 2004; Texeira et al., 2005). HLB causes a large scale economic impact to the citrus industry globally (Jagoueix et al., 1994). The symptoms of HLB include blotchy chlorosis, spotty leaves, diminutive growth, distorted-shaped fruit, and even plant death (Bové, 2006). There is no effective strategy that can eradicate it completely. The only way to control the HLB is to either chemically control the psyllids or remove the infected plants (Boina & Bloomquist, 2015). The transmission of phytopathogenic bacteria in plants can increase the level of reactive oxygen species (ROS) and peroxide, which play a vital role in the inhibition of the growth and development of bacteria (Li et al., 2009).

In the biological system, ROS and free radicals are produced due to biotic and abiotic stress (Xie et al., 2019; You & Chan, 2015). ROS and free radicals can damage and alter the functions of the membranes and biomolecules like proteins, nucleic acids, carbohydrates, and lipids, etc. (Birben et al., 2012). Antioxidant defense systems are known to reduce oxidative stress in living organisms. It has been reported that antioxidant defense proteins such as superoxide dismutase, DT-diaphorase, catalase, and peroxiredoxin, etc. cause growth and provide the shield against ROS, free radicals, and peroxides in pathogenic bacteria (Ighodaro & Akinloye, 2018; Nandi et al., 2019; Rhee, 2016).

Peroxiredoxins (Prxs) play a significant role in scavenging ROS and reduction of the peroxide level in bacteria (Dubbs & Mongkolsuk, 2007; Jara et al., 2007; Toledano & Huang, 2016). Prx is a highly conserved, thiol-based antioxidant enzyme that belongs to the peroxidase family. Prxs are classified into 1Cys and 2Cys based on the number of cysteine residues involved in catalysis. The 2Cys Prx is further subdivided into two groups; in typical 2Cys peroxiredoxins and atypical 2Cys peroxiredoxin (Chae et al., 1994; Wood et al., 2003). Prxs are linked with cell proliferation, differentiation,
apoptotic pathway DNA binding protein, and wound healing also (Auf Dem Keller et al., 2006; Kinnula et al., 2002).

The Prx family has a highly conserved active site region: PxxxTxxC motif (known as a catalytic motif) and a conserved Arg residue (Nelson et al., 2011). Prx declines more than 90% peroxide in the cytosol to maintain the cellular level (Hall et al., 2011). Tert-Butyl hydroperoxide (TBHP) is a very well-known substrate for the Prx (Bernier-Villamor et al., 2004; Ishida et al., 2014; Kučera et al., 2014; Singh et al., 2017). TBHP is an organic peroxide involved in the generation of ROS and free radicals (Kučera et al., 2014). In our earlier study, we have reported that TBHP is a substrate for both 1Cys Prx and 2Cys Prx (A. Singh et al., 2017). Mass spectrometry and immunoblot assay confirmed that TBHP could hyper oxidized the Prx (Ishida et al., 2014). Prx showed catalytic rates of $\sim 10^7 \text{ M}^{-1} \text{s}^{-1}$ to maintain the intracellular peroxide levels in several organisms (Hall et al., 2009, 2011; Perkins et al., 2014). Therefore, the identification of small molecules against Prx of CLas will interrupt the reduction of peroxide. This strategy can be helpful for the discovery of antimicrobial compounds against phytopathogenic bacteria.

In this study, the three-dimensional model of 2Cys Prx from CLas was predicted using SWISS-model. The predicted model was validated using Ramachandran plot, VERIFY-3D, ProSA, and ProQ. The fragmented molecules from selleckchem database were used for virtual screening and the top four molecules (B2AI, BLV, 1BAC, and BDLA) showed higher binding affinity than TBHP. Molecular docking results indicate that identified molecules bound at the active site of Prx via hydrogen bonds or hydrophobic interactions. Density functional theory calculations were performed to compute the chemical properties of the identified molecules. The molecular dynamics and MMPBSA was employed to determine the stability of Prx-TBHP and Prx-inhibitor(s) complex.

2. Materials and methods

2.1. Homology modelling

Peroxiredoxin (Prx) protein sequence from Candidatus Liberibacter asiaticus (CLas) was retrieved from NCBI protein (ALK06947.1) and BLAST tool was used to find the homologous structures (Altschul et al., 1990). The three-dimensional structure of Prx was predicted using SWISS-model, as done by Dhankhar et al. (Dhankhar et al., 2020; Schwede et al., 2003). The model was refined and energy minimized using ModLoop and Swiss PDB viewer, as done by Singh et al. (Fiser & Sali, 2003; Guex & Peitsch, 1997; Singh et al., 2017). The model was validated by Ramachandran plot generated by PROCHECK and MolProbity, as done by Pandit et al. (Chen et al., 2010; Laskowski et al., 1993; Pandit et al., 2018). The stereochemical qualities of the model were assessed using VERIFY-3D, ProSA, and ProQ, as done by Saini et al. (Eisenberg et al., 1997; Saini et al., 2019; Wallner & Elofsson, 2003; Wiedeinstein & Sippl, 2007). Moreover, the predicted model was also validated by molecular dynamics (MD) using GROMACS suite with GROMOS96 53a6 force field, as done by Kumari et al. (Gunsteren et al., 1996; Kumari et al., 2020; Van Der Spoel et al., 2005). The multiple sequence alignment of Prx with other homologous structures was performed using clustal omega and visualize using Espript 3.0, as done by Dalal et al. (http://esprid.ibcp.fr/ESPr ipt/cgi-bin/ESPr ipt.cgi) (Dalal et al., 2019; Gouet et al., 1999; Sievers et al., 2011).

2.2. Virtual screening

The fragments library having a total of 1015 fragment compounds were retrieved from selleckchem (https://www.selleckchem.com/screening/fragment-library.html). Tert butyl hydroperoxide (TBHP) a substrate of Prx was downloaded from PubChem database (PubChem ID: 6410) in sdf format (Kim et al., 2019). All the downloaded molecules were energy minimized and converted into pdbqt using Open Bable in PyRx0.8, as done by Malik et al. (Dallakyan & Olson, 2015; Malik et al., 2019; O’Boyle et al., 2011). AutoDock Vina in PyRx0.8 was used for virtual screening and nine distinct conformations were predicted for each molecule, as done by Kumar et al. (Kumar et al., 2020; Trott & Olson, 2010). The molecular grid was generated around Pro48, Thr52, Cys55, and Arg132 of protein. The grid points and grid dimensions were as: $X = 0.67$, $Y = -12.64$, and $Z = 2.58$ and $40 \angstrom$, $40 \angstrom$, and $40 \angstrom$. The generated poses of molecules were saved and visualized in PyMOL (DeLano, 2002). Four molecules (N-Boc-2-amino isobutyric acid (B2AI), BOC-L-Valine (BLV), 1-(boc-amino) cyclobutane carboxylic acid (1BAC), and N-Benzoyl-DL-alanine (BDLA)) showed higher binding affinity than tert butyl hydroperoxide (TBHP) were considered for further study.

2.3. Molecular properties and molecular docking

The pKCSM, an online tool, was used to compute the physicochemical properties such as molecular weight, the number of hydrogen bond acceptors, donors, and logP, as done by Dhankhar et al. (Dhankhar et al., 2020; Pires et al., 2015). Molecular docking of screened compounds and TBHP was done using AutoDock Tools and HADDOCK (De Vries et al., 2010; Kuruczcuoglu et al., 2018; Morris et al., 2009). Polar hydrogen atoms and gasteiger charges were added to TBHP, B2AI, BLV, 1BAC, and BDLA, as done by Singh et al. (Singh et al., 2018). The molecular grid for docking was kept around Pro48, Thr52, Cys55, and Arg132 of protein. The Lamarckian genetic algorithm was used to generate 50 conformations and the generated poses were inspected in PyMOL. Moreover, HADDOCK was also used for molecular docking of TBHP, B2AI, BLV, 1BAC, and BDLA, as done by Singh et al. (Singh et al., 2019). Pro48, Thr52, Cys55, and Arg132 of Prx were considered as active residues for molecular docking using HADDOCK.

2.4. Density functional theory calculations

Density functional theory (DFT) calculations of TBHP, B2AI, BLV, 1BAC, and BDLA were done using 6-311 G (d,p) basis set along with density functional theory Becke’s three-parameter hybrid functional combined with Lee-Yang-Parr
correlation functional (DFT/B3LYP) in Gaussian 16, as done by Saini et al. (Frisch et al., 2016; Lee et al., 1988; Saini et al., 2019; Schlegel, 1982). The polarity of ligands was assessed by molecular electrostatic potential (MEP). The Highest Occupied Molecular Orbital (HOMO) and Lowest Unoccupied Molecular Orbital (LUMO) were determined to assess the nucelophilic and electrophilic properties of the ligands, as done by Kesari et al. (Kesari et al., 2020). Further, HOMO and LUMO results were used to calculate the energy gap, electronegativity, hardness, and softness of the ligands.

2.5. Molecular dynamics simulation

Molecular dynamics (MD) simulation of Prx-Native, Prx-TBHP, and Prx-inhibitor(s) complexes were conducted to determine the stability at an atomic level. Molecular dynamics was performed using GROMOS96 53a6 force field in GROMACS on an Ubuntu-based workstation, as done by Singh et al. (Gunsteren et al., 1996; Singh et al., 2020; Van Der Spoel et al., 2005). The topology of small molecules (TBHP, B2AI, BLV, 1BAC, and BDLA) were generated using PRODRG (Schüttelkopf & Van Aalten, 2004). The partial atomic charges of ligands were computed using 6-311G (d,p) basis set along with DFT/B3LYP in Gaussian 16, as done by Dhankhar et al. (Dhankhar et al., 2020; Frisch et al., 2016; Lee et al., 1988; Schlegel, 1982). The systems were solvated in simple point charges (SPC) water model using the solvate tool in GROMACS. The protein complexes were kept in a triclinic box of volume 238.60 nm³ with a 1.0 nm distance from the edge of box. The systems were neutralized by the addition of 5 Na⁺ ions and energy minimized using the steepest descent algorithm for 50,000 iteration steps up to an energy cut of 1.0 kcal/mol⁻¹. The systems were equilibrated for a constant number of particles, volume, and temperature (NVT) and a constant number of particles, pressure, and temperature (NPT) for 1 ns at 300 K. Pressure and temperatures were regulated using Parrinello-Rahman barostat pressure coupling method and Berendsen thermostat, respectively (Berendsen et al., 1984; Parrinello & Rahman, 1981). Linear constraints Solver (LINCS) method was utilized to constrain covalent bonds (Hess et al., 1997). The final molecular dynamics was run for 100 ns and particle mesh ewald (PME) method was used to calculate the long-range electrostatic interactions (Abraham & Gready, 2011). In final MD coordinates were generated at regular intervals of 10 ps and the trajectories were used to generate the root mean square deviation (RMSD), radius of gyration (Rg), solvent accessible surface area (SASA), hydrogen bond numbers, and principal component analysis (PCA).

2.6. Mmpbsa Binding free energy calculations

Molecular Mechanics/Position-Boltzmann Surface Area (MMPBSA) method used to calculate the binding free energy of the Prx-TBHP, Prx-B2AI, Prx-BLV, Prx-1BAC, and Prx-BDLA (Kumari et al., 2014). The binding free energy of protein-ligand complexes were determined using g_mmpbsa as:

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

Here, $\Delta G_{bind}$, $\Delta G_{complex}$, $\Delta G_{protein}$, and $\Delta G_{ligand}$ denotes the binding free energy, free energy of the protein-ligand complex, total energy of protein and ligand in a solvent, respectively. Here, the trajectories consisted of 2000 frames generated at every 10 ps during an interval of 80 to 100 ns of molecular dynamics were used to estimate the binding affinity of protein-ligand(s) complexes. Further, per-residue decomposition analysis was performed from the 2000 snapshots extracted from the last 20 ns to explore the role of crucial important residues (Pro48, Thr52, Cys55, and Arg132) of Prx in Prx-ligand(s) complexes.

3. Results

3.1. Homology modeling

The amino acid sequence of 2Cys Prx was retrieved from the NCBI database. The homology model of Prx was generated by SWISS-model and energy minimized using SWISS PDB viewer. ModLoop was used to refine the disordered regions of the model. The Ramachandran plot generated by PROCHECK showed that 86.4% of amino acid residues were in favored regions, 13.6% of residues were in additional allowed regions, and no residue was in generously allowed and disallowed regions, as shown in Figure S1. The quality factor predicted by VERIFY-3D illustrated that 88.16% of amino acid residues had an average 3-D-1D score greater than or equal to 0.2, as shown in Figure S2A. ProSA showed a Z-score of −4.81 and −4.85 for the Prx model and X-ray structure of a peroxiredoxin (PDB ID: 4D73), respectively, as shown in Figures S2B and C. LG score of 4.9 predicted by ProQ for Prx indicates that the model was extremely good and reliable. Moreover, the structural superposition of Prx model with homologous structure showed an RMSD of 0.081 Å for 134 Cx atoms. Additionally, the RMSD by molecular dynamics revealed that the model got equilibration at 0.35 nm at 26 ns and remained stable up to the end of molecular simulation of 100 ns, as shown in Figure S2D. The validated Prx model consists of 5 α helices (α1- α5) and 7 β sheets (β1- β7), as shown in Figure S3. MSA results revealed that active site residues (Pro48, Thr52, Cys55, and Arg132) of Prx from CLas are conserved, as shown in Figure S4.

3.2. Virtual screening

The fragment library consists of 1015 compounds were downloaded in sdf format from selleckchem database. Tert butyl hydroperoxide (TBHP) was retrieved from the PubChem database in sdf format. The virtual screening was performed using AutoDock Vina and generated conformations were analyzed in PyMOL. Four molecules (B2AI, BLV, 1BAC, and BDLA) showed higher binding in the range of −5.5 to −6.4 kcal/mol than TBHP (-3.4 kcal/mol) for Prx, as shown in Table 1.
3.3. Molecular properties and molecular docking

The pkCSM online webserver was used to assess the physicochemical properties. B2AI, BLV, 1BAC, and BDLA were considered to predict the molecular properties, such as Lipinski’s rule of five. All the molecules exhibit a molecular weight in the range of 193.02 to 217.26 Da, as shown in Table S1. The maximum number of hydrogen bond donors and acceptors are 2 and 4, respectively. AutoDock Tools and HADDOCK were utilized for the molecular docking of TBHP and identified molecules with Prx, as shown in Tables 1 and S2.

AutoDock Tools and HADDOCK results showed that TBHP was found stable at the active site of Prx due to hydrogen bonds and hydrophobic interactions, as shown in Figures 1A and S5A. B2AI exhibited the highest binding affinity among identified molecules, as shown in Tables 1 and S2. B2AI formed eight hydrogen bonds with active site residues (Thr52, Lys121, Arg132, and Asp158) of Prx, as shown in Figure 1B. Prx-B2AI complex was also stabilized by hydrophobic interactions contributed by Pro48, Pro53, Cys55, Phe126, and Trp131, as shown in Figure S5B. BLV also formed a lower energy Prx-BLV complex as compared to TBHP with Prx. It formed ten hydrogen bonds with Thr52, Thr54, Lys121, Arg132, and Asp158, as shown in Figure 1C. Additionally, hydrophobic interactions due to Pro48, Pro53, Cys55, Val124, Phe126, Trp131, and Pro159 also stabilized the Prx-BLV complex, as shown in Figure S5C. 1BAC and BDLA were able to make hydrogen bonds with Thr52, Lys121, Arg132, and Asp158, as shown in Figures 1D and E. Moreover, hydrophobic interactions were due to Pro48, Pro53, Cys55, Val124, Phe126, Trp131, and Pro159 of Prx (Figures S5D and E).

3.4. Density functional theory calculations

Density functional theory (DFT) calculations were performed to study the molecular and electronic properties of TBHP, B2AI, BLV, 1BAC, and BDLA. Electrostatic potential maps were generated to assess the molecule’s high and low electron density regions (Figure 2). HOMO and LUMO plots of compounds were represented in Figure 3. The positive and negative phases of molecular orbitals were shown by red and green colors in HOMO and LUMO plots. The identified molecules exhibited a higher energy gap in the range of 2.66 to 3.13 eV than TBHP (1.53 eV), as shown in Table 2.

3.5. Molecular dynamics simulation

Molecular dynamics simulation was performed to determine the variation at the atomistic level in Prx-ligand(s) complexes. Therefore, in this study, we have examined several molecular dynamics parameters such as root mean square deviation (RMSD), radius of gyration (Rg), solvent accessible surface area (SASA), hydrogen bond formation, and principal component analysis (PCA) for Prx-TBHP and Prx-inhibitor(s) complexes.

The dynamics stability of backbone atoms of Prx-TBHP and Prx-inhibitor(s) complexes were assessed by RMSD. The RMSD plot showed an initial sharp up to 5 ns and became stable at 10 ns. Prx-ligand(s) complexes sustained equilibrium at 25 ns and remained stable up to the end of molecules simulation, as shown in Figure 4A. Prx-inhibitor(s) complexes showed lesser RMSD in the range of 0.24 to 0.32 nm than Prx-TBHP (0.29 to 0.35 nm). The average RMSD for Prx-TBHP, Prx-B2AI, Prx-BLV, Prx-1BAC, and Prx-BDLA complexes were 0.30 ± 0.03, 0.26 ± 0.02, 0.27 ± 0.02, and 0.28 ± 0.03 nm, respectively, as shown in Table 3. From Figure 4B, it can be clearly seen that all the identified molecules exhibited lesser RMSD in the range of 0.04 to 0.1 nm as compared to TBHP (0.12 nm). The average ligands RMSD of TBHP, B2AI, BLV, 1BAC, and BDLA were shown in Table 3. Overall, RMSD results indicated that Prx-inhibitor(s) complexes were more stable than Prx-TBHP complex.

The radius of gyration was determined to understand the compactness of protein-ligand(s) complexes during the molecular simulation. Prx-inhibitor(s) complexes revealed lesser Rg values as compared to Prx-TBHP complex, as shown in Figure 5. Prx-TBHP, Prx-B2AI, Prx-BLV, Prx-1BAC, and Prx-BDLA complexes showed average Rg values of 1.52 ± 0.01, 1.47 ± 0.01, 1.49 ± 0.01, 1.49 ± 0.01, and 1.50 ± 0.01 nm, as shown in Table 3. Rg results suggested that binding of inhibitor(s) to Prx formed a higher stable Prx-inhibitor(s) complex than Prx-TBHP complex. Solvent accessible surface area (SASA) plot illustrated that values of Prx-inhibitor(s) complexes were smaller than Prx-TBHP complex, as shown in Figure 6. The average SASA values of Prx-TBHP, Prx-B2AI, Prx-BLV, Prx-1BAC, and Prx-BDLA complexes were 90.35 ± 1.93, 85.70 ± 2.25, 86.08 ± 2.32, 87.33 ± 2.05, and 88.70 ± 2.08 nm², respectively, as shown in Table 3.

Intra protein hydrogen bonds were analyzed to check the stability of Prx-TBHP and Prx-inhibitor(s) complexes, as shown in Figure 7. From Figure 7A, it can be clearly seen that Prx-TBHP complex was stabilized due to 88 to 117 hydrogen bonds during the molecular simulation. Prx-B2AI complex exhibited hydrogen bond numbers in the range of 100 to 117, as shown in Figure 7B. Similarly, binding of other inhibitors (BLV, 1BAC, and BDLA) with Prx formed Prx-inhibitor(s) complexes due to 92 to 127 number of hydrogen bonds during the molecular dynamics, as shown in Figures 7C-E. The average intra protein hydrogen bonds for Prx-TBHP, Prx-B2AI, Prx-BLV, Prx-1BAC, and Prx-BDLA complexes were 107.4 ± 5.4, 114.0 ± 5.2, 112.9 ± 5.3, 110.8 ± 5.3, and 109.3 ± 5.6, respectively, as shown in Table 3.

Further, intermolecular hydrogen bonds were determined to investigate the stability of Prx-TBHP and Prx-

### Table 1. Virtual Screening and molecular docking results of TBHP, B2AI, BLV, 1BAC, and BDLA with Prx. Virtual screening and molecular docking of ligands with protein was done using AutoDock Vina and AutoDock Tools, respectively.

| S.No. | Compound | AutoDock (kcal/mol) | Vina | Tools | H Bond interactions |
|-------|----------|---------------------|------|-------|--------------------|
| 1     | TBHP     | ~3.4                | ~4.6 | ~5.2  | Thr52, Thr54, Arg132, and Asp158 |
| 2     | B2AI     | ~6.4                | ~6.0 | ~6.2  | Thr52, Lys121, Arg132, Thr157, and Asp158 |
| 3     | BLV      | ~6.2                | ~6.4 | ~6.4  | Thr52, Thr54, Lys121, Arg132, and Asp158 |
| 4     | 1BAC     | ~6.0                | ~6.4 | ~6.4  | Thr52, Lys121, Arg132, and Asp158 |
| 5     | BDLA     | ~5.5                | ~6.4 | ~6.4  | Thr52, Lys121, Arg132, and Asp158 |
inhibitor(s) complexes. Hydrogen bond plots showed that a minimum of 3, 8, 4, 5, and 5 hydrogen bonds stabilized the Prx-TBHP, Prx-B2AI, Prx-BLV, Prx-1BAC, and Prx-BDLA complex, respectively, as shown in Figure 8. Prx-TBHP complex displayed 3 to 5 hydrogen bonds throughout the molecular simulation (Figure 8A). It can be clearly seen that Prx-B2AI complex exhibited the highest number of hydrogen bonds than other Prx-inhibitor(s) complexes (Figure 8B). Prx-BLV complex sustained intermolecular hydrogen bonds in the range of 4 to 6 (Figure 8C). Similarly, Prx-1BAC complex maintained hydrogen bonds in the range of 5 to 8 (Figure 8D). From Figure 8E, it can be clearly observed that the hydrogen bonds in the range of 4 to 6 stabilized the Prx-BDLA complex.

Figure 1. Molecular docking results of Prx with TBHP and inhibitors. The protein is represented in the cartoon in white color and interacting residues of the protein are shown in the stick in green color. The ligands are represented in ball and stick format: A) TBHP (cyan), B) B2AI (pink), C) BLV (orange), D) 1BAC (yellow), and E) BDLA (magenta).
Overall, hydrogen bond results confirmed that the binding of inhibitor(s) to Prx resulted in the formation of the higher stable Prx-inhibitor(s) complexes than Prx-TBHP complex. Further, the superposition of Prx-TBHP and Prx-inhibitor(s) complexes at different times (0, 20, 40, 60, 80, and 100 ns) of molecular dynamics remained stable and

Figure 2. Molecular electrostatic potential (MEP) plots of TBHP (A), B2AI (B), BLV (C), 1BAC (D), and BDLA (E) generated by 6-311G (d,p) basis set along with DFT/B3LYP in Gaussian 16. The electron-rich and poor regions are shown in colors of RED < ORANGE < YELLOW < GREEN < BLUE.
Figure 3. HOMO and LUMO plots of TBHP (A, B), B2AI (C, D), BLV (E, F), 1BAC (G, H), and BDLA (I, J) predicted by 6-311 G (d,p) basis set along with DFT/B3LYP in Gaussian 16.
confirmed that ligand(s) bound Prx complexes sustained the interactions with the protein (Figure S6).

Essential dynamics was done to understand the motion of Prx-TBHP and Prx-inhibitor(s) complexes. Principal component analysis (PCA) depicts the dynamics differences along 456 eigenvectors for Prx-ligand(s) complexes. From Figure 9A, it can be clearly seen that the first ten eigenvectors cause 76.31%, 62.21%, 65.32%, 70.75%, and 71.27% eigenvalues for Prx-TBHP, Prx-B2AI, Prx-BLV, Prx-1BAC, and Prx-BDLA complexes, respectively. The Prx-inhibitor(s) complexes revealed very few motions than the Prx-TBHP complex, while the Prx-B2AI complex sustained the least motion among all the identified compounds. Prx-TBHP, Prx-B2AI, Prx-BLV, Prx-1BAC, and Prx-BDLA complexes showed the 50.4%, 28.8%, 30.8%, 38.7%, and 41.5% eigenvalues for first two eigenvectors. Therefore, two-directional movements along the first eigenvector (PC1) and second eigenvector (PC2) were generated and assessed, as shown in Figure 9B. Overall, PCA

Table 2. Density functional theory (DFT) analysis to predict the chemical reactive properties such as HOMO, LUMO, energy gap, electronegativity, hardness, and softness of ligands.

| S. No. | Compound | HOMO (eV) | LUMO (eV) | Energy Gap (eV) | Electro Negativity (eV) | Hardness (eV) | Softness (eV) |
|--------|----------|-----------|-----------|-----------------|-------------------------|---------------|---------------|
| 1      | TBHP     | −6.41     | −4.88     | 1.53            | 5.64                    | 0.76          | 1.31          |
| 2      | B2AI     | −7.77     | −4.64     | 3.13            | 6.20                    | 1.56          | 0.64          |
| 3      | BLV      | −7.58     | −4.73     | 2.85            | 6.12                    | 1.42          | 0.70          |
| 4      | 1BAC     | −7.63     | −4.84     | 2.79            | 6.23                    | 1.39          | 0.72          |
| 5      | BDLA     | −7.31     | −4.65     | 2.66            | 5.98                    | 1.33          | 0.75          |

Table 3. Average values of RMSD (protein and ligand), radius of gyration (Rg), solvent accessible surface area (SASA), and intra-H bond numbers of Prx-TBHP and Prx-inhibitor(s) complexes for the duration of 100 ns.

| S. No. | Compounds | Protein | Ligand | Rg (nm) | SASA (nm) | Intra-H bond numbers |
|--------|-----------|---------|--------|---------|-----------|----------------------|
| 1      | TBHP      | 0.30 ± 0.03 | 0.12 ± 0.01 | 1.52 ± 0.01 | 90.35 ± 1.93 | 107.4 ± 5.4          |
| 2      | B2AI      | 0.26 ± 0.02 | 0.07 ± 0.01 | 1.47 ± 0.01 | 85.70 ± 2.25 | 114.0 ± 5.2          |
| 3      | BLV       | 0.27 ± 0.02 | 0.08 ± 0.01 | 1.49 ± 0.01 | 86.08 ± 2.32 | 112.9 ± 5.3          |
| 4      | 1BAC      | 0.27 ± 0.02 | 0.08 ± 0.01 | 1.49 ± 0.01 | 87.33 ± 2.05 | 110.8 ± 5.3          |
| 5      | BDLA      | 0.28 ± 0.03 | 0.09 ± 0.01 | 1.50 ± 0.01 | 88.70 ± 2.08 | 109.3 ± 5.6          |

Figure 4. Root mean square deviation (RMSD) of protein-ligand(s) complexes and ligand(s) for the molecular dynamics of 100 ns done by using GROMOS96 53a6 force field in GROMACS. A) RMSD plots for Prx-TBHP (black), Prx-B2AI (red), Prx-BLV (green), Prx-1BAC (blue), and Prx-BDLA (pink) complex. B) Ligand RMSD: TBHP (black), B2AI (red), BLV (green), 1BAC (blue), and BDLA (pink).
results confirmed that the binding of inhibitor(s) to Prx formed the stable Prx-inhibitor(s) complexes. While Prx-B2AI complex was most stable among all Prx-inhibitor(s) complex as it sustained the least directional movements.

3.6. MMPBSA binding free energy calculations

MMPBSA binding free energy was calculated from the trajectories extracted from the last 20 ns of molecular dynamics. The binding affinity of Prx-TBHP, Prx-B2AI, Prx-BLV, Prx-1BAC, and Prx-BDLA complexes were $-64.12 \pm 5.49$, $-126.75 \pm 9.54$, $-117.81 \pm 4.04$, $-108.27 \pm 6.88$, and $-96.84 \pm 6.64$ kJ/mol, as shown in Table 4. The contribution of important residues of Prx in the binding of ligands was quantified using MMPBSA. The per residue interactions profile of Pro48, Thr52, Cys55, Lys121, Arg132, and Asp158 were calculated. The binding affinity of actively participated residues of Prx for the binding of inhibitor(s) was higher as compared to TBHP, as shown in Table S3. Overall, MMPBSA results confirmed that binding of inhibitor(s) with Prx results in the formation of lower energy stable Prx-inhibitor(s) complexes as compared to Prx-TBHP complex.

4. Discussion

CLas is a causative agent of citrus greening plant diseases, which can cause spotty leaves, irregular shaped fruits, distorted growth, and plant death (Bové, 2006). The only strategy that can be used to prevent psyllids by chemical treatment or removal of the whole plant (Boina & Bloomquist, 2015). The transmission of CLas induces the level of peroxide and reactive species in the host plant (Li et al., 2009). ROS and peroxides are known to be involved in the inhibition of the growth of bacteria due to damage of biomolecules like nucleic acids and proteins (Birben et al., 2012). Antioxidants defense proteins like superoxide dismutase, DT-diaphorase, catalase, and peroxiredoxin (Prx), etc., reduce oxidative stress and protect the bacteria (Ighodaro & Akinloye, 2018; Nandi et al., 2019; Rhee, 2016). In CLas, Prx play a pivotal role in reduction of peroxide level and scavenging ROS.

Several studies have characterized and identified novel potent molecules against several drug targets from Candidatus Liberibacter asiaticus (CLas) (Kumar, Dalal, Kokane, et al., 2020; Kumar, Dalal, Sharma, et al., 2020; Saini et al., 2018, 2019, 2021). Here, in this study, the three-dimensional structure of Prx was predicted for structure-based
virtual screening. The predicted model was validated by the Ramachandran plot generated by PROCHECK and MolProbity. Further, VERIFY-3D, ProSA, and ProQ results confirmed that the model is reliable. The fragments library was downloaded from selleckchem and converted into pdbqt. The virtual screening of ligands against Prx was performed using AutoDock Vina in PyRx. Four molecules (B2AI, BLV, 1BAC, and BDLA) showed higher binding affinity than TBHP were considered. All the identified molecules satisfied the Lipinski rule of five criteria. Further, the molecular docking results affirmed that all four molecules exhibited higher binding affinities than TBHP. B2AI showed the highest binding affinity among all the identified molecules and exhibits hydrogen bonds with Thr52, Lys121, Arg132, Thr157, and Asp158 of Prx. Prx-BLV, Prx-1BAC, and Prx-BDLA complexes were stabilized due to hydrogen bonds contributed by Thr52, Lys121,
Arg132, and Asp158. HOMO and LUMO energy gap results predicted by DFT/B3LYP suggested that identified molecules are reactive in nature.

Molecular dynamics simulation was employed to evaluate the stability of Prx-TBHP and Prx-inhibitor(s) complexes. The lesser RMSD values of Prx-inhibitor(s) complexes than Prx-TBHP complex suggested that binding of inhibitors with Prx tend to form the stable protein-ligand complexes. The Rg results illustrated that Prx-inhibitor(s) complexes were in stable conformations. SASA results indicated that Prx-inhibitor(s) complexes were more stable than Prx-TBHP complex. The presence of a higher number of hydrogen bonds in Prx-inhibitor(s) complexes than the Prx-TBHP complex also affirmed the outcomes of RMSD, Rg, and SASA results. PCA results revealed that inhibitors formed higher stable Prx-inhibitor(s) complexes than Prx-TBHP complex. Further,

Figure 8. Inter molecular hydrogen bonds bar graphs of Prx-TBHP and Prx-inhibitor(s) complexes. A) Prx-TBHP (black), B) Prx-B2AI (red), C) Prx-BLV (green), D) Prx-1BAC (blue), and E) Prx-BDLA (pink) complexes.
MMPBSA results confirmed that identified molecules interact with Pro48, Th52, Cys55, Lys121, Arg132, and Asp158 of Prx and resulted in the formation of a lower energy Prx-inhibitor(s) complex as compared to Prx-TBHP complex.

5. Conclusion

Huanglongbing (HLB) is a devastating citrus plant disease due to Candidatus Liberibacter asiaticus (CLas). The reactive species and peroxide are necessary to inhibit the growth and development of pathogenic bacteria in plants. Prx, an antioxidant defense enzyme, reduces the level of free radicals, reactive species, and peroxides to protect the CLas. Here in this study, various computational techniques such as molecular modeling, virtual screening, quantum mechanics, molecular dynamics, and MMPBSA have been employed to identify potent molecules against Prx. The series of fragments molecules were screened against 3D model of Prx. Molecular docking results revealed that four molecules (B2AI, BLV, 1BAC, and BDLA) interacted at the active site of Prx with a higher binding affinity than TBHP. The molecular dynamics results illustrated that the binding of inhibitor(s) with Prx formed the higher stable and compact Prx-inhibitor(s) complexes than Prx-TBHP complex. Further, MMPBSA analysis confirmed that these molecules interacted with active site residues (Pro48, Th52, Cys55, Lys121, Arg132, and Asp158) of Prx and resulted in the formation of a lower energy Prx-inhibitor(s) complex than Prx-TBHP complex. Therefore, the identified compounds can be further tested and optimized to develop novel antimicrobial compounds against Candidatus Liberibacter asiaticus.

Figure 9. Principal component analysis (PCA) of Prx-TBHP (black), Prx-B2AI (red), Prx-BLV (green), Prx-1BAC (blue), and Prx-BDLA (pink) for the molecular dynamics span of 100 ns. A) Eigenvector index versus eigenvalues for the first 10 eigenvectors for protein-ligand(s) complexes. B) Representation of 2D projection of motion of protein-ligand(s) complexes for projection on eigenvector 1 (PC1) and projection on eigenvector 2 (PC2).

Table 4. Molecular Mechanics/Position-Boltzmann Surface Area (MMPBSA) binding energy (kJ/mol) of Prx-TBHP and Prx-inhibitor(s) complexes calculated from the 2000 frames retrieved from the molecular simulation of 100 ns.

| S. NO. | Compound | Van der Waals energy | Electrostatic energy | Polar solvation energy | SASA energy | Binding energy |
|--------|----------|----------------------|----------------------|-----------------------|-------------|---------------|
| 1      | TBHP     | -85.31 ± 4.22        | -74.65 ± 7.68        | 101.67 ± 8.81         | -5.83 ± 0.23 | -64.12 ± 5.49 |
| 2      | B2AI     | -105.36 ± 6.35       | -133.77 ± 11.16      | 132.74 ± 12.96        | -20.36 ± 0.74 | -126.75 ± 9.54 |
| 3      | BLV      | -108.85 ± 5.47       | -123.87 ± 11.53      | 134.22 ± 11.80        | -19.31 ± 1.18 | -117.81 ± 4.04 |
| 4      | 1BAC     | -99.09 ± 4.94        | -120.38 ± 12.59      | 131.91 ± 13.08        | -20.71 ± 1.99 | -108.27 ± 6.88 |
| 5      | BDLA     | -95.06 ± 4.71        | -117.33 ± 9.55       | 135.39 ± 9.33         | -19.84 ± 0.55 | -96.84 ± 6.64 |
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