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Toxic effect of chemicals dumped in premises of UCIL, Bhopal leading to environmental pollution: An *in silico* approach

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ABSTRACT

Objective: To investigate the role of dumped residues in the loss of immunity using human immune proteins, which provides protection against *Mycobacterium tuberculosis*.
Methods: In this study, toxic chemicals were docked with immune proteins using AutoDock 4.0, and further, molecular dynamics simulations were performed for refinement of the docked complexes which were obtained from docking to confirm its stable behaviour over the entire simulation period.
Results: Results revealed that alpha-naphthol showed the maximum inhibition with glutathione synthetase protein, while butylated hydroxytoluene and carbaryl showed the maximum inhibition with p38 MAPK14 protein with binding free energy ΔG -5.06, -5.1 and -5.36 kcal/mol, respectively. Molecular dynamics simulation supported the greater stability of carbaryl and alpha-naphthol complexes with p38 MAPK 14 and glutathione synthetase protein as compared to butylated hydroxytoluene.
Conclusions: In summary, findings suggested that toxic exposure of carbaryl and alpha-naphthol as compared to butylated hydroxytoluene generated immunotoxicity and disrupted the functioning of immune system thus it may have caused an increase in susceptibility to *Mycobacterium tuberculosis* infection.

1. Introduction

Immune proteins are proteins which have an impact on, or play a role in, functioning of immune mechanism within the human body. They've the potential to provide protection against bacterial or viral illness. The immune process in our body either immediately kills the microbial pathogens through phagocytosis, or sets off the production of cytokines, which helps us the elimination of pathogens[1]. Isocyanates, a group of low molecular weight aromatic and aliphatic compounds containing the isocyanate group, are important raw materials with diverse industrial application, and their reactivity is due to the strain on the cumulative double bonds of isocyanates[2]. Methyl isocyanate, the smallest member of the isocyanate family, was released in Bhopal on the intervening night of 2nd and 3rd December 1984 from the Union Carbide India Ltd (UCIL) pesticides factory and around 500 000 inhabitants of Bhopal exposed to this toxic gas which exerts a wide spectrum of toxic effects including sensory, pulmonary toxicity and immune toxicity[3-6]. Pulmonary infection and infections of the upper respiratory tract are its most common effects[7]. These finding and evidence suggest that this toxic exposure affects the immune system and it also suggests that they modulate immune system function because this person becomes more susceptible to *Mycobacterium tuberculosis* (*M. tuberculosis*) infection[8,9].

While there are numerous reports showing the effect of methyl isocyanate on human health, very few reports are concerned about the dumped residues, *i.e.* toxic chemical residues (alpha-naphthol, butylated hydroxytoluene and carbaryl), in premises around UCIL Bhopal, which has led to the contamination of ground water, soil and air and can be fatal for people living in the vicinity. A survey conducted in 1997 by the National Environmental Engineering Research Institute, Nagpur in and around the UCIL premises[10] reported the presence of carbaryl, lindane, alpha-naphthol *etc*. Another study performed by using sixty-two samples of soil, surface and ground water (ponds, wells and hand pumps) obtained from different locations in and around Bhopal reported the

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presence of residues of 1-naphthol[11], which is the degradation product of carbamates. These reviews and findings suggested that survivors suffered with severe immunological suppression, as this susceptibility towards infectious disease is more in victims in comparison with non-victims.

Evidence from different cohort studies suggests that the immune system is a possible target of toxicity after exposure to a wide array of toxic industrial and environmental chemicals[12]. Street and Sharma[13] found that laboratory animals exposed to carbaryl at levels below those causing obvious toxicity significantly reduced antibody production. Other animal studies have found reduced proliferation of lymphocytes at levels that did not affect nervous system function[14]. Clinical evidence shows that defects in immune function and active immunosuppression are associated with increased susceptibility to infections to a variety of malignant diseases caused by bacterial agent *M. tuberculosis*[2,15].

We have previously studied the interaction of methyl isocyanate and their hydrolytic products to find their toxicity pattern in the inhibition of human immune protein[16,17]. Here in this study we tried to examine the loss of immunity, which provides protection against tuberculosis by the toxic chemicals like alpha-naphthol, butylated hydroxytoluene and carbaryl, dumped in premises around the UCIL Bhopal. Although numerous studies have done to predict the toxicity effect of methyl isocyanate but there is no such study which reports the effect of dumped toxic chemicals on inhibition of human immunoproteins. The binding energy of alphanaphthol with all the selected immune proteins were in accordance with earlier studies[18]. To study this we have performed *in silico* docking of these toxic chemicals with p38 MAPK14, CD40 ligand, urokinase plasminogen activator, and glutathione synthetase proteins, yielding interaction properties such as the binding energy, as well as the binding mode in the active site followed by molecular dynamic study to evaluate the binding stability of these protein-ligand complexes. The outcomes of study help to understand the molecular interaction pattern of these dumped toxic chemicals with human immune proteins involved in immunity against tuberculosis.

2. Materials and methods

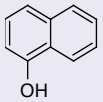
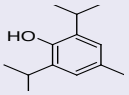
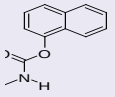
2.1. Protein preparation and active site residue identification

The crystal structure of urokinase plasminogen activator, glutathione synthetase, CD40 ligand and p38 MAPK 14 [Protein Data Bank (PDB) ID: 1W14, 2HGS, 3LKJ, 3OCG] was obtained from PDB (http://www.rcsb.org/pdb/) with resolution ranging from 2.10 to 2.50 Å. Further, obtained protein was prepared by the help of AutoDock tool, water molecules in the crystal were removed and gasteiger charges were added. Active site was identified with the help of PDB Japan[19].

2.2. Preparation of ligand

In present study, alpha-naphthol [compound identification (CID) No. 7005], butylated hydroxytoluene (CID No. 31404) and carbaryl (CID No. 6129) were used as a ligand. The 2D structures of these selected compounds were mentioned in Table 1. These compounds were retrieved from PubChem Compound Database (http://pubchem.ncbi.nlm.nih.gov/) as SDF format; further they are converted into the PDB format using open babel[20]. Preparation of ligand was also performed using the AutoDock tools. Docking simulations were carried out with the help of prepared ligands.

Table 1
Dumped residue used as ligand for docking.

Structure	PubChem CID number	Name
	CID 7005	Alpha-naphthol
	CID 31404	Butylated hydroxytoluene
	CID 6129	Carbaryl

2.3. Docking procedure

The molecular docking was performed to determine the binding orientations and conformations of the selected compound into the active site of protein. Thus, for the prediction of globally optimized conformation of the binding pose, AutoDock 4.0 was used for docking of compounds with the selected immune proteins using Lamarckian genetic algorithm[21]. In protein preparation, hydrogen atoms were added; while all non-polar hydrogen atoms were merged, addition of Kollman charge and atomic solvation parameter were assigned to the selected immune proteins using AutoDock tools. Removal of water molecule and heteroatoms was also performed. Grid points with 0.375 Å spacing were used using AutoGrid around the docking area for all the ligand atoms. The other protocols set for each docking run were: population size 150, maximum number of evaluation 2 500 000, rate of gene mutation 0.02, cross over rate 0.8, and the other remaining were set as default. The results obtained were clustered on the basis of cluster root-mean-square deviation (RMSD) and ranked by the lowest binding energy. The docked energy (ΔG) was the sum of the intermolecular and the internal energies. The pose which had the lowest estimated free energy of binding was chosen.

2.4. Molecular dynamics (MD) simulation

Based on the docking results, MD simulation studies were performed for the protein-ligand complex using the GROMOS96 43a1 force field of Gromacs 4.5.4 package[22,23]. The lowest binding energy dock conformation obtained from docking was used as initial conformation for this study. Further, the topology parameter of protein was generated by using the Gromacs programme. The topology parameters of ligands (alpha-naphthol, butylated hydroxytoluene and carbaryl) were built by the Dundee PRODRG server[24]. The complex was placed in a cubic box and solvated by simple point charge (SPC216) water molecules[25] followed by addition of sodium counter-ions to neutralize the system. Thus, solvated system (glutathione synthetase, alpha-naphthol and water) was neutralized by adding 8 Na ions and 6 Na ions respectively in the case of p38 MAPK14-butylated hydroxytoluene and p38 MAPK14-carbaryl complex. After neutralization of the system, energy minimization was performed first using the steepest descent followed by the conjugate gradient method to release conflicting contact. MD simulation studies consisted of equilibration and production

phases. After energy minimization process to equilibrate the system, the solute (protein, counterions, and ligands) were subjected to the position-restrained dynamics simulation (NVT and NPT) at 300 K for 100 ps. Finally, the system was allowed to production run at 300 K temperature and 1 bar pressure for 10 ns. And the atom coordinates were recorded at every 2 ps for analysis of simulation trajectory.

3. Results

3.1. Binding modes generated by molecular docking

Molecular docking studies have been carried out to get an insight into the inhibitory mechanism of dumped residue chemicals in the selected human immune proteins. Among the various conformers obtained from AutoDock, we choosed the docked conformer with the minimum binding energy. The details of number of intermolecular interactions, binding energies and amino acid interactions in the docked complexes of proteins were shown in Table 2 and their dock pose configurations were shown in Figure 1. All these residues were identified as active site as per PDB Japan database. According to the results as shown in Table 2, the dumped residues (alpha-naphthol[18], butylated hydroxytoluene and carbaryl) showed maximum inhibition with glutathione synthetase and p38 MAPK14 proteins. Alpha-naphthol showed maximum inhibition with glutathione synthetase protein while butylated hydroxytoluene and carbaryl showed maximum inhibition with p38 MAPK14 protein with binding free energy ΔG -5.06, -5.10 and -5.36 kcal/mol, respectively.

3.2. MD simulation of docked complexes

A combination of molecular docking and MD simulation techniques were used to predict more reliable receptor-ligand interaction mechanisms. In view of this, we performed MD simulation for 10 ns to assess the stability of protein-ligand complex.

3.2.1. Interaction of carbaryl and butylated hydroxytoluene with p38 MAPK14

Docking studies revealed that carbaryl was found to bind at the binding site of p38 MAPK14 with the lowest binding energy ΔG -5.36 kcal/mol, while butylated hydroxytoluene was found to have ΔG -5.10 kcal/mol. The major interaction shown in this binding was with amino acid residue Asp168 and Lys53; this minimum binding energy complex was used for carrying out MD simulation. We have analyzed the time dependent behaviour of MD trajectories of these two complexes, including RMSD for all backbone atoms and average fluctuations of these residues [root mean square fluctuation

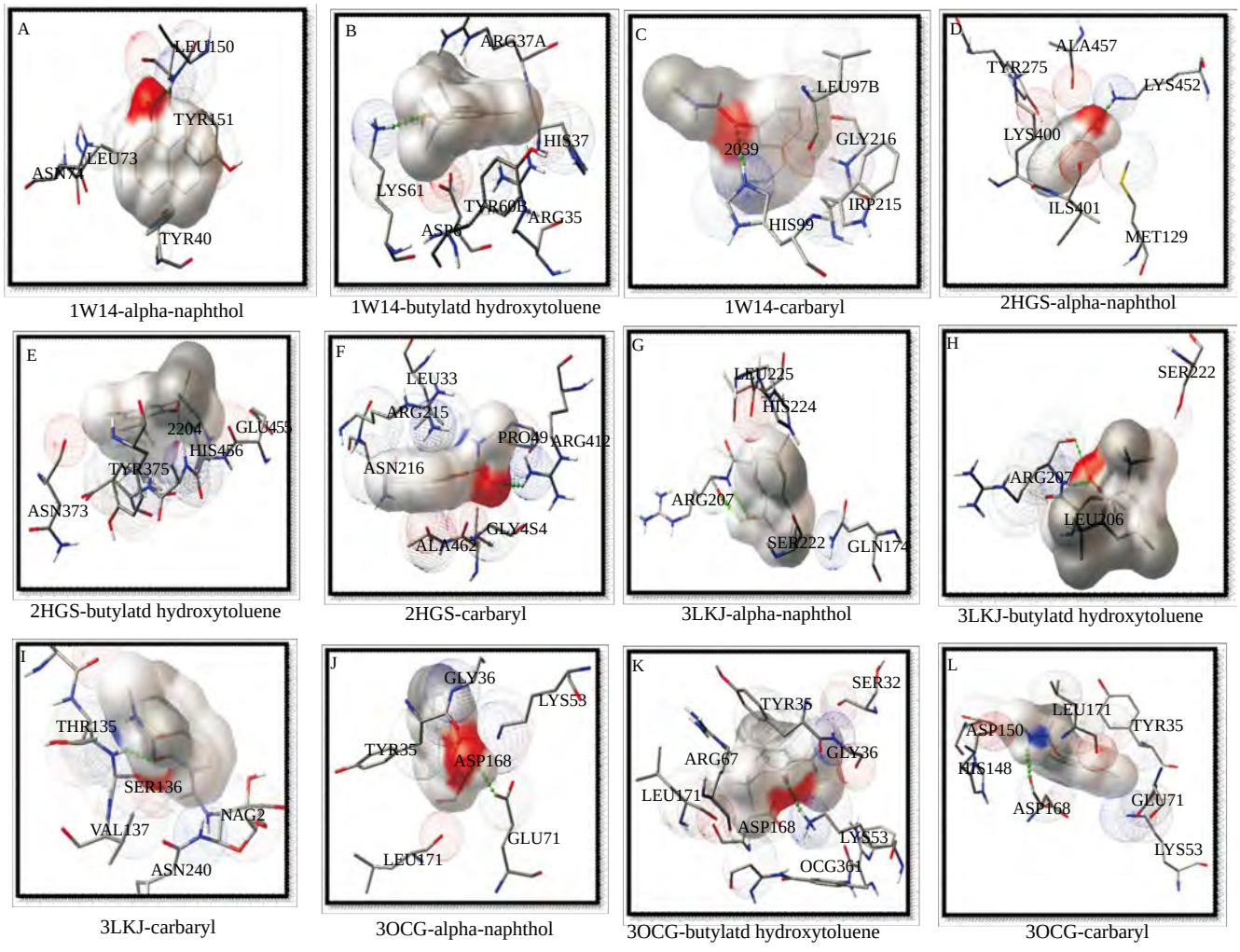


Figure 1. Protein-ligand interaction diagram.
A, B, C: Indicate interaction of plasminogen activator urokinase (1W14) with alpha-naphthol[18], butylated hydroxytoluene and carbaryl; D, E, F: Indicate interaction of glutathione synthetase (2HGS) with alpha-naphthol[18], butylated hydroxytoluene and carbaryl; G, H, I: Indicate interaction of CD40 ligand (3LKJ) with alpha-naphthol[18], butylated hydroxytoluene and carbaryl; J, K, L: Indicate interaction of p38 MAPK 14 (3OCG) with alpha-naphthol[18], butylated hydroxytoluene and carbaryl.

Table 2
Interaction of human immune proteins with dumped residue.

Name of protein PDB ID	Dumped residue	Binding energy (kcal/mol)	Hydrogen bonding	Bond length Å	Inhibition constant	vdW + Hbond + desolv energy
Plasminogen activator urokinase (1W14)	Alpha-naphthol	-4.72	TYR-151	1.812	344.19	4.95
	Butylated hydroxytoluene	-3.82	LYS-61	2.104	1.59	4.58
	Carbaryl	-3.52	HIS-99	2.039	2.63	4.01
Glutathione synthetase (2HGS)	Alpha-naphthol	-5.06	LYS-452	2.100	5.63	4.05
	Butylated hydroxytoluene	-4.06	ALA-457	2.204	1.05	4.91
	Carbaryl	-4.73	ARG-412	2.168	343.19	5.49
CD40 ligand (3LKJ)	Alpha-naphthol	-4.64	ARG-207	1.927 2.168	395.22	4.85
	Butylated hydroxytoluene	-4.24	ARG-207	2.209 2.200	774.97	5.15
	Carbaryl	-4.10	SER-136	2.043	182.51	4.81
p38 MAPK 14 (3OCG)	Alpha-naphthol	-4.64	GLU-71	1.750	394.99	4.64
	Butylated hydroxytoluene	-5.10	LYS-53	1.867	182.79	5.86
	Carbaryl	-5.36	ASP-168	1.976	117.43	5.96

(RMSF)] along with the radius of gyration. We also performed 10 ns simulation of unbound protein p38 MAPK14 for comparing the results between bound and unbound forms.

RMSD of the backbone atom was calculated as a function of time to assess the conformational stability of protein during the simulation. Figure 2 shows that the RMSD pattern of 3OCG-carbaryl complex was stable in the simulation and shows less fluctuations during the entire simulation time as compared to the 3OCG-butylated hydroxytoluene complex which showed larger fluctuations.

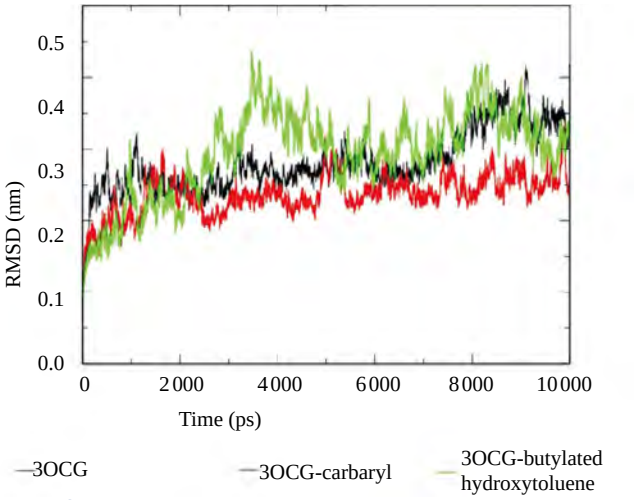


Figure 2. Plot of RMSD of backbone of 3OCG unbound (black) with 3OCG-carbaryl (red) and 3OCG-butylated hydroxytoluene complex (green).

In 3OCG-carbaryl complex, the RMSD of backbone showed minimum fluctuations as compared to unbound p38 MAPK14 protein. Initially it was found to be 0.9 nm up to 1 000 ps and after 3 000 ps it showed stable behavior till the end of simulation with a mean value of 0.25 nm. The backbone RMSD profile of 3OCG-butylated hydroxytoluene complex showed large fluctuations initially from 1 000 to 2 000 ps with a mean value of 0.25 nm and after 3 000 ps showed larger fluctuations up to the end of simulation. These results showed that the trajectories of MD simulation after equilibrium was reliable for post analysis. Analysis of 3OCG-cabaryl complex trajectory indicated that carbaryl showed remarkable stability in p38 MAPK14 pocket during MD simulation.

To investigate the thermodynamic stability of the complexes in the simulation, their potential energy fluctuation was analyzed and found to be constant value -1.445e+06 during the entire simulation length (Figure 3). The radius of gyration for the native p38 MAPK14 protein, 3OCG-butylated hydroxytoluene complex and 3OCG-carbaryl complex was analyzed to determine the effect of toxic compounds on the folding of p38 MAPK14 protein. The radius of gyration value of 3OCG-cabaryl complex was found to be nearly the same with continuous up and down during simulation as compared to 3OCG-butylated hydroxytoluene complex, which indicated very little conformational change in the secondary structure of p38 MAPK14 protein when bound to 3OCG-carbaryl complex (Figrue 4). The number of H-bonds formed during MD simulation between p38 MAPK14 protein and toxic chemicals was also calculated. A variable profile was observed which fluctuated between 0 and 3; carbaryl and butylated hydroxytoluene both formed one stable hydrogen bond during the entire simulation time (Figures 5 and 6). To identify local mobility or flexible region in the protein, the RMSF of protein residues was analyzed. These values were obtained from the trajectory of the last 1 000 ps when the simulation was equilibrated as shown in Figure 7. As is clear from the RMSF profiles of 3OCG complex, the fluctuations of residues in the internal cavity were lower than the others, which indicated that the binding site during MD simulation remained approximately rigid.

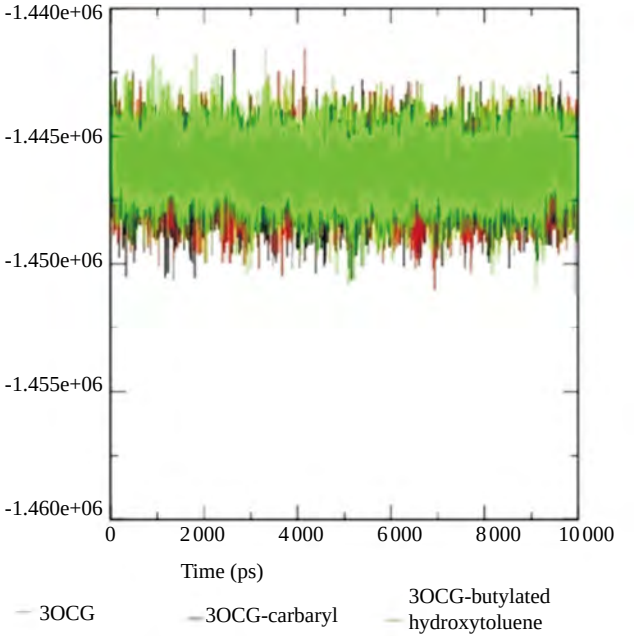


Figure 3. Potential energy profile of 3OCG unbound (black) with 3OCG -carbaryl (red) and 3OCG-butylatedhydroxytoluene complex (green) during 10 000 ps simulation.

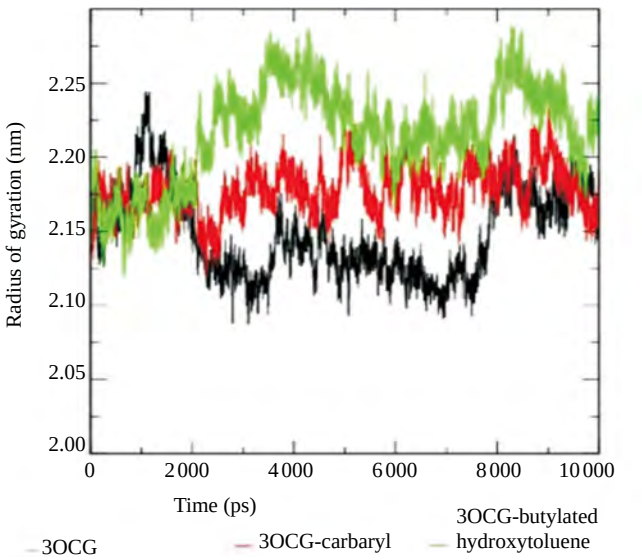


Figure 4. Plot of radius of gyration during 10000 ps simulation of 3OCG protein unbound (black) with 3OCG-carbaryl (red) and 3OCG-butylated hydroxytoluene complex (green).

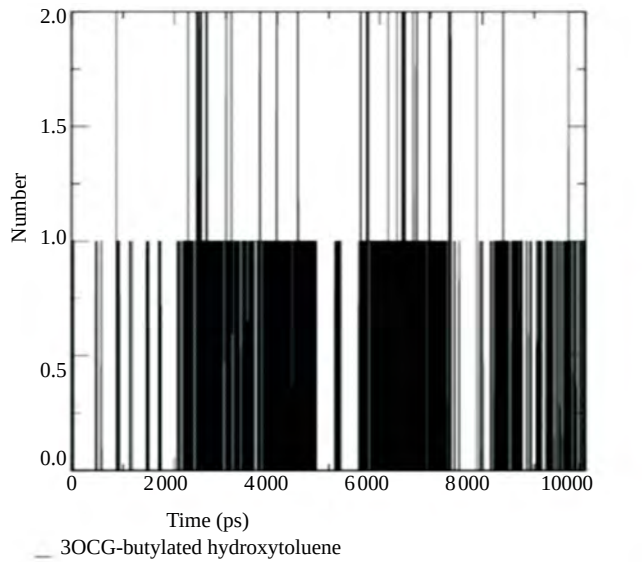


Figure 5. Number of H-bonds formed between butylated hydroxytoluene and 3OCG during 10000 ps simulation.

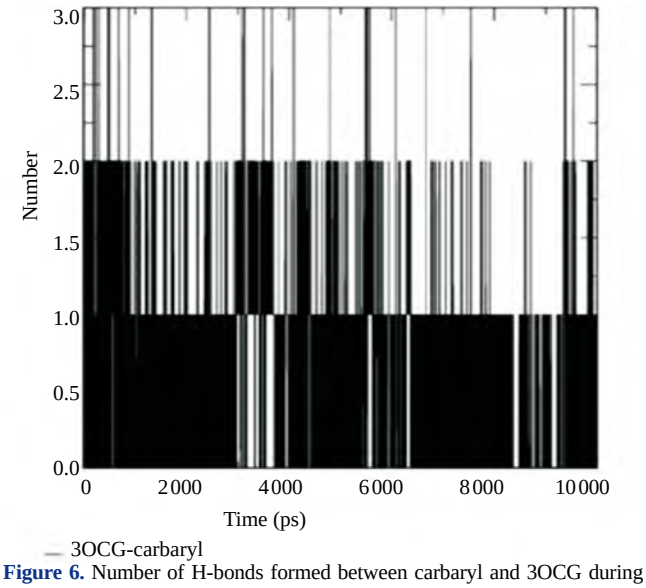


Figure 6. Number of H-bonds formed between carbaryl and 3OCG during 10000 ps simulation.

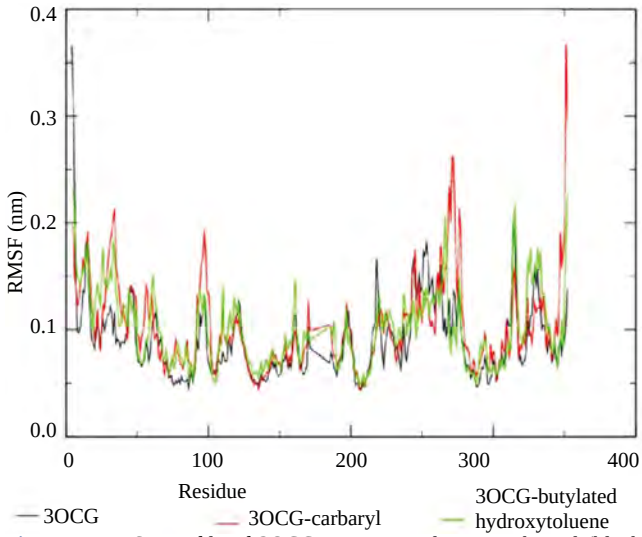


Figure 7. RMSF profile of 3OCG protein residues in unbound (black) with 3OCG-carbaryl (red) and 3OCG-butylated hydroxytoluene complex (green).

3.2.2. Interaction of alpha-naphthol with glutathione synthetase

Molecular docking study revealed that alpha-naphthol interacted with glutathione synthetase interface residue with a binding energy ΔG -5.06 kcal/mol and formed a hydrogen bond with Lys452 residue[18]. Further, MD simulation was performed to access the conformational stability of 2HGS-alpha-naphthol complex and to study the changes in dynamics at this interface. We also performed a 10 ns simulation of unbound glutathione synthetase protein for comparing the results between bound and unbound forms of alpha-naphthol. Figure 8 shows that the RMSD profile of backbone atoms was always less than 0.3 nm during the entire simulation time of 10000 ps. Some fluctuations occurred up to 4000 ps and the trajectory remained stable after 5000 ps till the end of simulation with a mean value of 0.2 nm, which indicated that alpha-naphthol showed remarkable stability in the glutathione synthetase pocket during MD simulation. Potential energy fluctuation was also analyzed to check the thermodynamic stability of the complex, and it was found to be a constant value -1.663e+06 for the complex during the entire simulation length as shown in Figure 9. The stability of backbone atoms of glutathione synthetase protein in alpha-naphthol complex was assessed by studying the radius of gyration as a function of time as shown in Figure 10. The radius of gyration of backbone atoms decreased upon binding with alpha-naphthol, which indicated very few conformational changes in the secondary structure of the protein. The number of hydrogen bonds formed between 2HGS and alpha-naphthol complex during MD simulation was also calculated, and a variable profile was observed which fluctuated between 0 and 2 with a mean value of 1, as shown in Figure 11. Local mobility or flexible region in the protein was identified by the RMSF obtained from the last 1000 ps trajectory, and was shown in Figure 12. As seen in the figure, the residue involved in complex formation did not show higher fluctuation in the alpha-naphthol bound complex as compared to the unbound form throughout the MD simulation, and fluctuations in internal cavity residues were lower than those in the others, which indicated that the binding site during MD simulation remained approximately rigid.

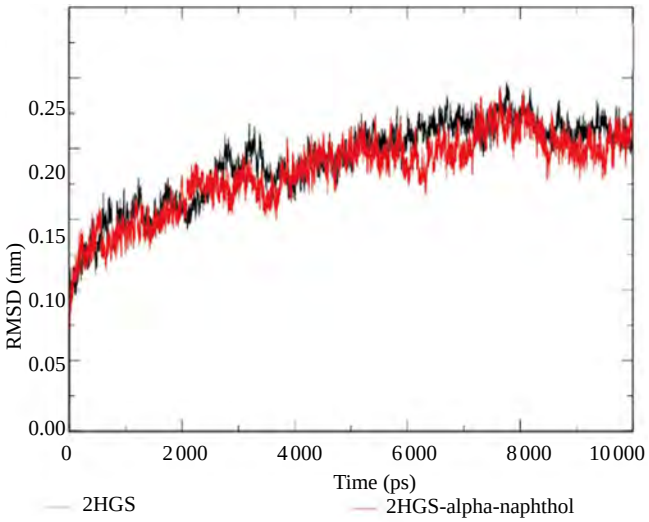


Figure 8. Plot of RMSD of backbone of 2HGS unbound (black) and 2HGS-alpha-naphthol complex (red).

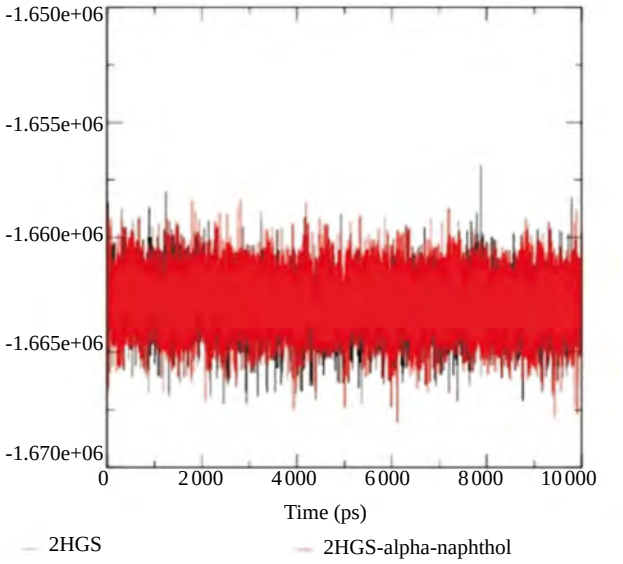


Figure 9. Potential energy profile of 2HGS unbound (black) and 2HGS-alpha-naphthol complex during 10 000 ps simulation.

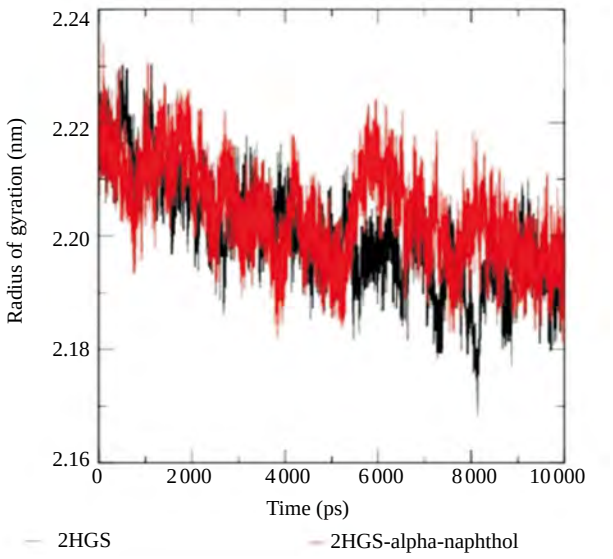


Figure 10. Plot of radius of gyration during 10 000 ps simulation of 2HGS protein unbound (black) and 2HGS-alpha-naphthol complex (red).

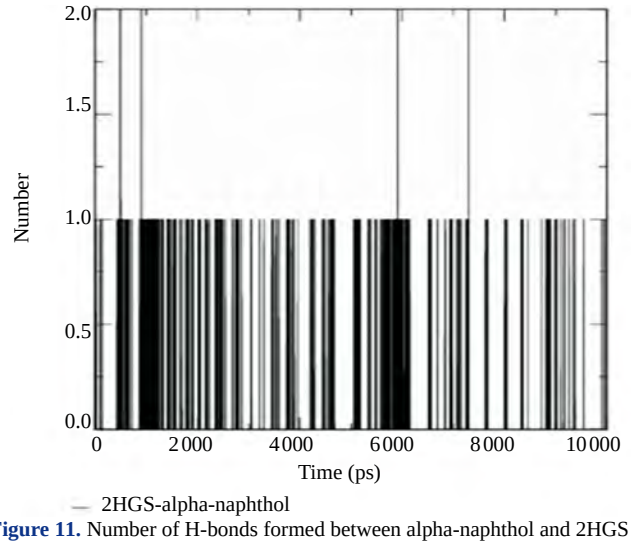


Figure 11. Number of H-bonds formed between alpha-naphthol and 2HGS during 10 000 ps simulation.

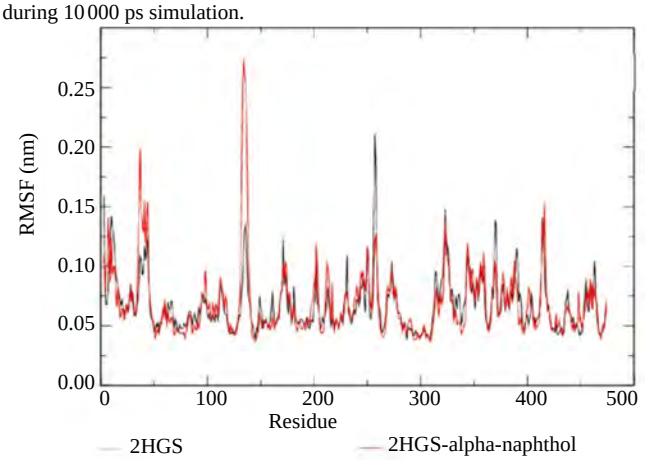


Figure 12. RMSF profile of 2HGS protein residues in unbound (black) with 2HGS-alpha-naphthol complex (red).

4. Discussion

According to docking results, carbaryl and butylated hydroxytoluene show maximum inhibition with p38 MAPK14 protein. Further, MD study of these complexes reveals that carbaryl shows perfect binding with p38 MAPK14 protein and it also has the maximum negative binding energy ΔG -5.36 kcal/mol. It is reported that Th1 mediated cellular immune response plays an important role in the host clearance of *M. tuberculosis* infection and granuloma formation[26-28]. p38 MAPK14 is an 8 kDa intracellular signal transduction protein whose activation is a prerequisite for the *in vitro* production of several cytokines like interleukin-1, 6, 8 and TNF-α leading to the induction of macrophage. The macrophage has potent antimicrobial substance, including reactive oxygen species and nitric oxide, which have the potential to degrade the ingested microbe[29,30]. Thus, the destruction or any inhibition in this protein leads to the evasion of an immune response in the host and they become more susceptible to *M. tuberculosis* infection.

Alpha-naphthol, another toxic chemical used in previous study, showed maximum binding with the glutathione synthetase protein. In this study, the MD simulation trajectory of their docked complex also showed its stability behaviour. It is reported that glutathione synthetase also plays a major role in regulating immune cell function by regulating the Th1 and Th2 type immunity[31]. Glutathione synthetase shows antioxidant property and has potential to protect cell from toxic effects generated by the reactive oxygen intermediate and reactive nitrogen intermediate generated by activated macrophages for the protection against *M. tuberculosis*. It also has immune enhancing effect leading to

the growth inhibition of *M. tuberculosis*[32,33].

The above study revealed that carbaryl and alpha-naphthol may disrupt the normal functioning of p38 MAPK14 and glutathione synthetase proteins, which play a role in providing immunity against *M. tuberculosis*. Thus, any disturbance in the normal functioning of these proteins can lead an individual to become prone to *M. tuberculosis* infection.

In the present study, the molecular docking of the dumped toxic chemicals was performed and from the various docked poses, we selected the best binding pose on the basis of binding energy. Based on the above study, we can reveal that the carbaryl and alpha-naphthol may disrupt the normal functioning of immune proteins providing immunity against *M. tuberculosis*, causing the survivors living in the vicinity of UCIL, Bhopal to become more prone to *M. tuberculosis* infection due to immunosuppression.

Conflict of interest statement

We declare that we have no conflict of interest.

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