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Brevilin A Binds with Neh1 Domain of NRF2 and Inhibits its Translocation into Nucleus in Cancer Cells

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ABSTRACT

Nuclear factor erythroid 2-related factor 2 (NRF2) is a transcriptional factor which is constitutively activated in various cancers where it confer survival advantages by transcribing cytoprotective genes involved in cancer initiation, progression, metastasis and development of drug resistance. Here in this study, we have identified and validated Brevilin A (BLN-A), a potent anticancer bioactive compound as potential NRF2 inhibitor using *in silico* and *in-vitro* studies. We predicted NRF2 structure by AlphaFold, validated it by different approaches, performed its molecular docking, and MD simulation by computational methods. Our findings showed that BLN-A binds with Neh1 (DNA-binding) domain of NRF2 through multiple interactions including hydrogen bonds and hydrophilic interactions. Molecular dynamic simulations accomplished by RMSD (root mean square deviation), RMSF (root mean sequer fluctuation) and RG (radius of gyration) exhibited the stability, flexibility and compactness of BLN-A and NRF2 complex over 100 ns. Our western blot analysis showed that BLN-A inhibited the translocation of NRF2 into nucleus in a dose-dependent manner in A549 lung cancer and Hey-T30 taxol-resistant ovarian cancer cells. Moreover, BLN-A exhibits drug likeness properties. Therefore, BLN-A could be developed into a potent NRF2 inhibitor for multiple therapeutic effects.

INTRODUCTION

Nuclear factor erythroid 2-related factor 2 (NRF2) is one of the major player in cancer initiation, progression, metastasis and multi-drug resistance development, as it is constitutively activated in various cancers (Telkoparan-Akillilar *et al*., 2021). It is a master regulator and intracellular transcriptional factor that protect cells from oxidative and electrophilic stress. A number of genes are regulated by NRF2 which encode detoxifying factors and anti-apoptotic proteins essential for cancer cell survival (Sajadimajd and Khazaei, 2018). Human NRF2 protein contains 605 amino acids and comprised of seven NRF2-ECH homology conserved (Neh1-Neh7) domains. NRF2 stability is controlled by Neh1, Neh2 and Neh6 domains. Whereas Neh3-Neh5 are transactivation domains of NRF2. Neh7 domain inhibits targeted genes transcription of NRF2 by binding with a nuclear receptor (Panieri *et al*., 2020). It is well known that cancer cells have high level of reactive oxygen species (ROS) which play essential role in cancer cell survival, proliferation and development of drug resistance (Dong *et al*., 2023). This high level of ROS in turn activates NRF2 in cancer cells (Panieri and Saso, 2019). Therefore, the inhibition of NRF2 would be highly progressive approach for development of promising anticancer drugs.

Brevilin A (BLN-A) is a sesquiterpene lactone component of *Centipeda minima* (Fig. 1). It is also a major component of Centipedae herba, a traditional Chinese medicine, which is dried whole plant of *Centipeda minima* (Su *et al*., 2020). BLN-A possesses various pharmacological activities such as anti-inflammatory, anti-viral, anti-bacterial, and anticancer (Zhang *et al*., 2019; Liu *et al*., 2022). BLN-A has been extensively studied for its anticancer mechanism in various human cancers including colon cancer (You *et al*., 2018), lung cancer (Khan *et al*., 2020), breast cancer (Saleem *et al*., 2020),

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colorectal cancer (Meng *et al*., 2023), and glioblastoma (Wang *et al*., 2018). Moreover, BLN-A has been reported to overcome taxol resistance in lung cancer cells (Ding *et al*., 2022). Although BLN-A exhibited anticancer activity through multiple mechanisms in various cancer, yet ROS generation has been reported to be the primary event in induction of apoptosis.

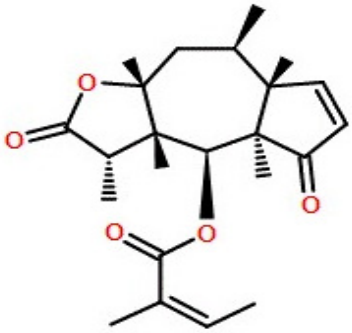

Fig. 1. Chemical structure of Brevilin A.

Keeping in view the diverse biological and pharmacological activities of BLN-A, especially ROS inducing activity, the present study was conducted to evaluate the inhibitory effect of BLN-A against ROS responsive transcriptional factor NRF2.

In the present study, we predicted NRF2 structure and found its inhibition by potential bioavctive compound BLN-A for the first time by *in silico* and *in-vitro* studies along with drug likeness.

MATERIALS AND METHODS

Cell lines and reagents

A549 and Hey-T30 cancer cell line were purchased from ATCC, USA. BLN-A was purchased from Tauto Biotech. Co., Ltd. China. All reagents required for cell culture and western blot were purchased from Research Time Scientific. Antibodies were purchased from Affinity Biosciences, China.

NRF2 structure modelling and validation

NRF2 crystal structure having complete amino acid residues in any source organism has not been published yet. In human beings, it is comprised of 605 amino acid residues (Lee and Hu, 2020). A short peptide of 16 amino acid residues of NRF2 is bound with highly conserved region of KEAP1 crystal structures (PDB ID: 4IFL, 2FLU) by multiple hydrogen bonds (Madden and Itzhaki, 2020).

AlphaFold is an artificial intelligence algorithm accessed on Nov, 2021 by DeepMind in collaboration with EMBL's European Bioinformatics Institute. It predicts the three-dimensional protein structure with unprecedented accuracy from its amino acid residues sequence based on convolutional neural network and gradient descent optimization (Jumper *et al*., 2021). We got the 605 amino acid sequence of human NF2L2 gene (ID: Q16236) from UNIPROT Database and submitted to AlphaFold (https://alphafold.ebi.ac.uk/) for NRF2 3D structure prediction. Sometimes the predicted structure contains unreliable regions with non-consistent templates. So, we refined the predicted structure by a free accessed GalaxyWEB server (https://galaxy.seoklab.org/) (Lee *et al*., 2019).

To verify these predicted structures, we performed Ramachandran plot from PROCHECK. To further verify it, we superimposed a short experimental NRF2 peptide (PDB ID: 2FLU) with AlphaFold's predicted structure to check its accuracy. In addition, we performed its protein-protein docking with KEAP1 of (2FLU) after removing NRF2 short peptide (16 amino acids already complexed with its structure) by online server ClusPro (https://cluspro.org) to check either it bind with NRF2 binding region of KEAP1 or not. As AlphaFold is best and strong database for homology modeling of 3D protein structure based on trained neural network. It gives >90% accuracy and revolutionized in the field of *in silico* drug designing (Adelusi *et al*., 2022). So, we proceeded our further studies of molecular docking and MD simulation by using AlphaFold predicted structure.

Molecular docking

BLN-A was obtained in 3-dimonsional sdf format from PubChem Database, a free online server for chemical compounds. It was converted into pdbqt format by adding Gasteiger charges merged with non-polar hydrogens and rotatable bonds as explained by our previous study (Gul *et al*., 2024).

Three-dimensional structure of NRF2 was modeled by AlphaFold protein structure Database in pdb format (AF-Q16236-F1). MGL tools were used to prepare protein for docking. Pdbqt structure of protein for molecular docking was prepared by removing all water molecules and adding polar hydrogens to compute proper protonation of targeted residues and energetically optimized the protein structure. Kollman united atom charges were assigned to the protein. All these changes were made to convert protein into PDBQT format as described by us previously (Gul *et al*., 2024).

Protein and ligand were converted into pdbqt format as an input for molecular docking. The grid positioned to cover whole protein in the box with 126x118.1x128.47 Å sizes for x, y and z axis dimensions respectively and 8 exhaustiveness and molecular docking was performed by

AutoDock Tools (Bamidele *et al*., 2020). For visualization, ligand interactions with protein amino acid residues, Biovia Discovery studio visualizer 2020 and LigPlot+ were used.

MD simulation

Molecular dynamics (MD) simulations was performed by GROMACS version 2023.3 to understand bimolecular interactions and conformational dynamics by mimicking controlled experimental physiological conditions and generating testable hypothesis results. Identification of potent inhibitors by molecular docking and MD simulation-based studies has been proven worthy in drug designing (Padhi *et al*., 2021).

In GROMACS, CHARMM27 force field was applied and ligand topology was generated by pdb2gmx. MD simulation was performed by defining the box in which protein is solvated by TIP3P water model. Protein get charged by adding NH3+ and COO- at protein termini. Neutralization of system was done by adding Na+ and Cl- ions. Energy minimization was performed by 50,000 steps by cutoff short range electrostatic forces. System equilibration was achieved at constant temperature (300k) and pressure of 1bar for 1000 ps (Harini *et al*., 2024; Rajguru *et al*., 2022). Finally, MD simulation analysis was performed over 100 ns and values of RMSD (root mean sequer daviation), RMSF (root mean sequer fluctuation) and Rg (radius of gyration) were calculated. Origin 2023 software was used to analyze the results of MD simulation of ligand and protein complex.

Cell culture

A549 cells were cultured in DMEM while HEY-T30 cells were cultured in RPMI-1640 culture medium supplemented with 10% FBS. The cells were maintained in CO_2 incubator at 37°C in humidified environment.

Western blot

A549 and Hey-T30 cells were cultured in 6 well plate and treated with BLN-A. After treatment, the adherent and floating cells were collected and washed with PBS (cold). Protein were extracted from control and BLN-A treated cells by RIPA buffer (Khan *et al*., 2020; Li *et al*., 2019). Protein concentrations were determined by using enhanced BCA protein Assay kit according to the given instructions. Nuclear extracts were prepared. Mixing of equal amount of 2X loading buffer at 100°C for 5 min denatured protein samples. In the final step, 40µg of these protein samples were loaded on 10% SDS-PAGE and immunoblotting was performed (Maryam *et al*., 2017)

Drug likeness profiling of BLN-A

For evaluation of pharmacokinetics properties of BLN-A, a web browser SwissADME (http://www.swissadme.ch) was used to get bioavailability radar and BOILED-EGG model of BLN-A.

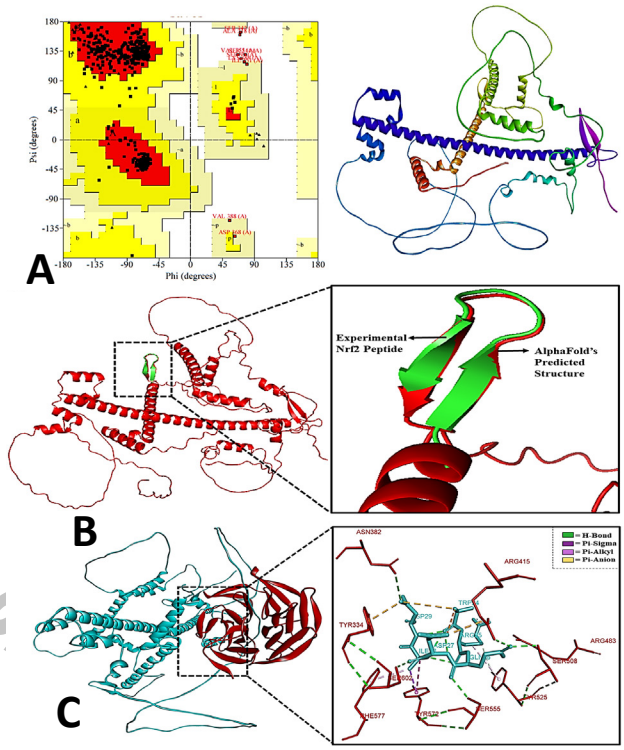

Fig. 2. NRF2 structure prediction, analysis and validation. (**A**) AlphaFold's predicted structure analysis by Ramachandran Plot revealed 95.2% residues in most favoured region energetically (**B**) Significant structural alignment of predicted NRF2 complete structure with experimental partial KEAP1 binding domain of NRF2 (RMSD=0.39Å) for validation (**C**) Protein-protein docking of predicted NRF2 structure with KEAP1 (2FLU) confirmed its efficient binding with NRF2 binding domain of KEAP1.

RESULTS

NRF2 structure modeling and validation

We predicted NRF2 structure (UniProt ID: AF-Q16236-F) from amino acids sequence by artificial intelligence based program, AlphaFold is shown in Figure 2A. GalaxyWEB server corrected all un-reliable residues and angles. Ramachandran Plot by PROCHECK verified that 95.2% residues lies in most favorable regions. Further verification showed that a short experimental NRF2 peptide superimposed with AlphaFold's predicted structure with 0.39Å RMSD value as shown in Figure 2B and showed its accuracy. Moreover, its protein-protein docking with

pure KEAP1 by ClusPro showed that it's KEAP1 binding domain firmly bind with NRF2 binding region of KEAP1 via several hydrogen bonds as shown in Figure 2C. All these verifications enabled us to further proceed our *in silico* study with this predicted NRF2 structure.

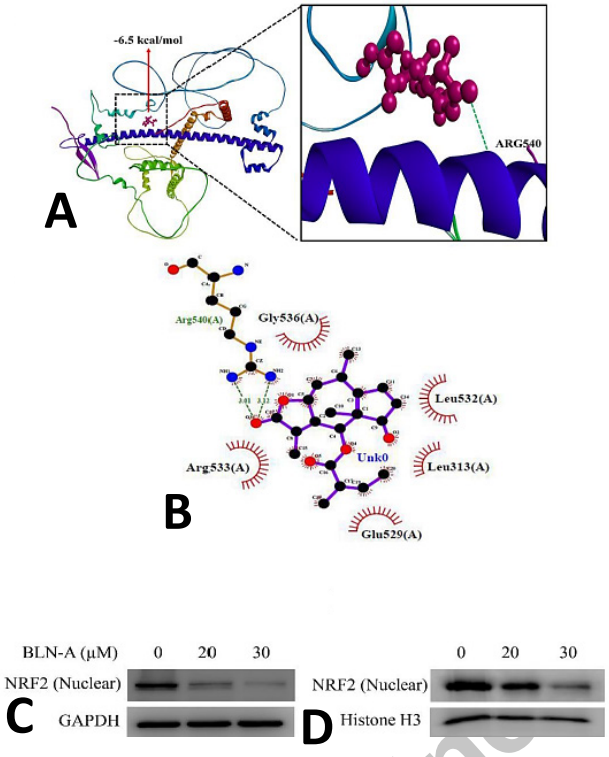

Fig. 3. BLN-A is predicted to bind with NRF2 and inhibits its nuclear translocation. (**A**) Molecular docking studies showed that BLN-A bind with DNA- binding domain of NRF2 by H-bond. (**B**) 2D- representation obtained by Ligplot+ revealed hydrogen bonding and other hydrophilic interactions among BLN-A and NRF2. (**C**) NFR2 expression in nuclear extract of BLN-A treated A549 cells. (D) NRF2 expression in nuclear extracts of BLN-A treated Hey-T30 cells.

Molecular docking of BLN-A with NRF2 and intermolecular interactions

While investigation of any potential drug candidate, analysis of its binding affinity and interaction with protein has prime importance. Molecular docking is an *in silico* technique for analyzing docking based molecular interactions (Ilyas *et al*., 2022). In our study, we performed molecular docking of BLN-A as a ligand with NRF2 protein to determine its inhibition potential against NRF2 as novel drug candidate. Results showed that BLN-A docked with NRF2 by -6.5 kcal/mol, binding energy. Two and three dimensional prediction of docked BLN-A and NRF2 in Figure 3 depictes that BLN-A binds with amino acid residues of NRF2 with hydrogen and multiple hydrophobic interactions. Figure 3A showed that BLN-A form one hydrogen bond with ARG540 which lies in DNA binding domain of NRF2. While 2D- visualization by Ligplot+ in Figure 3B showed that BLN-A forms two hydrogen bonds with same amino acid ARG540 by 3.01 and 3.32Å bond lengths at different NH2 termini and hydrophobic interaction with GLU529, LEU532, ARG 533 and LEU313 amino acid residues of NRF2 protein.

BLN-A inhibits NRF2 nuclear translocation

Since BLN-A has been predicted to bind with Neh1 domain of NRF2, we were interested to see if BLN-A really inhibits NRF2 activation. For this we treated HEY-T30 and A549 cancer cells with BLN-A for 24 h and evaluated the expression of NRF2 in nuclear extracts by immunoblotting. Our data showed that 20 and 30μM concentration of BLN-A inhibited the nuclear translocation of NRF2 in A549 and hey-T30 cells as shown in Figure 3C, D, respectively. Thus, our *in-vitro* findings are in line with *in silico* findings.

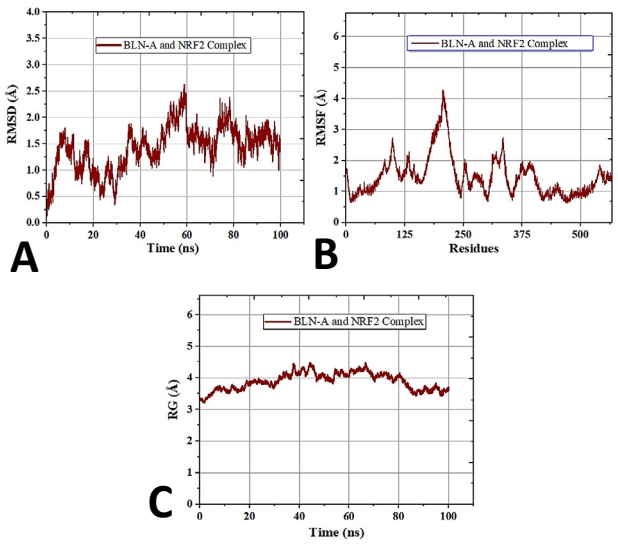

Fig. 4. MD simulation analysis of BLN-A and NRF2 complex: (**A**) RMSD, (**B**) RMSF, (**C**) RG values remained within optimal range depicting stability and compactness of BLN-A and NRF2 complex over 100ns.

Molecular dynamic simulations

After molecular docking, the binding stability and dynamic characteristics of BLN-A and NRF2 protein were investigated by MD simulation over 100 ns based on evaluation criteria such as RMSD, RMSG and RG values. Figure 4A demonstrating the dynamics of RMSD values of NRF2-BLN-A complex. The average deviations observed

over 100 ns was 1.42Å. The maximum deviation of RMSD values was about 2.58 Å at 59 ns while the minimum value was 0.3 Å at 29 ns. Very little to and fro deviations were observed with no steep change. Hence, throughout the 100 ns time duration, the deviations sustained between 1 Å to 2 Å indicating the significant stability of the complex.

Figure 4B illustrates the conformational fluctuations of the complex by RMSF values. The average RMSF values observed over 100 ns was 1.48 Å throughout 100 ns time period. A steep high value of RMSF was observes at TYR 206 amino acid, while the minimum at LEU471 residue. Besides this steep high value, the remaining trend remain equilibrated over 100 ns. Similarly, Figure 4C exhibiting the compactness of protein in complex by RG values. The average RG value observed was 3.8 Å over 100 ns time. But no steep high or low values were observed over 100 ns. These variations remains between 3.2 to 4 Å throughout the simulation time duration with significant smooth trend. The results of MD simulations over 100 ns showed that BLN-A and NRF2 complex is significantly stable, flexible and compact.

Toxicological profiling and drug-ability of BLN-A

Two key ADMET parameters including blood brain barrier and gastrointestinal absorption were predicted by boiled-egg model through SwissADME online server. Figure 5A is indicating that BLN-A has accessibility and absorption in both gastrointestinal tract and blood brain barrier as it is inside the egg-yolk region. While the red dot (-PGP) is indicating that BLN-A cannot be easily effluxed from central nervous system by P-glycoprotein. The bioavailability radar in Figure 5B is indicating the excellent oral administration of BLN-A as its molecular weight, lipophilicity, flexibility and polarity were in optimum range (pink area).

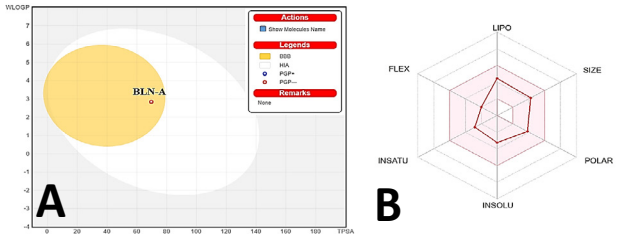

Fig. 5. Boiled-egg modal and bioavailability radar of BLN-A: (**A**) Represents that BLN-A accessibility to blood brain barrier (BBB) and gastrointestinal adsorption. (**B**) Bioavailability radar showing LIPO (Lipophilicity), FLEX (flexibility), INSATU (in saturation), INSOLU (insolubility), POLAR (polarity), and SIZE (molecular weight) properties of BLN-A are within optimum range (Pink region).

DISCUSSION

NRF2 is a transcriptional factor that transcribe multiple genes involve in cancer (Telkoparan-Akillilar *et al*., 2021). In current study, we modeled NRF2 structure and identified BLN-A as a promising NRF2 inhibitor for the first time. This was achieved by performing *in silico* and *in-vitro* studies. For that purpose, we predicted three-dimensional structure of NRF2 by AlphaFold (Liu *et al*., 2022). The predicted structure was verified by Ramachandran plot which gives its structural assessment. It showed its excellent quality with high confidence scores (Saikat *et al*., 2020). Further verification by superimposing the experimental NRF2 peptide with predicted NRF2 structure was done with RMSD value 0.39 Å which is very less than standard value 2 Å (Saba *et al*., 2022). Similarly, protein-protein docking of NRF2 and KEAP1showed that both protein docked with their respective domains via multiple hydrogen bonds (Ooi *et al*., 2018). Finally, these validations results made this predicted NRF2 structure suitable for further studies.

Molecular docking was performed between BLN-A as a ligands and NRF2 as protein. As NRF2 is transcription factor and binds to DNA to transcribe multiple oncogenes and multidrug resistance genes. Neh1 is DNA binding domain of NRF2 composed of amino acids range from 434 to 561 (Liu *et al*., 2021). Our molecular docking data showed that BLN-A binds with ARG540, GLU529, LEU532, and ARG 533 amino acids residues indicating that BLN-A interacts with DNA binding domain of NRF2 protein by hydrogen bonds and hydrophobic interactions to inhibit its transcriptional activity (Ulasov *et al*., 2022). MD simulation findings confirmed the stability, flexibility and compactness of docked BLN-A and NRF2 complex by RMSD, RMSF and RG values. We found 1.42Å, 1.48Å average RMSD, RMSF values of complex respectively, over 100ns. These average RMSD and RMSF values of complex were less than the standard optimal range ~3Å and indicates its structural and conformational stability and flexible alignment (Saba *et al*., 2022). Lesser the values of RMSD and RMSF, greater will be the stability and flexibility of complex (Martínez, 2015). Similarly, smooth and equilibrated trend of RG scores was observed which indicates the compactness of complex by stable folding and unfolding dynamics over 100ns (Gul *et al*., 2024).

As natural bioactive compounds are prime source of anti-cancer drug discovery. A number of natural compounds such as luteolin, brusatol, brucein D and apigenin have been investigated to inhibit NRF2 and regulate therapeutic response of anticancer drugs (Zhang *et al*., 2023). Similarly, we found in our *in-vitro*

investigation by western blot that BLN-A inhibited the nuclear translocation of NRF2 in taxol resistant cell line of ovarian cancer (HEY-T30 cells) and A549 cells so that its transcriptional activity to transcribe anti-oxidant enzymes can be suppressed. As anti-oxidant enzymes provide chemoprevention to cancer cells by elevated ROS level. Inhibition of NRF2 can increase ROS above the threshold level causing ROS-mediated death of cancer cells (Xue *et al*., 2020).

Principle screening step of drug likeness in drug discovery is important to reduce the efforts in empirical experimentation and enhance the success rate. This can be accomplished by monitoring the physicochemical and pharmacokinetics properties of candidate drug in the human body that have an impact on oral bioavailability (Abdullahi *et al*., 2022). Our research team member has already reported some of these properties of BLN-A for drug screening (Rizwana *et al*., 2023). As lipophilicity, solubility and intestinal absorption are correlated for oral bioavailability of any drugs. BLN-A has shown highest values of intestinal absorption with excellent lipophilicity and solubility (Lagorce *et al*., 2017). These results were found by BOILED-EGG model and drug bioavailability radar (Olasupo *et al*., 2020). Hence, the results of our findings suggests that BLN-A can be a promising NRF2 inhibitor (Fig. 6).

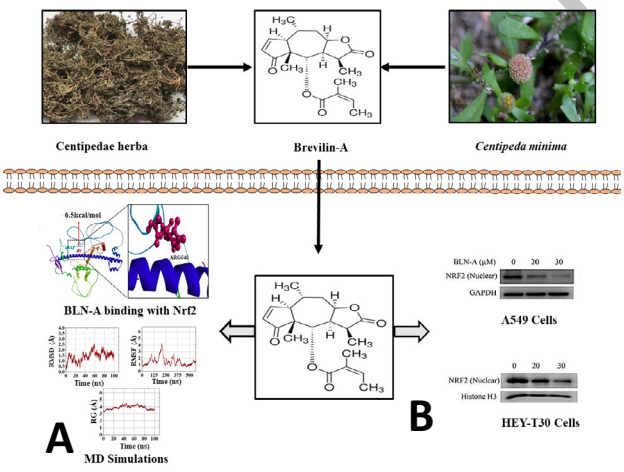

Fig. 6. A schematic representation of BLN-A-induced NRF2 inhibition. (**A**) BLN-A binds with DNA-binding domain of NRF2 and form stable complex validated by MD simulation studies. (**B**) BLN-A significantly suppress nuclear translocation of NRF2.

CONCLUSION

In conclusion, we have shown that BLN-A is a promising NRF2 inhibitor and exhibits good drug-likeness properties. The BLN-A mediated NRF2 inhibition has been found to be associated with direct binding of BLN-A with Neh1 or DNA binding domain as evident from molecular docking data and inhibition of its nuclear translocation as evident from Western blot data. Further *in-vitro* and *in-vivo* investigations are required to validate and develop BLN-A into a potent NRF2 inhibitor.

DECLARATIONS

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Statement of conflict of interests

The authors have declared no conflict of interest.

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