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# Inhibition of the NEMO/IKK $\beta$ association complex formation, a novel mechanism associated with the NF- $\kappa$ B activation suppression by *Withania somnifera*'s key metabolite withaferin A

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## Abstract

**Background:** Nuclear Factor kappa B (NF- $\kappa$ B) is a transcription factor involved in the regulation of cell signaling responses and is a key regulator of cellular processes involved in the immune response, differentiation, cell proliferation, and apoptosis. The constitutive activation of NF- $\kappa$ B contributes to multiple cellular outcomes and pathophysiological conditions such as rheumatoid arthritis, asthma, inflammatory bowel disease, AIDS and cancer. Thus there lies a huge therapeutic potential beneath inhibition of NF- $\kappa$ B signalling pathway for reducing these chronic ailments. *Withania somnifera*, a reputed herb in ayurvedic medicine, comprises a large number of steroidal lactones known as withanolides which show plethora of pharmacological activities like anti-inflammatory, antitumor, antibacterial, antioxidant, anticonvulsive, and immunosuppressive. Though a few studies have been reported depicting the effect of WA (withaferin A) on suppression of NF- $\kappa$ B activation, the mechanism behind this is still eluding the researchers. The study conducted here is an attempt to explore NF- $\kappa$ B signalling pathway modulating capability of *Withania somnifera*'s major constituent WA and to elucidate its possible mode of action using molecular docking and molecular dynamics simulations studies.

**Results:** Formation of active IKK (I $\kappa$ B kinase) complex comprising NEMO (NF- $\kappa$ B Essential Modulator) and IKK $\beta$  subunits is one of the essential steps for NF- $\kappa$ B signalling pathway, non-assembly of which can lead to prevention of the above mentioned vulnerable disorders. As observed from our semi-flexible docking analysis, WA forms strong intermolecular interactions with the NEMO chains thus building steric as well as thermodynamic barriers to the incoming IKK $\beta$  subunits, which in turn pave way to naive complex formation capability of NEMO with IKK $\beta$ . Docking of WA into active NEMO/IKK $\beta$  complex using flexible docking in which key residues of the complex were kept flexible also suggest the disruption of the active complex. Thus the molecular docking analysis of WA into NEMO and active NEMO/IKK $\beta$  complex conducted in this study provides significant evidence in support of the proposed mechanism of NF- $\kappa$ B activation suppression by inhibition or disruption of active NEMO/IKK $\beta$  complex formation being accounted by non-assembly of the catalytically active NEMO/IKK $\beta$  complex. Results from the molecular dynamics simulations in water show that the trajectories of the native protein and the protein complexed with WA are stable over a considerably long time period of 2.6 ns.

**Conclusions:** NF- $\kappa$ B is one of the most attractive topics in current biological, biochemical, and pharmacological research, and in the recent years the number of studies focusing on its inhibition/regulation has increased

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manifolds. Small ligands (both natural and synthetic) are gaining particular attention in this context. Our computational analysis provided a rationalization of the ability of naturally occurring withaferin A to alter the NF- $\kappa$ B signalling pathway along with its proposed mode of inhibition of the pathway. The absence of active IKK multisubunit complex would prevent degradation of I $\kappa$ B proteins, as the I $\kappa$ B proteins would not get phosphorylated by IKK. This would ultimately lead to non-release of NF- $\kappa$ B and its further translocation to the nucleus thus arresting its nefarious acts. Conclusively our results strongly suggest that withaferin A is a potent anticancer agent as ascertained by its potent NF- $\kappa$ B modulating capability. Moreover the present MD simulations made clear the dynamic structural stability of NEMO/IKK $\beta$  in complex with the drug WA, together with the inhibitory mechanism.

## Background

NF- $\kappa$ B (Nuclear Factor kappa B) is a ubiquitous transcription factor involved in the regulation of cell signalling responses. It is a key regulator of cellular processes involved in the immune response, differentiation, cell proliferation, and apoptosis [1,2]. NF- $\kappa$ B is secreted predominantly in cytoplasm in the form of an inactive complex with I $\kappa$ B inhibitor proteins. Binding to I $\kappa$ B (Inhibitor of kappa B) prevents NF- $\kappa$ B:I $\kappa$ B complex from translocating to the nucleus, thereby maintaining NF- $\kappa$ B in an inactive state. NF- $\kappa$ B signalling is generally considered to occur through NF- $\kappa$ B activation being initiated by stimuli like proinflammatory cytokine TNF (tumor necrosis factor) alpha and bacterial lipopolysaccharide (LPS). Signalling pathways lead to activation of the beta subunit of the IKK (I $\kappa$ B kinase) complex, which then phosphorylates I $\kappa$ B proteins leading to their degradation and subsequent release of NF- $\kappa$ B. The freed NF- $\kappa$ B dimers translocate to the nucleus where it binds to the target genes. The constitutive activation of NF- $\kappa$ B contributes to multiple cellular outcomes and pathophysiological conditions such as rheumatoid arthritis, asthma, inflammatory bowel disease [3], AIDS [4] and cancer [5]. Thus there lies a huge therapeutic potential beneath inhibition of NF- $\kappa$ B signalling pathway for reducing menace of these chronic ailments [6].

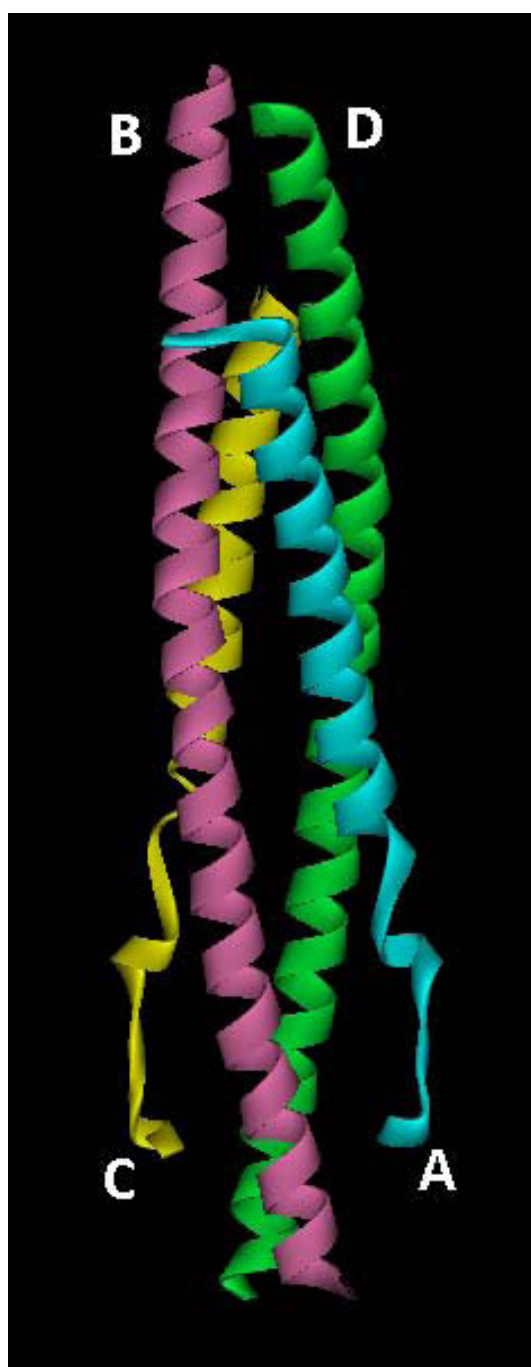
Degradation of I $\kappa$ B is a tightly regulated event that is initiated upon specific phosphorylation by activated IKK. IKK is a multisubunit complex that contains two kinase subunits, IKK $\alpha$  (IKK1) and IKK $\beta$  (IKK2), and a regulatory subunit, NEMO (NF- $\kappa$ B Essential Modulator) or IKK $\gamma$  [7]. In the classical NF- $\kappa$ B signalling pathway, IKK $\beta$  is both necessary and sufficient for phosphorylation of I $\kappa$ B $\alpha$  on Ser 32 and Ser 36, and I $\kappa$ B $\beta$  on Ser 19 and Ser 23. Thus inhibition of NEMO/IKK $\beta$  complex assembly by employment of small molecule inhibitors can offer a modest mode of inhibition of NF- $\kappa$ B activation while providing additional favors of oral administration and decreased immunogenicity.

*Withania somnifera*, also known as “ashwagandha”, “Queen of Ayurveda”, “Indian ginseng”, and “winter cherry”, has been an important herb in the Ayurvedic

and indigenous medical systems for more than 3,000 years [8]. Its roots have been used as herb remedy to treat a variety of ailments and to promote general wellness. It has received much attention in recent years due to the presence of a large number of alkaloids and steroidal lactones known as withanolides [9,10]. Many of Ashwagandha's pharmacological activities have been attributed to two primary withanolides: withaferin A (WA) and withanolide D. The principal withanolide in the Indian variety of the plant is WA. This drug is known to have anti-inflammatory [11], antitumor [12], antibacterial [13], antioxidant [14], anticonvulsive [15], and immunosuppressive properties [16]. It has the potential to increase tumor sensitization to radiation and chemotherapy while reducing some of the most common side effects of these conventional therapies [17]. Long-term effects of *W. somnifera* on adjuvant-induced arthritis in rats have also been reported [18]. Most recently, these were shown to potentiate apoptosis of tumor cells by suppression of NF- $\kappa$ B activation [19-21], protect against UV-induced skin cancer [22] and enhance neurite regeneration and memory [23,24]. Thus, many studies have been reported depicting the effect of WA on suppression of NF- $\kappa$ B activation, but the mechanism behind this effect is still eluding the researchers. The study conducted here is an attempt to elucidate a possible mode of action of *Withania somnifera*'s major constituent WA on NF- $\kappa$ B signalling pathway using molecular docking studies.

## Structural aspects of NEMO/IKK $\beta$ association domain

The structural features of the receptor macromolecule [PDB: 3BRV] have been described in detail elsewhere [25] by the depositors of the crystal structure to the Protein Data Bank. Briefly, the protein is a 4-helix bundle of NEMO and IKK $\beta$  domains each consisting of two chains B, D and A, C respectively as shown in Figure 1. NEMO density extends from residues 49 to 109 in chain B and from 49 to 109 in chain D. The IKK peptide density extends from residues 705 to 743 in chain A and from residues 701-744 in chain C. N and C termini of NEMO chains B and D form dimerization



**Figure 1** Ribbon structure of NEMO/IKK $\beta$  complex. Chains B (pink) and D (green) constitutes the NEMO domain while chains A (cyan) and C (yellow) represent secondary structure of IKK $\beta$ .

patches on the ends where the two IKK peptides attach themselves. The IKK peptides are almost helical except for an unwound stretch ranging from 732 to 742. The interactions between side chain tryptophans and main chain amides cause constriction in the backbone resulting in this unwinding region. Each of the IKK $\beta$

peptide chain associate loosely with the corresponding NEMO chains at their N-terminus but they are stringently attached to the two NEMO chains at the C-terminus. The authors have assigned three regions within the IKK $\beta$  peptide, designated as helical (705–731), linker (732–736), and the NBD (NEMO Binding Domain) (737–742). Residues 85–101 of dimeric NEMO form a flat slit paving way to two broad and extensive IKK-binding pockets; each pocket being occupied by the IKK peptide linker and the NBD. The IKK peptide forms intermolecular hydrogen-bond interactions (Ser85:Q730 and Glu89:S733) with NEMO in the NEMO's specificity pocket. Three large IKK side chains inside the NEMO pocket which form consolidated intermolecular hydrophobic interactions (Leu93:F734, Phe92:T735, Met94:F734, Phe97:W739, Ala100:W741, and Arg101:W741) are responsible for formation of NEMO-IKK $\beta$  complex.

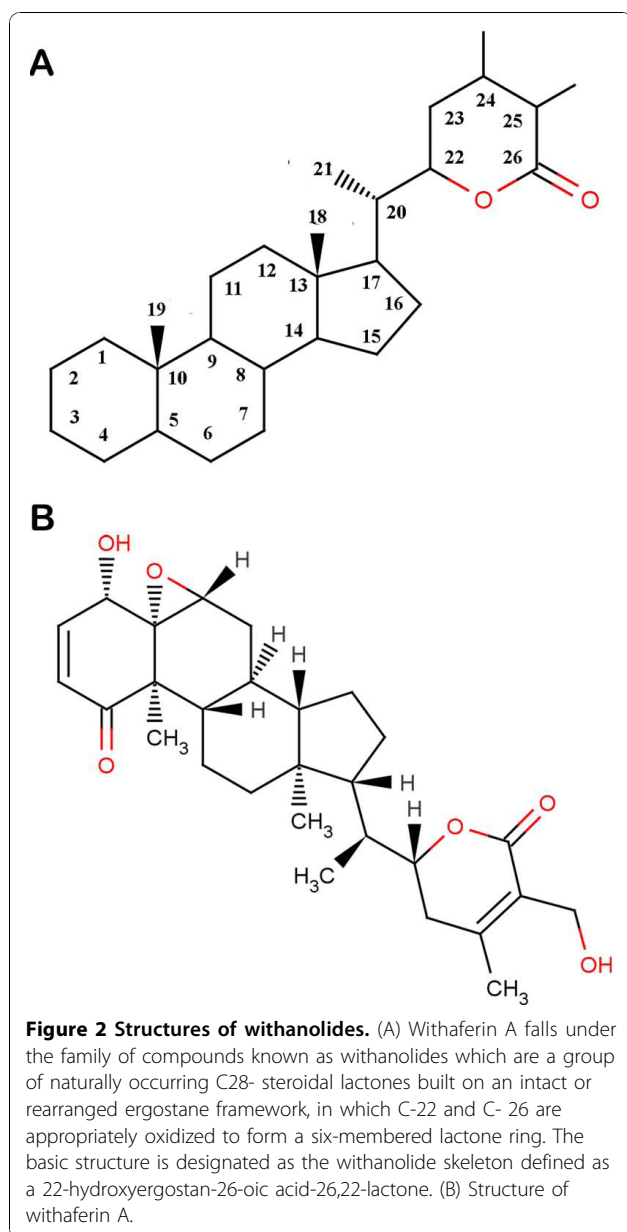
## Methods

### Ligand and receptors

The crystal structure of the NEMO/IKK $\beta$  association domain [PDB: 3BRV] was obtained from the Protein Data Bank (PDB) [26]. The crystal structure contained many missing atoms which were supplemented by the repairCommands module of AutoDock. Before docking, the protein crystal structure was cleaned by removing the water molecules. H-atoms were added to these target proteins for correct ionization and tautomeric states of amino acid residues. The modified structure so obtained was used for all the flexible docking studies while only NEMO chains were used for performing semi-flexible dockings. The ligand molecule withaferin A [PubChem:265237] was retrieved from NCBI-PubChem Compound database [27]. Figure 2 shows the basic skeleton of withanolides along with the structure withaferin A. The energy of the ligand molecule and receptors were minimized in Steepest Descent and Conjugate Gradient methods using Accelrys Discovery Studio (Version 1.7, Accelrys Software Inc.), the most comprehensive suite of modeling and simulation solutions for drug discovery available. Each of the minimization methods were carried out with CHARMM force field.

### Semi-flexible docking

AutoDock 4.0 suite was used as molecular-docking tool in order to carry out the docking simulations [28]. Several studies report the comparison of AutoDock with various docking programs. AutoDock has been found to be able to locate docking modes that are consistent with X-ray crystal structures [29,30]. AutoDock helps to simulate interactions between substrates or drug candidates as ligands and their macromolecular receptors of known three dimensional structures, allowing ligand flexibility described to a full extent elsewhere [28]. In our docking simulations we



first used only the NEMO domain of the NEMO-IKK $\beta$  association domain structure for performing semi-flexible docking, with the ligand WA made flexible while keeping the receptor macromolecule being rigid. Flexibility of the ligand helps it explore six spatial degrees of freedom for rotation and translation and an arbitrary number of torsional degrees of freedom. A random perturbation to each is applied at each time step, and the interaction energy was evaluated for the new location and conformation [31].

#### Flexible docking

One of the novel features of AutoDock 4.0 allows side chains in the protein as well as in the ligand to be flexible. Thus flexibility of receptor molecule was also

exploited in docking studies by making use of AutoDock flexres scripts. It is worth assumable that the incoming ligand arriving at the binding sites of the two chains would try to make its own associations with the residues in order to minimize the energy of the system. To facilitate this notion, the key residues of NEMO and IKK $\beta$  chains which form H-bonds with corresponding residues in the two chains as reported [25] were made flexible in order to observe their mode of interactions with the ligand and to account for the subsequent rearrangements. The peptide bonds of the amino acids chosen to be flexible were kept "inactive" or non-rotatable. The Graphical User Interface program "AutoDock Tools" was used to prepare, run, and analyze the docking simulations. Protein was prepared for docking simulations by assigning of Kollman united atom charges, solvation parameters and polar hydrogens to the receptor PDB file. Water molecules were removed from its PDB file to make it a free receptor. Since ligands are not peptides, Gasteiger charge was assigned and then nonpolar hydrogens were merged. AutoDock assigns the rigid roots to the ligand automatically saving time as compared to manual picking. Five bonds in the ligand were made "active" or rotatable. Atomic solvation parameters were assigned to the receptor using default parameters.

#### Grid design

Auto-Tors, an auxiliary program using Interactive queries to define rotatable torsion angles, is used to assign all rotatable dihedrals in the ligands and to remove non-polar hydrogen atoms, uniting their partial charges with their bonded carbon atoms. AutoGrid, which calculates grids of interaction energy based on the interaction of the ligand atom probes with receptor target, is used to obtain the grid maps required prior to docking. Each probe consists of an atom type present in the ligand being docked. The pre-calculated grid maps store the potential energy arising from the interaction with the macromolecule. The user defined three dimensional grid must surround the region of interest in the macromolecule, and the ligand was limited to this search space during docking. In the present study, the location and dimensions of the grid box are chosen such that it incorporates the amino acid stretch (85-101) of NEMO domain involved in binding with the IKK $\beta$  chains for the formation of active IKK complex. The energy scoring grid was prepared as a 60, 60, and 60 Å $^{\circ}$  (x, y, and z) cube. The spacing between grid points was 0.375 angstroms.

#### The genetic algorithm

The Lamarckian Genetic Algorithm (LGA) was chosen to search for the best conformers. Using mathematical concepts designed to simulate the conditions

influencing biological evolution, genetic algorithms are able to search conformational space by “mutating” a ligand in order to find its lowest energy conformation in the “environment” of a fixed protein. Searches driven by this energy funnelling have been shown to provide a good indication as to the optimum protein-ligand interactions and therefore conveying the structure most likely to be found *in vivo* [32]. The default parameters for the Lamarckian genetic algorithm [28] were used as the search protocol except for the maximum number of energy evaluations, which were changed to 2.5 million. During the docking process, a maximum of 20 conformers was considered for each compound. The population size was set to 150 and the individuals were initialized randomly. Maximum number of generations were kept as 1000, maximum number of top individual that automatically survived set to 1, mutation rate of 0.02, crossover rate of 0.8. Step sizes for translations, quaternions and torsions were kept same as defaults. An efficient and accurate energy assessment of the ligand conformations is as important to the success of a docking simulation as the power of the search algorithm. AutoDock uses a variation on the AMBER’95 force field [33] with terms empirically determined by linear regression analysis from a set of protein-ligand complexes with known binding constants [28,34]. Gibbs Free energy ( $\Delta G$ ) is calculated as a sum of six energy terms of dispersion/repulsion, hydrogen bonding, electrostatic interactions, deviation from covalent geometry, internal ligand torsional constraints, and desolvation effects.

#### Selection and representation of docking modes

AutoDock reports the best docking solution (lowest docked free energy) for each GA run and also performs a cluster analysis in which the total number of clusters and the rank of each docking mode (cluster rank) are reported. Docking modes were selected on the basis of two criteria: ligand’s proximity to the N-terminal Glutamine and extent of its interactions with the IKK $\alpha$  and IKK $\beta$  interacting hydrophobic amino acid side chains of NEMO. For a 10 GA run there would be up to 10 total docking modes from which the lowest energy-docking mode was chosen that met the above two criteria. All the AutoDock docking runs were performed in Intel Core 2 Duo P8400 CPU @ 2.26 GHz of Sony origin, with 3 GB DDR RAM. AutoDock 4.0 was compiled and run under Windows VISTA operating system. The output from AutoDock and all modeling studies as well as images were rendered with PyMOL [35] and Accelrys ViewerLite 5.0. PyMOL was used to calculate the distances of hydrogen bonds as measured between the hydrogen and its assumed binding partner.

#### Confirmation of the docking results

The docking results obtained using AutoDock were also confirmed using ParDOCK [36], which is an all atom energy based monte carlo docking protocol. Docking using ParDOCK requires a reference complex (target protein bound to a reference ligand) and a candidate molecule along with specific mention of the centre of mass of the cavity on which the ligand is to be docked.

#### MD simulations in water

The AMBER v.10 package [37] was used to prepare the protein and the ligand files as well as for the Molecular Dynamics (MD) simulations. The binding complex of NEMO/IKK $\beta$ /WA obtained using ParDOCK and the free protein simulated in this study were neutralized by adding appropriate number of sodium counter-ions and was solvated in a octahedron box of TIP3P water with a 10 Å distance between the protein surface and the box boundary [38]. The partial atomic charges for the ligand were obtained using “antechamber” [39] module of Amber.

The energy minimization and MD simulations of NEMO/IKK $\beta$  and its complex with WA were carried out with the aid of the SANDER module of the AMBER 10 program. First of all, the simulated binding complex was effected with a 1000 step minimization using the steepest descent algorithm followed by a 2000 step minimization using conjugate gradient to remove bad steric contacts. Topology and parameter files for the protein were generated using “ff03” and for the drug using “gaff” based on the atom types of the force field model developed by Cornell et al [33]. Then the system was equilibrated beginning with the protein atom restrained simulations having 20 ps equilibration dynamics of the solvent molecules at 300 K and a harmonic potential with a 10 kcal/mol restraint force. Next step involved the equilibration of the solute molecules with a fixed configuration of the solvent molecules in which the system was slowly heated from T = 10 to 300 K in three intervals of 20 ps each. The entire system was then equilibrated at 300 K for 70 ps before a sufficiently long MD simulation (for 2.6 ns) at room temperature. The MD simulations were performed with a periodic boundary condition in the NPT ensemble at T=298.15 K with Berendsen temperature coupling [40] and constant pressure P=1 atm with isotropic molecule-based scaling. The SHAKE algorithm [41] was applied to fix all covalent bonds containing hydrogen atoms. We used a time step of 2 fs and a nonbond-interaction cutoff radius of 10 Å. The Particle Mesh Ewald (PME) method [42] was used to treat long-range electrostatic interactions. The coordinates of the trajectory was sampled every 1 ps for analysis of the energy stabilization and RMSD values of the protein as well as that of



the complex. MD simulations were performed on a 320 processors SUN Microsystems clusters at Supercomputing Facility (SCFBio) at IIT Delhi.

## Results and discussion

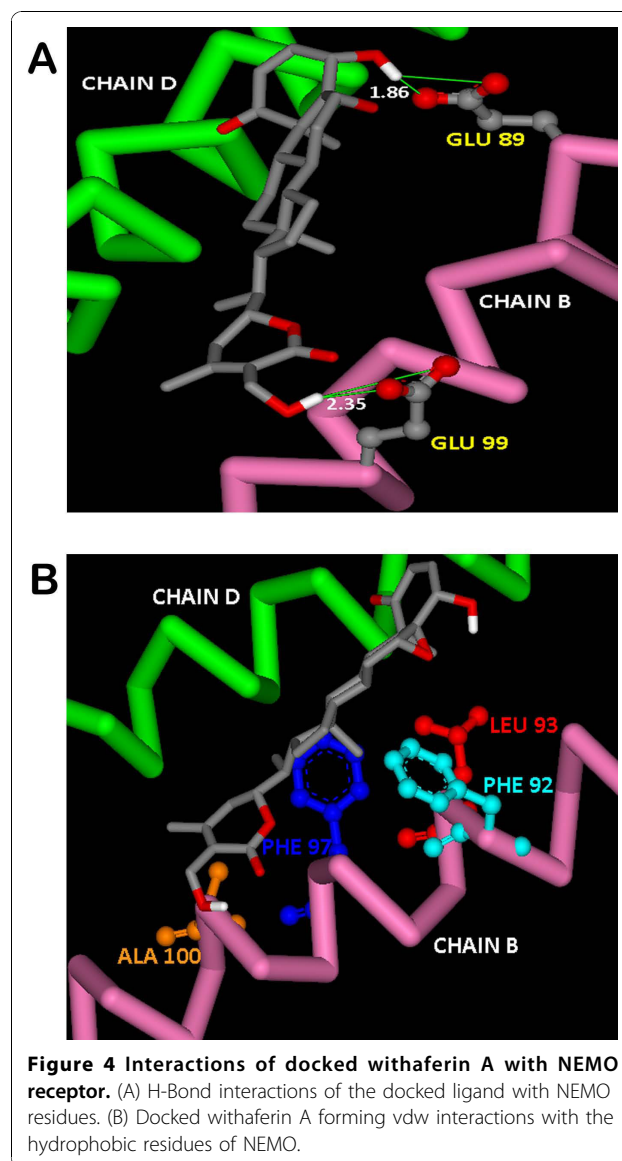
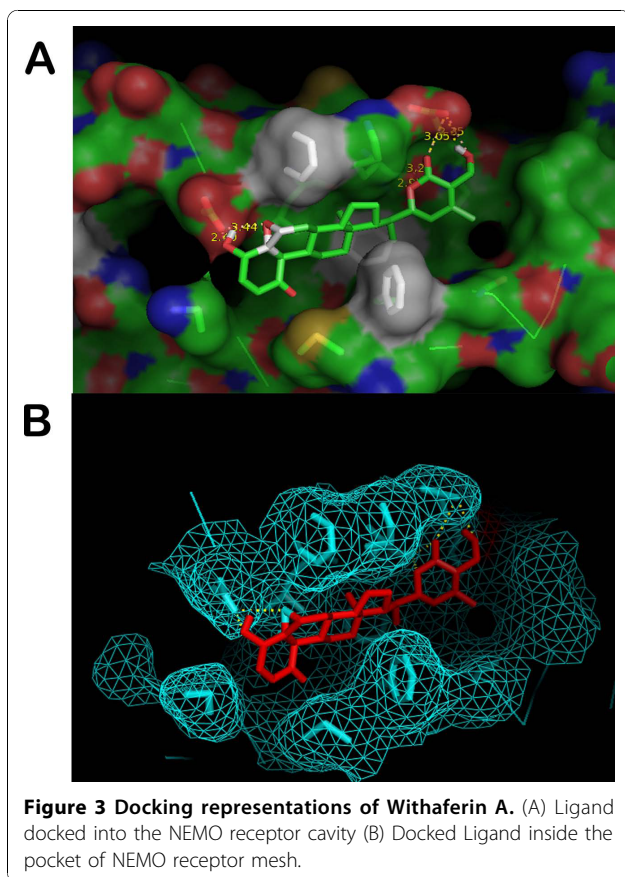
### Semi-flexible docking of WA into NEMO

One possible mode of action which is proposed here for WA to act as a NF- $\kappa$ B activation suppressor is by non-formation/disruption of the complex between NEMO and IKK $\beta$ . In order to explore the possibility of non-formation of the complex, we first carried out molecular docking studies with only the NEMO chains of the protein crystal structure. Before docking to be carried out, the structures of receptor macromolecules were minimized in Steepest Descent and Conjugate Gradient methods using Accelrys Discovery studio. Macromolecular receptors minimized using Conjugate Gradient have comparatively lower potential energy values than those obtained from Steepest Descent and are thus utilized further for carrying out the docking studies. Figure 3A shows the docked ligand WA to the selective NEMO receptor. WA gets buried inside the pocket of NEMO as depicted by mesh representation in Figure 3B. For this particular configuration the binding energy of WA with NEMO is -9.44 Kcal/mol (Table 1).

**Table 1 Energies obtained after docking of withaferin A into NEMO**

| Property              | Quantity       |
|-----------------------|----------------|
| Binding Energy        | -9.44 Kcal/mol |
| Intermolecular energy | -9.68 Kcal/mol |
| Total internal energy | -1.13 Kcal/mol |

The binding of WA to NEMO is characterized by H-bonding between a terminal hydroxyl group of the ligand and the side chain carboxyl group of Glu 89 (Figure 4A). The residue Glu 89 has been reported as one of the critical residues being involved in H-bond formation with Ser 733 of IKK $\beta$  resulting in complex formation with NEMO [25]. The lengths of the two H-bonds formed are 1.86 and 2.93 Å. The other end of



WA also forms H-bond with the side chain carboxyl group of Glu 99 with a bond distance of 2.35 Å. It has also been reported that the residues Phe 92, Leu 93, Met 94, Phe 97, Ala 100 and Arg 101 of NEMO are responsible for intermolecular hydrophobic interactions. In the present docked structure, WA is forming van der Waals interactions with Phe 92, Leu 93, Phe 97 and Ala 100 of NEMO (Figure 4B). These non-covalent interactions help stabilize the binding of the ligand with the macromolecule by lowering the energy. Strong interactions formed by WA with these particular residues would result in building steric as well as thermodynamic barriers to the incoming IKK $\beta$  subunits, thus providing hindrance for binding to NEMO at these particular residues. This obstruction would pave way to unlikely complex formation capability of NEMO to IKK $\beta$  yielding in either the formation of a deformed complex or at extreme no complex formation at all. Moreover the deformed complex would be thermodynamically much less stable as compared to the native complex owing to the non-availability of WA occupied hydrophobic interaction forming residues of NEMO. In both cases the NEMO/IKK $\beta$  complex would not get assembled to its catalytically active form. The absence of active IKK multisubunit complex would prevent degradation of I $\kappa$ B proteins, as the I $\kappa$ B proteins would not get phosphorylated by IKK. This would ultimately lead to non-release of NF- $\kappa$ B and its further translocation to the nucleus thus arresting its nefarious acts.

#### Flexible docking of WA into active NEMO/IKK $\beta$ complex

Docking of WA performed on the catalytically active NEMO/IKK $\beta$  complex also provided interesting results. The binding of WA to NEMO provides significant evidence in support of the proposed mechanism of NF- $\kappa$ B activation suppression by inhibition or disruption of active NEMO/IKK $\beta$  complex formation. Four clusters were obtained for Genetic Algorithm run for generation of twenty models. Large negative binding energies were obtained for all the clusters as evident from Table 2. The lowest binding energy was more than twice as lower as that of the energy obtained from binding of WA to the segregated NEMO receptor. As shown in Figure 5 for Cluster 1, the hydroxyl group of WA

disrupts SER733:GLU89 H-bond present in the active complex by itself hydrogen bonding with SER733 hydroxyl group. The same hydroxyl group of the ligand is also involved in H-bonding with THR735, which is one of the critical residues in complex formation. The other terminal hydroxyl group of WA is also involved in forming H-bonds with SER740 and ASP738 of chain A. NEMO and IKK $\beta$  chains of active NEMO/IKK $\beta$  complex are held together by strong intermolecular hydrophobic interactions between LEU93:PHE734, THR735:PHE92, PHE734:MET94, TRP739:PHE97, TRP741:ALA100, TRP741:ARG101. But withefrin A disrupts majority of these hydrophobic interactions by placing itself in between the binding chains, As depicted in Figure 6 for Cluster 1, WA itself starts forming strong intermolecular interactions with these critical residues.

The energy values for the different clusters shown in Table 2 can be explained on the basis of the representative cluster structures, which are the ones having lowest energy in the respective cluster (Figure 7). All the hydrogen bonds being formed in cluster 1 (Ser 733, Thr 735, Asp 738 and Ser 740) were lost in cluster 2 (Figure 7A) which explains the increase in energy for this cluster as compared to cluster 1. But the amount of energy lowered is not that much significant owing to the presence of strong van der Waals interactions with Phe 92, Leu 93 and Phe 97 of chain B. In cluster 3 (Figure 7B), though there is formation of few hydrogen bonds with residues of chain A, the van der Waals interactions are also markedly reduced resulting in further decrease in energy. In cluster 4 (Figure 7C), due to the absence of any hydrogen bond formation in addition to very weak van der Waals interactions, the energy is recorded as minimum of all the four clusters.

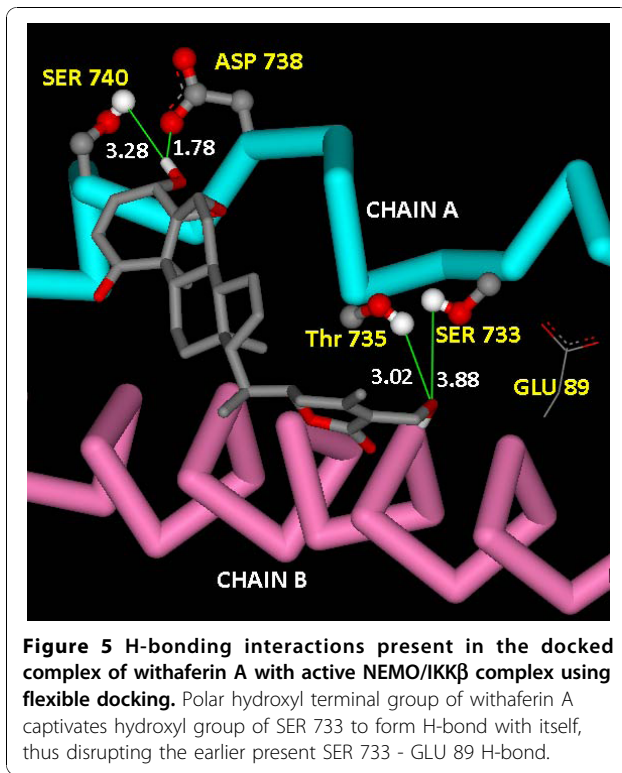
The results obtained (Figure 5 &6) from docking of WA into the active complex, with a majority of H-bond and hydrophobic interaction forming residues kept flexible, clearly show that WA blocks the intermolecular hydrophobic interactions between NEMO and IKK $\beta$  at the residues which are significantly involved in formation of the active complex. The large value of binding energy for Cluster 1(-19.33 Kcal/mol) involved in binding of WA to the complex consolidates the thermodynamic stability of the binding. These

**Table 2 Clustering results obtained from docking of withaferin A into NEMO/IKK $\beta$  complex**

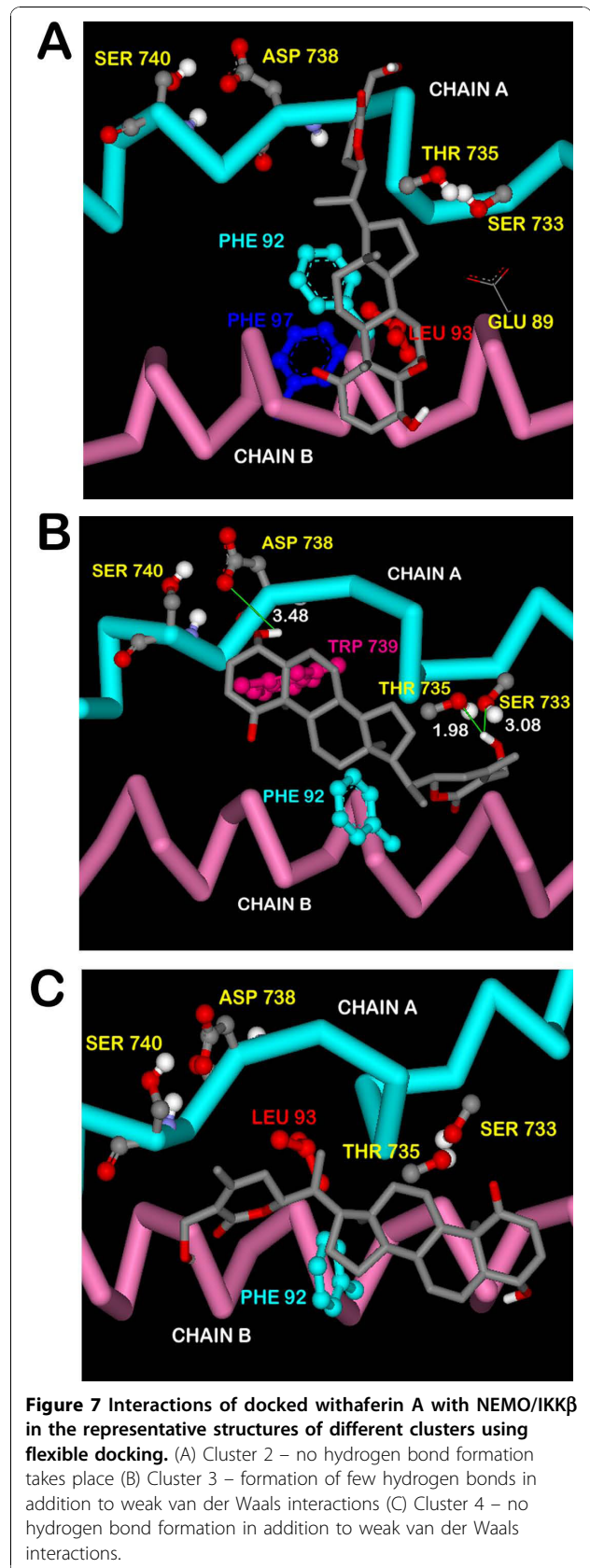
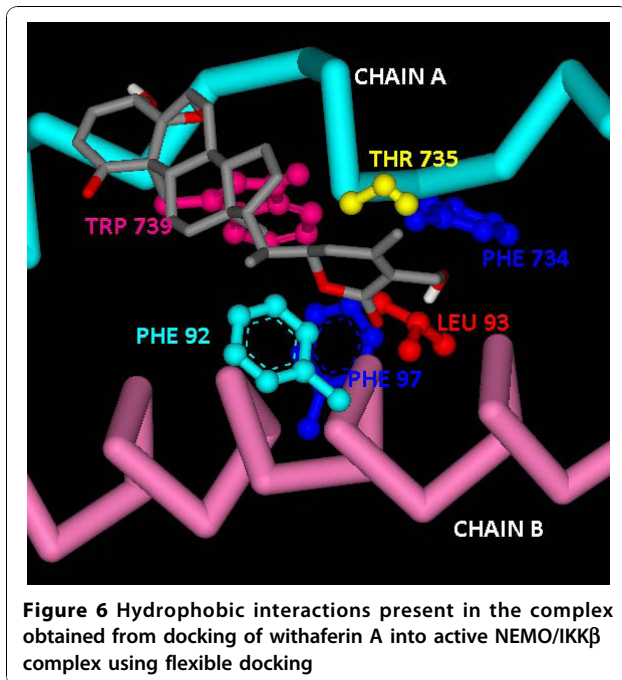
| Receptor                 | No. of AutoDock clusters <sup>a, b</sup> | Cluster rank <sup>b</sup> | No. of structures in the cluster | Lowest binding energy of cluster | Energy range within cluster |
|--------------------------|--|---------------------------|----------------------------------|----------------------------------|-----------------------------|
| NEMO/IKK $\beta$ complex | 4 (20)                                   | 1                         | 8                                | -19.33                           | -19.33 to -17.24            |
|                          |  | 2                         | 8                                | -19.12                           | -19.12 to -16.53            |
|                          |  | 3                         | 3                                | -18.6                            | -18.6 to -16.99             |
|                          |  | 4                         | 1                                | -18.33                           | -18.33                      |

<sup>a</sup> Number of GA runs are shown in parentheses

<sup>b</sup> Clustering is done with RMS tolerance of 5.0 Å



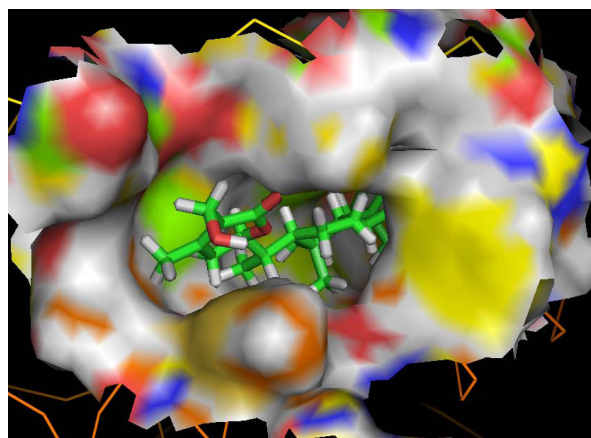
results substantiate the hypothesis that WA possess the potential to disarray the active complex by disrupting the stability of attachment of NEMO and IKK $\beta$  chains, being accounted by hydrophobic and H-bond interactions.



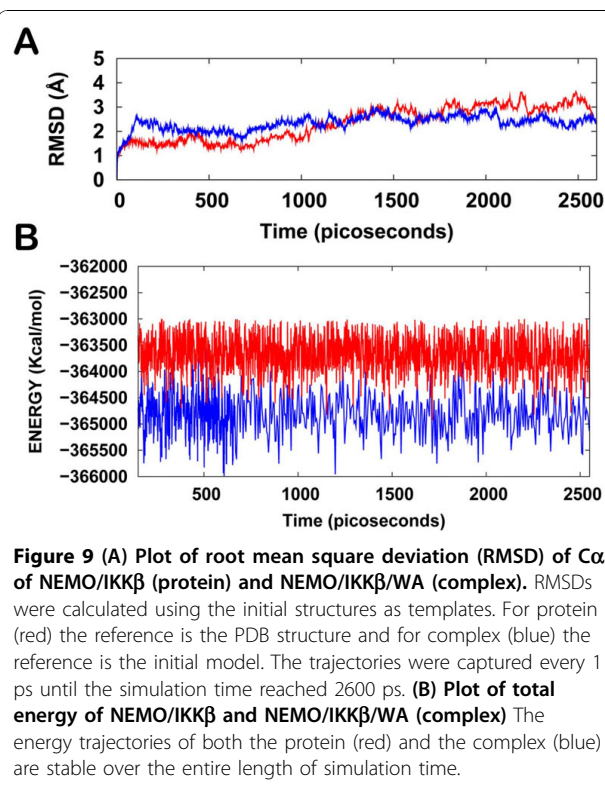


### MD simulations in water

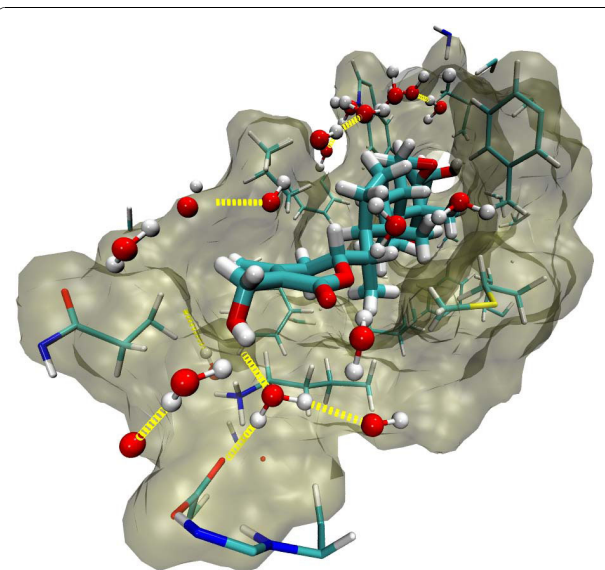
The NEMO/IKK $\beta$ /WA protein-drug binding complex with the binding energy of -9.33 kcal/mol obtained using ParDOCK (Figure 8) was used for carrying out MD simulations. After the MD simulation, we calculated RMSDs between C $\alpha$  of NEMO/IKK $\beta$  complex trajectories recorded every 1 ps and C $\alpha$  of their X-ray crystal structure. The RMSDs for the trajectories of the NEMO/IKK $\beta$  complexed with WA were also calculated using its initial model as a reference structure. The results in Figure 9A show that the RMSD of the complex has achieved a stationary phase during the later stage of the simulation and is always less than 3 Å for the entire simulation length suggesting the stability of the complex, while the RMSDs of the protein from its initial X-ray PDB structure kept increasing. It was also found that the energy of the complex (blue) is always lower than that of the protein (red) alone throughout the length of the simulation (Figure 9B). This rule out the possibility of the complex getting activated in the presence of WA. It was also observed that the water molecules act as intermediate bridges thus playing a vital role in facilitating the binding of ligand to the protein (Figure 10). The MD simulations were also carried out for WA complex with NEMO. It was observed that RMSD for this complex kept increasing with marked fluctuations (Figure 11). Thus the WA complex with NEMO alone seems to be quite unstable. The simulation length used in the entire study were long enough to allow rearrangement of side chains of the native as well as the drug complexed protein to find their most stable binding mode. In conclusion, the present MD simulations made clear the dynamic structural stability of NEMO/IKK $\beta$  in complex with the drug WA, together with the inhibitory mechanism. These results would be valuable for



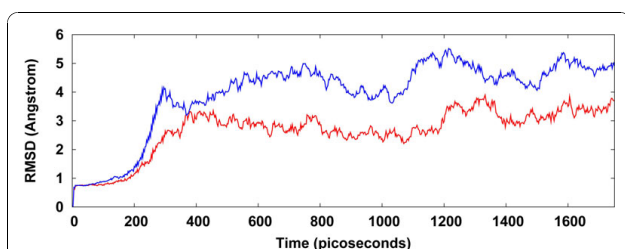
**Figure 8** Docking representation of the drug WA inside the cavity of NEMO/IKK $\beta$  obtained using ParDOCK.



**Figure 9** (A) Plot of root mean square deviation (RMSD) of C $\alpha$  of NEMO/IKK $\beta$  (protein) and NEMO/IKK $\beta$ /WA (complex). RMSDs were calculated using the initial structures as templates. For protein (red) the reference is the PDB structure and for complex (blue) the reference is the initial model. The trajectories were captured every 1 ps until the simulation time reached 2600 ps. (B) Plot of total energy of NEMO/IKK $\beta$  and NEMO/IKK $\beta$ /WA (complex) The energy trajectories of both the protein (red) and the complex (blue) are stable over the entire length of simulation time.



**Figure 10** A simulated snapshot of WA/NEMO/IKK $\beta$  complex showing water molecules coordinating the ligand and the protein. The water molecules act as intermediate bridges facilitating the binding of WA to NEMO/IKK $\beta$ .



**Figure 11** Plot of root mean square deviation (RMSD) of C $\alpha$  of NEMO (protein) and NEMO/WA (complex). RMSDs were calculated using the initial structures as templates. For protein (red) the reference is the PDB structure and for complex (blue) the reference is the initial model. The trajectories were captured every 1 ps until the simulation time reached 1750 ps.

further designing non-covalent type inhibitors with high specificity and potent activity.

## Conclusions

NF- $\kappa$ B is one of the most attractive topics in current biological, biochemical, and pharmacological research, and in the recent years the number of studies focusing on its inhibition/regulation has increased manifolds. Small ligands (both natural and synthetic) are gaining particular attention in this context. Our computational analysis, provided a rationalization of the ability of naturally occurring WA to alter the NF- $\kappa$ B signalling pathway. The large value of binding energy involved in binding of WA to the active NEMO/IKK complex consolidates the thermodynamic stability of the binding. Our docking results obtained substantiate the hypothesis that WA has the potential to inhibit the formation of active NEMO/IKK complex by either resulting in non-formation of the complex or by disrupting the stability of attachment of NEMO to IKK $\beta$  chains. Conclusively our results strongly suggest that withaferin A is a potent anticancer agent as ascertained by its potent NF- $\kappa$ B modulating capability.

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## Authors' contributions

AG, VSB and DS designed the methods and experimental setup. AG carried out the implementation of the various methods. AS and AP assisted AG in this process. AG and DS wrote the manuscript. All authors have read and approved the final manuscript.

## Competing interests

The authors declare that they have no competing interests.

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