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Exploration of phytochemical compounds from Millets as NRF2 activators using molecular docking and molecular dynamics simulation approaches

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Oxidative stress plays vital role in progression of several diseases like cardiovascular, neurological and gastrointestinal disorders. Millets, on regular consumption, are known to have beneficial effects. They are also rich in phytochemicals. In the current study, these phytochemicals are aimed against the complex of KEAP1-NRF2 and activating NRF-2 which is a potent inducer of anti-oxidant enzymes like heme-oxygenase and superoxide dismutase. Molecular docking was conducted to study compounds that can form an interaction similar to 1VX (co-crystalized ligand). CDOCKER interaction energies of isovitexin, vitexin, kaempferol, protocatechuic acid, and vanillic acid were better than 1VX. The best three molecules- isovitexin, vitexin and kaempferol, based ontheir CDOCKER interaction energy, were subjected to molecular dynamic simulation for 100 ns which revealed their stability at active site. Further MM-PBSA binding free energy of phytochemicals was calculated. Values of isovitexin, vitexin, kaempferol and 1VX are -32.04 Kcal/mol, -24.81Kcal/mol. -4.17 Kcal/mol, and -10.25 Kcal/mol, respectively, which indicate high degree of binding compared to co-crystallized ligand 1VX. Results indicated that selected compounds- isovitexin, vitexin, kaempferol can act as NRF2 activators which further can be confirmed through *in vitro* and *in vivo* studies.

Keywords: Isovitexin, Kaempferol, Millets, NRF2, Vitexin

Oxidative stress is defined as imbalance between reactive oxygen species, reactive nitrogen species and anti-oxidants contributing to defence mechanisms[1]. Free radicals are extremely reactive atoms with one or more unpaired electron in their outer orbital and produced when oxygen interacts with definitive molecules[2]. The terms reactive oxygen species (ROS) and reactive nitrogen species (RNS) indicate reactive radical and non-radical derivatives of oxygen and nitrogen, respectively. ROS and RNS play vital role in ageing and progression of several diseases like cardiovascular disease, respiratory diseases, cancer and complications of diabetes mellitus[3,4].

Endogenous defence mechanisms to counter free radicals are classified into enzymatic and non-enzymatic defences. The major antioxidant enzymes are superoxide dismutase, catalase, and glutathione peroxidase. Non enzymatic anti-oxidants include bilirubin, α-tocopherol (vitamin E), and β-carotene[5,6]. These antioxidant levels inside the cell are synchronized by the gene expression of various transcription factors, stimulated under stressed conditions[7,8]. Many investigations around the globe suggest that nuclear factor erythroid 2-related factor (NRF2) in mammalian cells seems to play an important role in the maintenance of normal cellular physiological conditions, whenever there is a damage from oxidative stress by inducing several anti-oxidant enzymes[9]. NRF2 is a transcription factor, mainly regulates the expression of genes that combat with free radicles and decrease oxidative stress[10]. Kelch-like ECH associated protein (KEAP1) inhibited the activity of NRF2 by preventing its binding to the antioxidant response element[11]. Phytochemicals such as polyphenols, flavonoids, steroids, are secondary metabolites of plants that are commonly present in different parts of plants. They have shown beneficial effects in the treatment of diseases like diabetes, cardiovascular disease, cancer and obesity[12]. Phytochemicals have property of scavenging ROS and showed anti-oxidant property in both *in vitro* and *in vivo* studies. Apart from this, they can activate NRF2 promoting its translocation into nucleus[13].

Millets been used as a food stuff from centuries in continents of Asia and Africa because of their health benefits[14,15]. It constitutes various essential

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biomolecules like protein, carbohydrate, vitamins, etc. required for healthy lifestyle[16,17]. India, being one of the top producers of millet, is producing different assorted types of millet such as foxtail millet (*Setaria italica*), pearl millet (*Pennisetum glaucum),* finger millet (*Eleusine coracana*), kodo millet (*Paspalum scrobiculatum*), proso millet (*Panicum miliaceum*), little millet (*Panicum sumatrense*) and barnyard millet (*Echinochloa esculenta*)[18]. They are not only rich in various biomolecules but also have various disease preventive properties like minimizing the risk of colon and colorectal cancer, reduction in cholesterol and triglycerides levels, antidiabetic activity, treating celiac disease, attenuates hepatic damage, and many more because of the presence of various bioactive molecules[19]. In the present study, we aimed to investigate the potential of phytochemicals of millets to activate NRF2 by inhibiting KEAP1-NRF2 complex through molecular docking and molecular dynamic simulation studies.

Materials and Methods

Molecular docking studies

Preparation of protein

The crystallographic structure of KEAP1-NRF2 crystal structure with co-crystallized ligand 1VX (PDB ID: 4L7D) with human origin was downloaded from the RCSB protein data bank (https://www.rcsb.org/structure/4L7D). 1VX is NRF2 activator and a direct inhibitor of the KEAP1-NRF2 complex formation. To optimize the protein structure for further analysis, we employed the BIOVIA Discovery Studio (DS) 2022 software "prepare protein" feature, ensuring the inclusion of loop construction and protonation. Furthermore, we eliminated heteroatoms, and the native inhibitors from the protein structure to create a refined and suitable starting point for our research.

Preparation of ligand

Phytochemicals sourced from the millets were identified and collected through a comprehensive review of existing literature and databases[20,21]. These compounds were then download from PubChem in the form of 3D structural data files. To ensure their suitability for subsequent docking analysis, the 3D structures of these ligands were processed by utilizing the "Small Molecule" tool within BIOVIA DS 2022.

Molecular docking

The concept of structure based drug designing was implemented to understand the receptor-ligand binding through technique of molecular docking. The prepared protein and ligand were docked using tool CDOCKER, a grid-based molecular dynamics simulation. For the molecular docking study, the binding site of co-crystallized ligand to the KEAP1-NRF2 was selected as the active site of interest. The specific coordinates for this binding site on NRF2 was selected as a grid in 3D Cartesian space X: -22.79; Y: 39.11; Z: -36.57 and 1VX was used as positive control[22]. Furthermore, interpretation of docked complexes through scoring function and CDOCKER interaction energies was done to assess the interactions between the ligands and the receptor[23,24].

Molecular dynamics simulation and Molecular Mechanics-Poisson Boltzmann Surface Area (MM-PBSA)-based binding free energy calculation

Molecular dynamics (MD) simulation studies were performed using Biovia Discovery Studio software, 2022 based on the CHARMm molecular mechanics. Top ranked poses without any restraints were used for the study. The complexes were solvated based on previous literature[25,26]. Minimization was carried out based on the previous literature[27]. After heating and equilibration method, NAMD was used for the final production and simulation time of 100,000 ps (100 ns) was set. Whole process was set with Langevin Dynamics (temperature) and Langevin Piston (pressure). 2 fs was used as time step for the integration. Multiple-time step algorithm was used to integrate the long- and short-range forces with Impulse/Verlet-I[28]. Results were saved at 40ps. The output trajectory files were used to analyze parameters such as root mean square deviation (RMSD), radius of gyration (rg) and root mean square fluctuations (RMSF).

The binding free energies for each protein-ligand complex were calculated using MM-PBSA. The free energy of the protein-ligand binding (ΔG binding) was calculated using equation stated below.

$$\Delta G \text{ binding} = \Delta G \text{ complex} - [\Delta G \text{ protein} + \Delta G \text{ ligand}]$$

Results and Discussion

Molecular docking

Millets have shown several beneficial effects like antidiabetic, anti-cancer, antiatherogenic, and antibacterial properties[29]. Regular consumption of millets, can reduce occurrence of gastrointestinal disorders, cardiovascular disease, and several other ailments[30]. Recent studies have showed beneficial

effects of phytochemicals of millets against diabetes and hypertension through *in silico* studies[21]. In this study, we have deployed bioactives from millets against NRF2, a potent inducer of anti-oxidant defense mechanism against free radicals. To our knowledge, this is the first study of phytochemicals of millets exploring their ability to activate NRF2 (Fig. 1).

The CDOCKER interaction energy obtained after docking of the compounds into KEAP1-NRF2 (PDB ID: 4L7D) are presented in Table 1. Hydrogen bonds and vander wall interactions play an important role in the binding of ligand to the protein. Apart from hydrogen bond, vander wall forces also contribute to binding of ligand to the protein despite their weakest nature in terms of energy[31]. *In silico* results revealed that many compounds showed better affinity against NRF2 in comparison with 1VX. Isovitexin (Fig. 2A) exhibited the highest affinity to the active site followed by vitexin (Fig. 2B) and kaempferol (Fig. 2C). 1VX formed two hydrogen bonds ARG A:415, SER A:602 at the active site (Fig. 2D) and formed several vander wall interactions, pi–pi stacking, pi-alkyl, alkyl and pi-sigma interactions at SER A:508, ALA A:556, GLY A:509, GLY A:462, GLY A:603, GLY A:364, SER A:555, TYR A:334, SER A:363, ASN A:382, ARG A:380, ASN A:414, PHE A:577, TYR A:572 (Fig. 2D). 1VX showed CDOCKER interaction energy of -49.8822 Kcal/mol. Among all phytochemicals, isovitexin showed highest CDOCKER interaction energy of -77.5467 Kcal/mol.

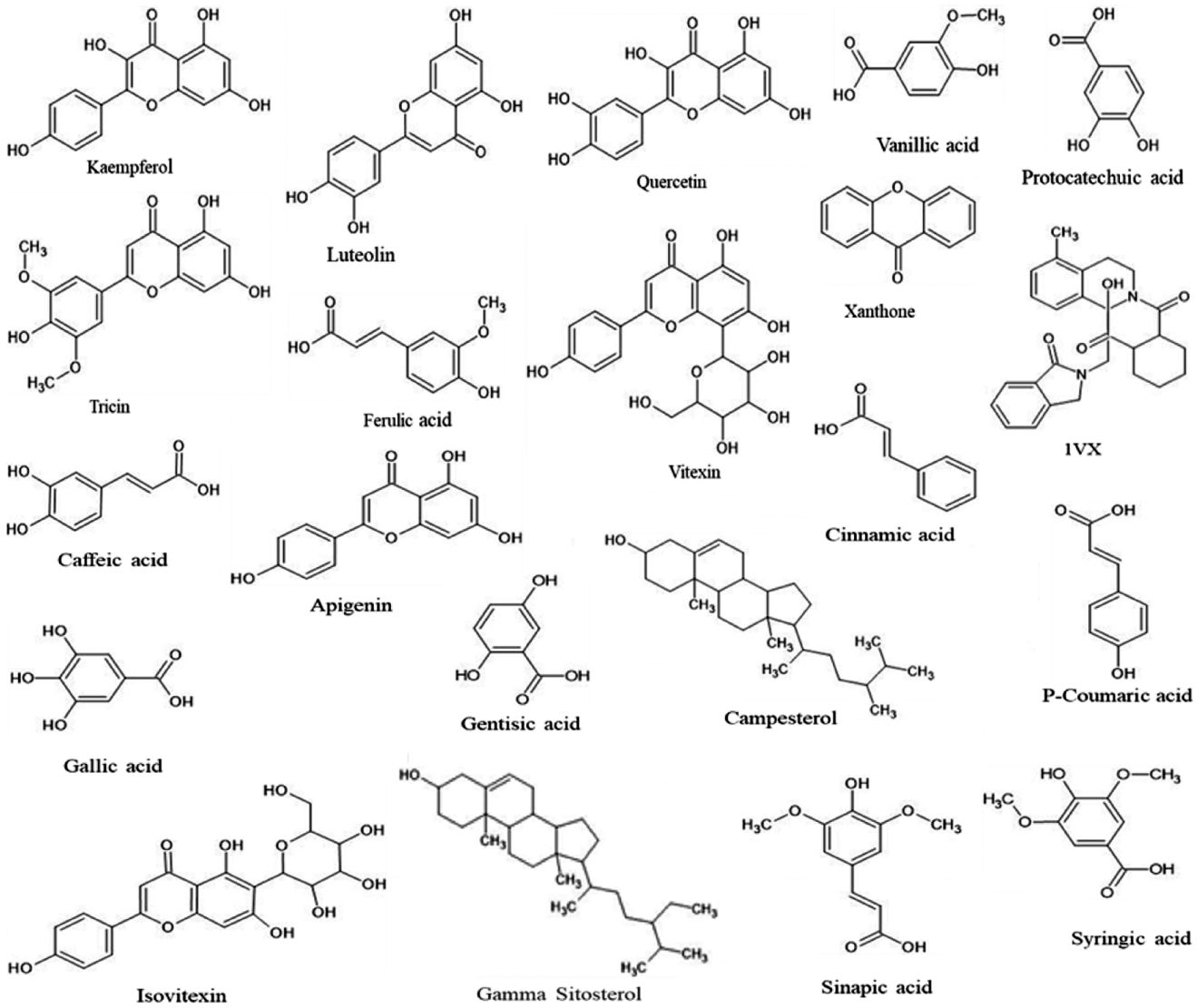

Fig. 1 — Structures of various phytochemicals from millets and 1VX used in present study

Table 1 — Results of the docking of compounds showing hydrogen bond and hydrophobic interactions of Millets on the crystal structure of KEAP 1-NRF2

Ligands	Molecular Docking Parameters		
	CDOCKER interaction energy (-KCAL/ MOL)	Hydrogen bond interaction	Hydrophobic interaction
IVX	49.8822	ARG A:415, SER A:602	SER A:508, ALA A:556, GLY A:509, GLY A:462, GLY A:603, GLY A:364, SER A:555, TYR A:334, SER A:363, ASN A:382, ARG A:380, ASN A:414, PHE A:577, TYR A:572
Caffeic acid	36.7794	GLY A:462, SER A:508	ALA A:556, TYR A:572, PHE A:577, SER A:602, TYR A:334, GLY A:603, SER A:363, GLY A:364, GLY A:509, ARG A:415, ARG A:483
Apigenin	36.6296	LEU A:365, VAL A:604, GLY A:364,GLY A:603, ARG A:483, GLY A:509, ILE A:416	ALA A:366 GLY A:464, VAL A:463, GLY A:417, GLY A:462 ARG A:415, SER A:508, PHE A:478, TYR A:525, SER A:555
Campesterol	42.1001	-	PHE A:577, TYR A:572, GLY A:462, ALA A:510, GLY A:464, VAL A:463, GLY A:417, LEU A:557, ILE A:416, GLY A:509, LEU A:365, VAL A:604, ARG A:415, GLY A:364, ALA A:556, GLY A:603, SER A:363, ASN A:414, SER A:602, TYR A:334
Cinnamic acid	32.8094	SER A:508, GLY A:462, GLY A:509	GLY A:364, SER A:363, GLY A:603, TYR A:334, ALA A:556, SER A:602, PHE A:577, ARG A:483. ARG A:415
P-Coumaric acid	35.3668	SER A:602, GLY A:603, ARG A:415, SER A:508	PHE A:577, TYR A:334, TYR A:572, SER A:555, GLY A:509, ARG A:415, ARG A:483, GLY A:603, SER A:363
Gallic acid	41.2643	GLY A:509, GLY A:462, GLY A:364, GLY A:603	ARG A:415, ARG A:483, ALA A:556, SER A:508
γ- Sitosterol	43.37	-	TYRv572, ALA A:556, GLY A:364, ARG A:415, GLY A:603, GLY A:462, GLY A:509, GLY A:558, VAL A:604, LEU A:557, LEU A:65, VAL A:463, GLY A:417, ILE A:416, ALA A:366, SER A:363, SER A:602, TYR A:334, PHE A:577
Gentisic acid	32.01	ARG A:415, GLY A:462	GLY A:603, SER A:602, ALA A:556, TYR A:572, GLN A:530, SER A:555, TYR A:525, SER A:508, GLY A:509
Isovitexin	77.5467	SER A:602, GLY A:364, GLY A:603, LEU A:365, VAL A:604, GLY A:462, VAL A:463, GLY A:509	GLN A:530, SER A:555, TYR A:572, PHE A:577, TYR A:334, SER A:363, ALA A:556, GLY A:605, ILE A:416, LEU A:557, GLY A:464, ARG A:483, ARG A:415, SER A:508, TYR A:525
Kaempferol	61.698	GLN A:530, ARG A:483, GLY A:509, GLY A:462	ILE A:461, ARG A:415, ALA A:556, SER A:508, PHE A:478, GLY A:574, TYR A:572, SER A:555, TYR A:525
Luteolin	36.7917	VAL A:463, GLY A:462, ILE A:416, GLY A:608, GLY A:364, SER A:363,	GLY A:464, GLY A:417, ALA A:366, VAL A:604, LEU A:369, GLY A:609, TYR A:334, ALA A:556, ARG A:415, GLY A:509
Quercitin	48.25	VAL A:415, ILE A:416, LEU A:365	GLY A:509, VAL A:463, GLY A:462, GLY A:364, VAL A:604, TYR A:572, GLY A:603, ALA A:556, SER A:602, TYR A:334, PHE A:577
Sinapic acid	43.72	SER A:602, SER A:508, ARG A:415, GLY A:462	TYR A:334, GLY A:603, SER A:363, ASN A:414, GLY A:364, ILE A:461, TYR A:525, GLY A:509, ARG A:483, SER A:555 ALA A:556, PHE A:577, TYR A:572
Syringic acid	38.50	SER A:555, ARHG A:415, GLY A:462	TYR A:572, SER A:602, SER A:363, GLYV603, ALA A:556, GLY A:509, ARG A:483, SER A:508, GLN A:530, TYR A:525
Tricin	42.37	ARG A:415, ALA A:510, GLY A:464, VAL A:604, LEU A:365	SER A:555, SER A:5008, ARG A:483, TYR A:525, VAL A:463, GLY A:462, GLY A:511, VAL A:512, GLY A:605, ALA A:366, GLY A:364, SER A:363, GLY A:603, TYR A:334, SER A:602

(*Contd*.)

Table 1 — Results of the docking of compounds showing hydrogen bond and hydrophobic interactions of Millets on the crystal structure of KEAP 1-NRF2 (*Contd*.)

Ligands	Molecular Docking Parameters		
	CDOCKER interaction energy (-KCAL/ MOL)	Hydrogen bond interaction	Hydrophobic interaction
Vanillic acid	53.83	GLN A:530, GLY A:462	TYR A:572, SER A:555, ALA A:556, GLY A:509, SER A:508, PHE A:478, ARG A:483, ARG A:415, TYR A:525
Xanthone	23.48	ASN A:414, ARG A:415	ALA A:556, GLY A:364, ARG A:380, ASN A:387, ASN A:382, TYR A:334, SER A:602, PHE A:577, GLY A:603, SER A:363
Protocatechuic acid	50.58	GLN A:530	ARG A:483, ARG A:415, PHE A:478, TYR A:572, SER A:555, ALA A:556, GLY A:509 TYR A:525, GLY A:462, SER A:508, ILE A:461
Ferulic acid	39.8392	SER A:602, GLY A:603, GLY A:462, SER A:508	TYR A:334, SER A:363, ALA A:556, GLY A:364, ARG A:483, ARG A:415, GLY A:509, PHE A:577, TYR A:572
N-coumaryl	43.8436	TYR A:334, VAL A:463, VAL A:604, ILE A:529	SER A:363, ALA A:556, GLY A:608 GLY A:364, GLY A:508, GLY A:462, ILE A:416, GLY A:417, VAL A:418, VAL A:469, GLY A:464, VAL A:606, GLY A:558, GLY A:609, ALA A:366, LEU A:557, ARG A:415, LEU A:369, SER A:602, PHE A:577
Vitexin	62.59	GLN A:530, SER A:555, ASN A:414, SER A:602, TYR A:334	ARG A:380, ARG A:415, TYR A:525, TYR A:572, GLY A:364, GLY A:603, ALA A:556, PHE A:577, SER A:363

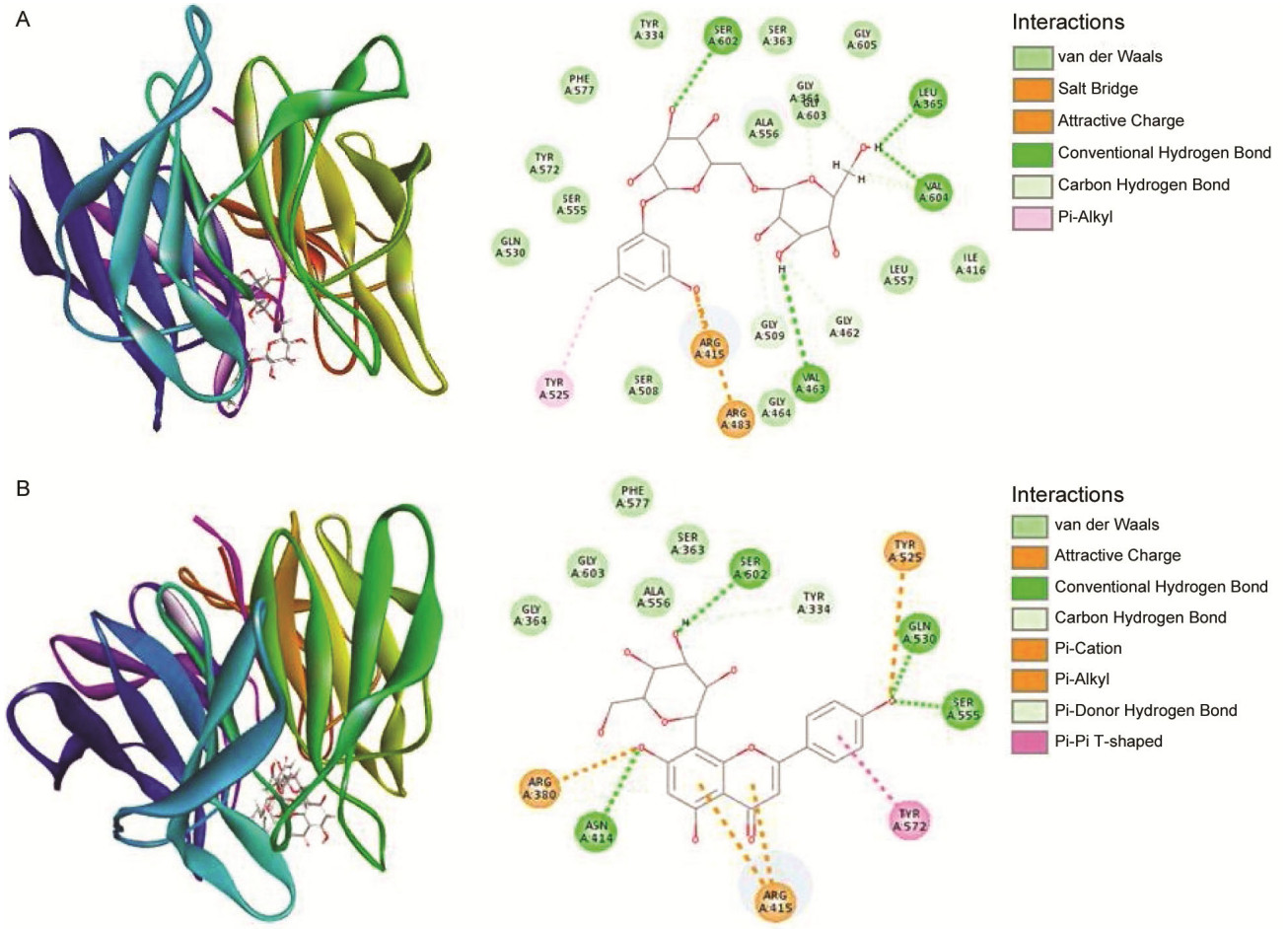

Fig. 2 — 3D and 2D representation of the predicted binding mode of (A) isovitexin; and (B) vitexin

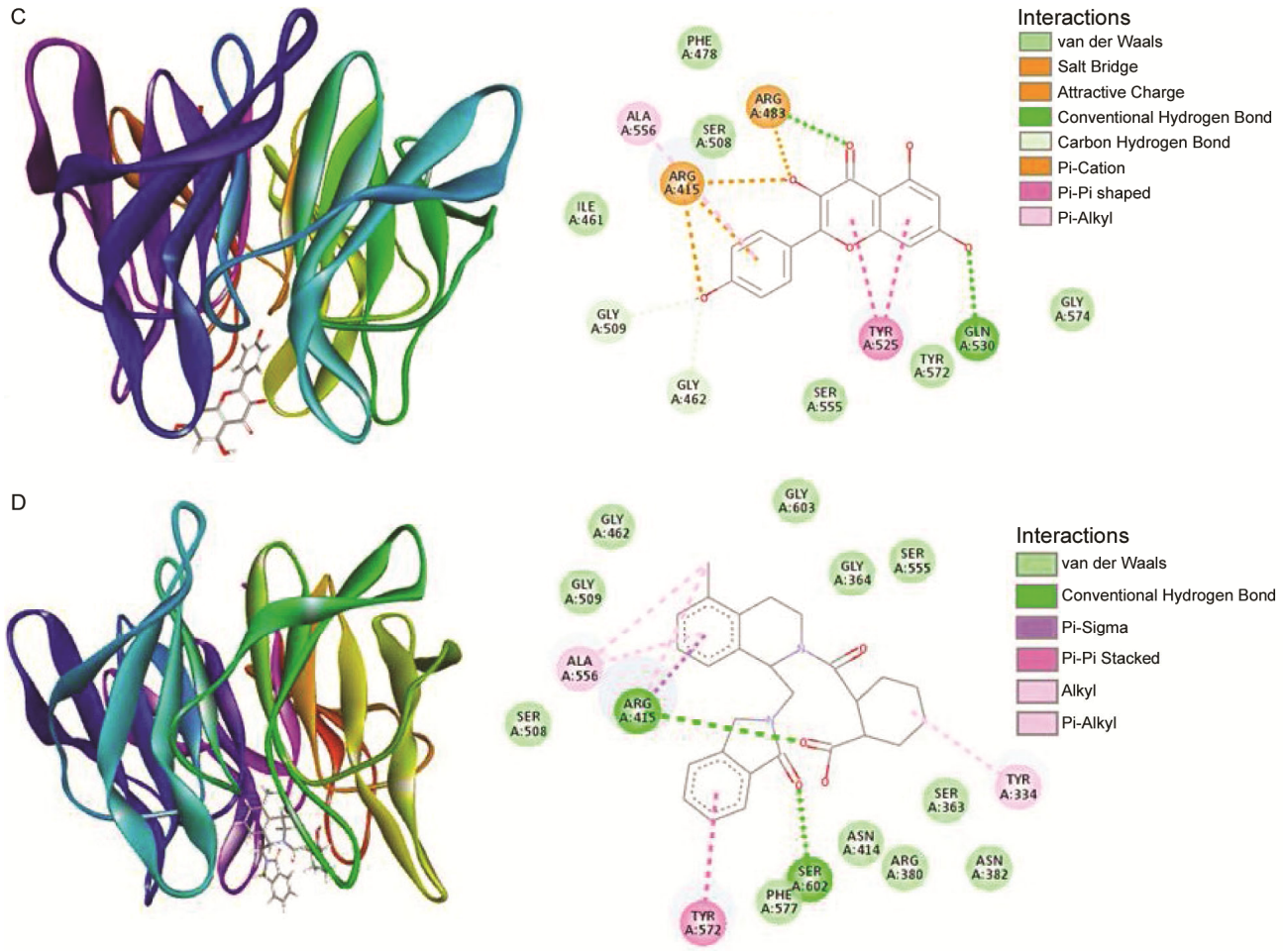

Fig. 2 —3D and 2D representation of the predicted binding mode of (C) kaempferol; and (D) 1VX inside the active site of KEAP 1-NRF2

Isovitexin formed eight hydrogen bonds, SER A:602, GLY A:364, GLY A:603, LEU A:365, VAL A:604, GLY A:462, VAL A:463, and GLY A:509. It formed attractive charges at ARG A:415 and ARG A:483 and formed pi-alkyl interaction at TYR A:525. Remaining interactions include vander wall interactions at GLN A:530, SER A:555, TYR A:572, PHE A:577, TYR A:334, SER A:363, ALA A:556, GLY A:605, ILE A:416, LEU A:557, GLY A:464, and SER A:508 (Fig. 2A). Vitexin showed CDOCKER interaction energy of -62.59 Kcal/mol forming hydrogen bond interactions at GLN A:530, SER A:555, ASN A:414, SER A:602, TYR A:334 and hydrophobic interactions at ARG A:380, ARG A:415, TYR A:525, TYR A:572, GLY A:364, GLY A:603, ALA A:556, PHE A:577, SER A:363 (Fig. 2). Kaempferol showed CDOCKER interaction energy of-61.698 Kcal/mol with four hydrogen bond interactions at GLN A:530, ARG A:483, GLY A:509, GLY A:462 and hydrophobic interactions at residues ILE A:461, ARG A:415, ALA A:556, SER A:508, PHE A:478, GLY A:574, TYR A:572, SER A:555, TYR A:525 (Fig. 2C).

Molecular dynamics simulation

Root-mean square deviation (RMSD)

RMSD reveals the degree of the positional change of the molecular structure over time. After simulation studies, the graph obtained by calculating RMSD indicates the structural changes in the structure specifically the deviation between several structures can be best interpreted. RMSD calculation represents spatial differences of the molecules present in the protein backbone during the simulation[32,33]. Isovitexin has showed highest CDOCKER energy interaction, average RMSD value (Fig. 3A), observed for the isovitexin was calculated to be 1.89 Å. The trajectory remains almost same throughout the entire simulation period after 100000 ps without any deviation. Kaempferol exhibited an average RMSD value of 1.76 Å, trajectory is same as isovitexin without any

deviation till 100 ns. Vitexin showed a spike at 18000 ps and had no any deviation till 80000 ps and had a spike after that and average RMSD value of 2.93 Å indicating its flexibility and less compactness in the structure. Positive control 1VX showed increased trajectory values of RMSD with average value of 3.3Å.

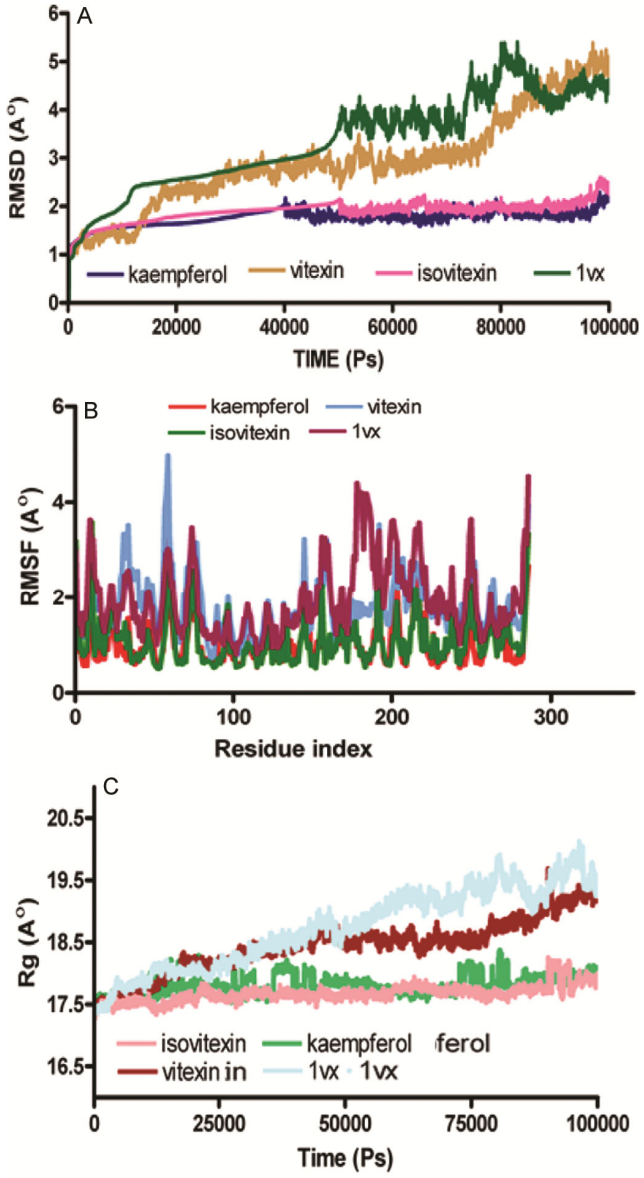

Fig. 3 — Molecular dynamic simulation graph (A) RMSD; (B) RMSF; and (C) Rg of phytochemicals and 1VX

Isovitexin and kaempferol showed no fluctuations indicating compactness in the protein structure.

Root mean square fluctuation (RMSF)

The RMSF value represents the mobility and flexibility of a structure[34,35]. To examine the binding efficiency of phytochemicals and 1VX, the RMSF values for C-α atoms of all the residues were measured based on 100 ns trajectory data. Values were analyzed for phytochemicals and 1VX. Results are represented in the Fig. 3B. The average RMSF value for 1VX, Isovitexin, vitexin and kaempferol are 1.96 Å, 1.08 Å, 1.83 Å and 1.04 Å, respectively. Co-crystallized ligand and vitexin showed much fluctuations compared to isovitexin and kaempferol. The RMSF values of key amino acid residues, namely, SER363, ARG380, ASN382, ARG415, ARG483, TYR525, GLN530, SER508, SER555 and SER602 did not fluctuate significantly indicating that there was no much deviation at the active site after binding of phytochemicals.

Radius of gyration (Rg)

Rg analysis represents compactness of the protein structure. If the protein is in properly folded state, the values of Rg will be same without much deviation[36,37]. We observed that during the simulation period of 100 ns the average Rg value for 1VX, isovitexin, vitexin and kaempferol are 18.74 Å, 17.67Å, 18.44 Å and 17.81 Å, respectively (Fig. 6C). Isovitexin and kaempferol showed no fluctuation in Rg value throughout the entire period of simulation. Vitexin showed constant value till 80000 ps and drift in the value was seen after 80000 ps.

MM-PBSA of the complex

MM-PBSA free binding energies were calculated for each protein-ligand complex (Table 2). The binding free energy of phytochemicals isovitexin, kaempferol, vitexin and 1VX are -32.04 Kcal/mol, -4.17 Kcal/mol, -24.81Kcal/mol and -10.25 Kcal/mol, respectively. Binding energies of isovitexin and vitexin were better compared to 1VX indicating stable thermodynamic complexes.

Table 2 — Results of the MM-PBSA analysis of compounds of millets along with MCC950

Ligands	Energy Parameters (Kcal/mol)			
	Final binding energy ($G_{binding}$)	Complex Energy ($G_{complex}$)	Ligand Energy (G_{Ligand})	Protein Energy ($G_{protein}$)
Kaempferol	-4.17	-12,462.21	-206.05	-12,251.98
Isovitexin	-32.04	-12,354.97	-89.33	-12,233.60
Vitexin	-24.81	-12,451.67	-234.32	-12,192.53
1VX	-10.25	-12,175.99	30.65	-12,196.39

Conclusion

Present study was aimed to study the bioactives from millets to inhibit KEAP1-NRF2 complex and activating NRF2. Docking analysis was done to identify compounds interaction with active site. Isovitexin, kaempferol, vitexin showed good interaction. Molecular dynamic simulation showed that these compounds were stable after interacting with protein. These results confirm that they can be better drug candidates as NRF-2 activators. Further, *in vitro* and *in vivo* studies are needed for a clear understanding of their activity.

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Conflict of interest

All authors declare no conflict of interest.

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