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Exploring *in silico* affinity of flavonoids and tannins to human fibroblast growth factor-inducible14 (Fn14), a member of TNF receptor super family

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Abstract:
The Fn14 and TWEAK are the receptor and ligand respectively and their mutual recognition and binding was reported to induce pathogenesis of cancer and chronic autoimmune diseases. We had identified Fn14 as a novel target of low linear energy transfer (LET) ionizing radiation in mice population. In the present study we generated the novel homology model of human Fn14, optimized its energy and validated for authenticity by checking Ramachandran plot and also by calculating the RMSD. Based on our earlier findings with *Hippophae rhamnoides*, a group of flavonoids and tannins were screened for their docking potential with Fn14 at the site where its natural ligand TWEAK was binding. The comparative docking analysis showed that the order of docking, from best to least, was Genistein, Rutin, Gallic acid ethyl ester and Quercetin, respectively. The findings predicted the radio-modifying action of flavonoids and tannins. The study has immediate applications in development of non-toxic drugs/ nutraceuticals that may protect human population from harmful effects of radiation in various situations, such as nuclear accidents, occupational exposure, diagnosis or radiotherapy.

Key words: *Hippophae rhamnoides*, Genistein, Radiation, Rutin, Gallic acid Ethyl ester, Quercetin, TWEAK.

Background:
Low linear energy transfer (LET) ionizing radiation exposure causes multiple pathologies, dose dependent carcinogenicity and lethality. The threat of unwanted exposure to radiation is increasing with increase in the use of ionizing radiation in industry, warfare, medicine, diagnosis and therapy. Identification of radiation targets, their role in pathogenesis and development of agents which could counter the radiation hazards (generally termed as radioprotective drugs/ radiation countermeasures) is an important field of research. The challenge is so enormous that despite decades of research so far no drug, meant for whole body radiation protection, has been approved for human use. The only drug, WR2721, approved for use with radiotherapy displays multiple toxicities including neurotoxicity. Earlier, we had identified the novel radiation target protein, the fibroblast growth factor-inducible 14 (Fn14) or tumor necrosis factor receptor super family member 12A (TNFRSF12A), which showed radiation dose dependent increased transcription in the liver of whole body ^{60}Co-gamma-irradiated mice **[1].** The *fn14* or *TNFRSF12A* is a growth-factor-inducible immediate-early-response gene and codes for a type I trans-membrane protein Fn14, which is 102-amino-acid long. The Fn14 belongs to tumor necrosis factor (TNF) receptor super family. TWEAK (TNF-homologue with apoptosis inducing activity), is a member of the TNF super family and is the specific binding ligand of Fn14. The extracellular ligand-

binding region of Fn14 is a single cysteine-rich domain (CRD) and comprises 53 amino acid residues **[2].** The receptor-ligand recognition between Fn14 and TWEAK induces a variety of cellular processes such as inflammation, immune responses, tissue repair, carcinogenesis etc. **[3, 4].** The constitutive low expression of Fn14 in livers was associated with normal slow hepatocytes turnover without activating the oval cells. On the other hand increased expressions of TWEAK and Fn14 were reported in case of massive liver injury and were associated with uncontrolled proliferation of oval cells or situations associated with hepatocellular carcinoma **[5].** We also reported that adverse effects of radiation were prevented by treating the mice with tannins and flavonoids rich extract of *Hippophae rhamnoides* before irradiation **[6, 7],** which presumably acted via inhibiting the Fn14-TWEAK interaction **[1].**

The objective of this study was to investigate the *in silico* affinity of the flavonoids (quercitin, genistein and rutin) as well as tannins (gallic acid and ellagic acid) towards the human Fn14 region which was binding to the natural ligand TWEAK. The approach was made in two essential steps, first a homology model of human Fn14 protein was developed because no crystallographic structure for human Fn14 is available so far, and second the binding of various antioxidants (tannins and flavonoids) was examined to the TWEAK specific sites on the CRD domain of Fn14.

Methodology:

Model generation of TNFRSF12A

Sequences of human tumor necrosis factor receptor super family member 12A (TNFRSF12A) with Uniport ID: Q9NP84 consists 129 amino acids were obtained from Uniport database (www.uniprot.org). To get tertiary structure of TNFRSF12A, sequence alignment was performed by using online Basic Local Alignment Search Tool for Protein (BLASTp) against Protein Data Bank (PDB) (http://www.pdb.orgN/ pdb/ home/home.do).In the output result no proper homologous entries were found and therefore, TNFRSF12A protein was modeled using Iterative Threading Assembly refinement (I-Tasser sever) (http: //zhanglab.ccmb.med.umich.edu/I-TASSER/). Multiple templates were used in the iterative structural assembly simulation method **[8, 9].** To get the best model, minimum confidence score protein was selected for further study.

Energy minimization and structure validation

The best selected model of TNFRSF12A was subjected to GROMACS 4.5.3 Package for energy minimization **[10, 11]** and the structure energy minimization was done by using OPLS-AA/L force field **[12].** In subsequent step, the structure was embedded in SPC216 water molecules cubic box **[13].** The charged states of ionizable groups, which usually occur in the normal state at pH 7.0, were neutralized by adding respective ions in the system. The ion treatment was followed up by energy minimization. The equilibrium of the system was maintained according to the protocol in two phases. The first phase included NVT ensemble in which a short 100 picoseconds (ps) position restrained molecular dynamics simulation (MDS) at 300k was done by using a Berendsen thermostat for ensuring proper stabilization of the temperature. In the second phase of NPT ensemble, loops position-restrained MDS at 300K and 1 bar was done by using a Parrinello-Rahman barostat pressure coupling to stabilize the system in relation to pressure and density **[14].**

At the end, unrestrained 10 nanoseconds (ns) MDS was done on the NPT ensemble for both structures. The output obtained was further subjected to quality checks, numerical graphs and interpretation of data by using Xmgrace software. The stereo chemical quality and parameter of modeled structures as well as minimized generated structures were scrutinized by PROCHECK and WHATIF **[15, 16],** ERRAT was used to determine non-bonded interaction between different atom types in structures **[17].** VERIFY3D **[18, 19]** was used to check the compatibility of amino acids in models. Finally the secondary structural changes and conformational analysis were performed in Profane in PDBsum **[20].**

Selection of inhibitors

Based on our previous report **[6, 7]** the active constituents of leaf extract from *Hippophae rhamnoides* i.e., flavonoids (Quercitin, Rutin and Genistein) and tannins (Gallic acid and Ellagic acids), were selected. 3D structures of Ellagic Acid (CID: 5281855), Genistein(CID:5280961), Rutin (CID:5280805) & Gallic acid ethyl ester (CID:13250) and Quercetin (CID:5280343) were downloaded from Pubchem (http:// pubchem.ncbi.nlm.nih. gov /).

Docking of inhibitors

All inhibitors of TNFRSF12A were tested to find out the best inhibitor, which could bind to the site of its natural ligand protein TWEAK. Docking was performed by using Autodock 4.2.0 in the platform of MGLTool 1.5.4. **[2, 21].** AutoGrid was used to generate grid maps. The grid box dimension was 60X60X60 and spacing between the grid points was 0.375 Å. Each job consisted 50 independent runs and the generated log files were analyzed using MGLTool **[22].**

Result & Discussion:

Molecular dynamics simulation analysis and structure validation of TNFRSF12A

Sequences of human tumor necrosis factor receptor super family member 12A (TNFRSF12A) with Uniport ID: Q9NP84 consists 129 amino acids were obtained from Uniport database (www.uniprot.org). Best model structure, which had -3.01 confidence score amongst the top five predicted models, by I-Tassar Sever, was subjected for MD simulations to get a stable structure. The structures were compared and the main-chain root mean square deviations (RMSD) were calculated as a function of time. The resulting RMSD profiles are shown in **(Figure 1A)**. Major structural change occurred during the initial few picoseconds at RMSD of ~0.55 nm, subsequently, the system got equilibrated and structural deviations were minimized. The main-chain root mean square fluctuations (RMSF), indicated that the initial 250 C-terminal atoms fluctuated more **(Figure 1B)** out of the 1975 atoms of structure. All catalytic site residue atoms had similar fluctuation pattern. However, the amino acids falling in the interacting site showed minimum fluctuations, indicating that it was a promising site for docking **(Figure 1C).**

Fifty successive structures were generated with 200ps time difference. Between each structure trajectory was 10ns. The stereo chemical quality of each amino acid of modeled Fn14

(TNFRSF12A) was minimized and the structure was renamed as TNFRSF12Am. For both the structures, Ramachandran plot was used to measure RMSD **(Figure 1D).** PROCHECK based evaluation results showed better stereo chemical quality in comparison to initially modeled TNFRSF12A**.** ERRAT calculated the overall quality factor for non-bonded atomic interactions. The higher ERRAT score meant better quality of structure**.** The ERRAT score for TNFRSF12Am and TNFRSF12A structure were 41.538 and 23.967 respectively. The ERRAT score for TNFRSF12Am structure shows an enhancement in atomic interaction after molecular dynamics. Simulated structures were evaluated by VERIFY3D. The simulated structures showed better sequence-to-structure agreement in comparison to initial proteins as shown in **Table 1 (see supplementary material).** The overall quality G-factor scores for TNFRSF12Am and TNFRSF12A were -0.71 and -0.64 respectively, indicating that minimized one had good quality in comparison to initial model.

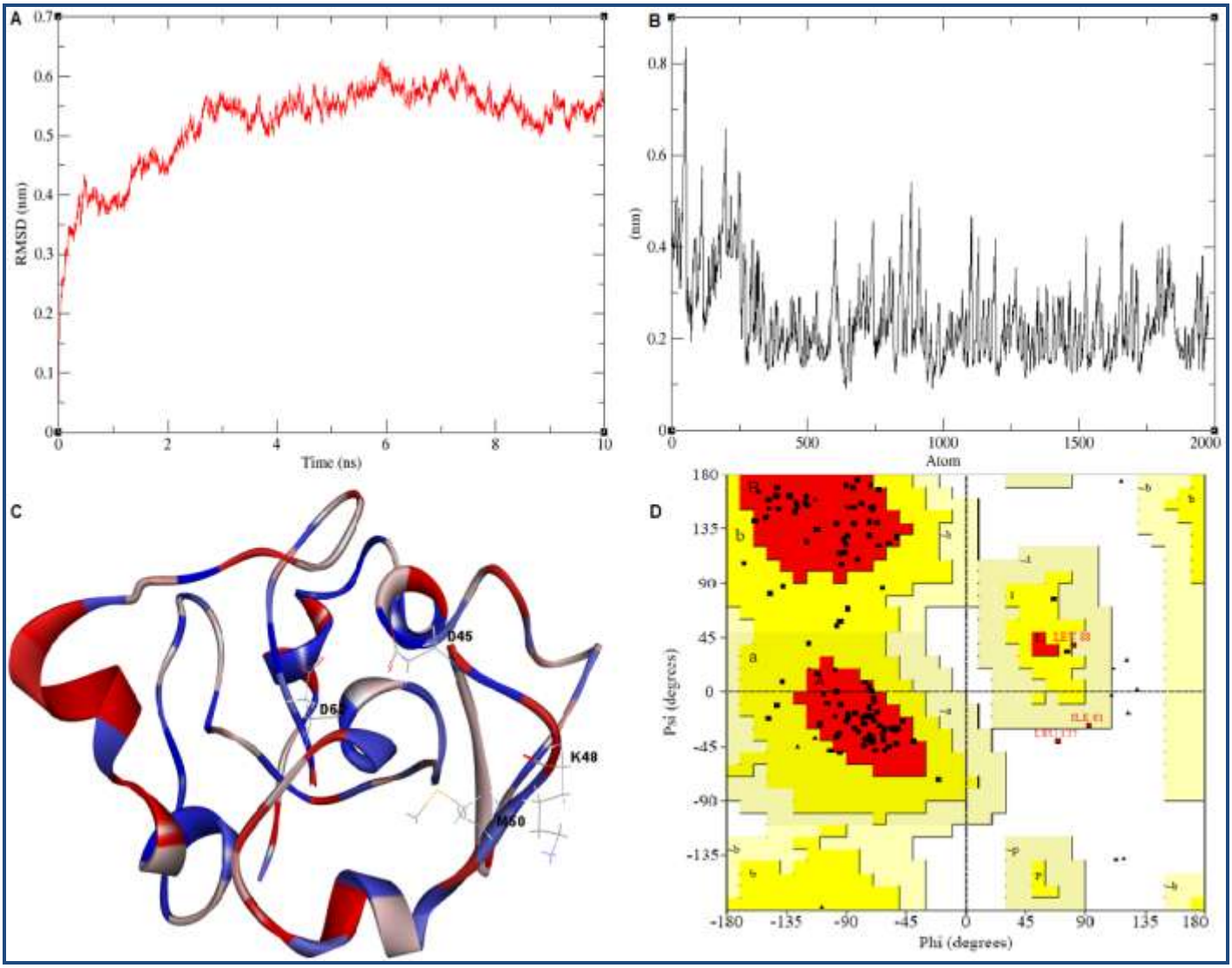

Figure 1: The calculated of Root Mean Square Deviations (RMSD) plot **(A)** Root Mean Square Fluctuations (RMSF) plot; **(B)** of TFNR12Am; **(C)** Ribbon structure of TFNR12Am showing (D45, Lys48, M50 and D62) crucial interacting amino acids in stick form; **(D)** The secondary structural investigation for the model structure TFNR12Am on Ramachandran plot**.**

Docking analysis

All the compounds except Ellagic acid (**Figure 2A-D, Table 2 (see supplementary material)**) docked into the TWEAK interacting site of TNFRSF12Am. The docking site contains Asp 45, Lys48, Met50 and Asp62 amino acids which are reportedly crucial for TWEAK interaction. The binding energy and the number of their interacting hydrogen bonds are presented in **Table 2**. After comparative docking analysis it was learnt that Rutin and Genistein showed better inhibition in comparison to other compounds. It was observed that the order of docking, from best to least, was Genistein, Rutin, Gallic acid ethyl ester and Quercetin.

Flavonoids and tannins are widespread in plant kingdom and *in vitro* studies as well as clinical trials to show that dietary intake of flavonoids, prevented tumor progression **[23].** Our model predicted that radiation induced liver pathologies, which are induced by over expression of Fn14-TWEAK interaction, can be prevented by treatment with Genistein, Rutin, Gallic acid and Quercitin. Further our model showed that ellagic acid was not acting through binding with Fn14 and

TWEAK. This study predicted that radiation induced harmful/lethal/carcinogenic effects could be prevented by blocking the binding of TWEAK on Fn14 by using phytochemicals/ nutraceuticals containing Genistein, Rutin, Gallic acid ethyl ester and Quercetin. The flavonoids and tannins are dietary constituents and are non-toxic. These can be developed into drugs meant for whole body radiation protection. The immediate applications of this study could be that supplementation of radiotherapy treatment protocols with these flavonoids and tannins, and/or nutraceuticals rich in Gallic acid, Rutin, quercitin, genistein, may counter the harmful effects of radiation.

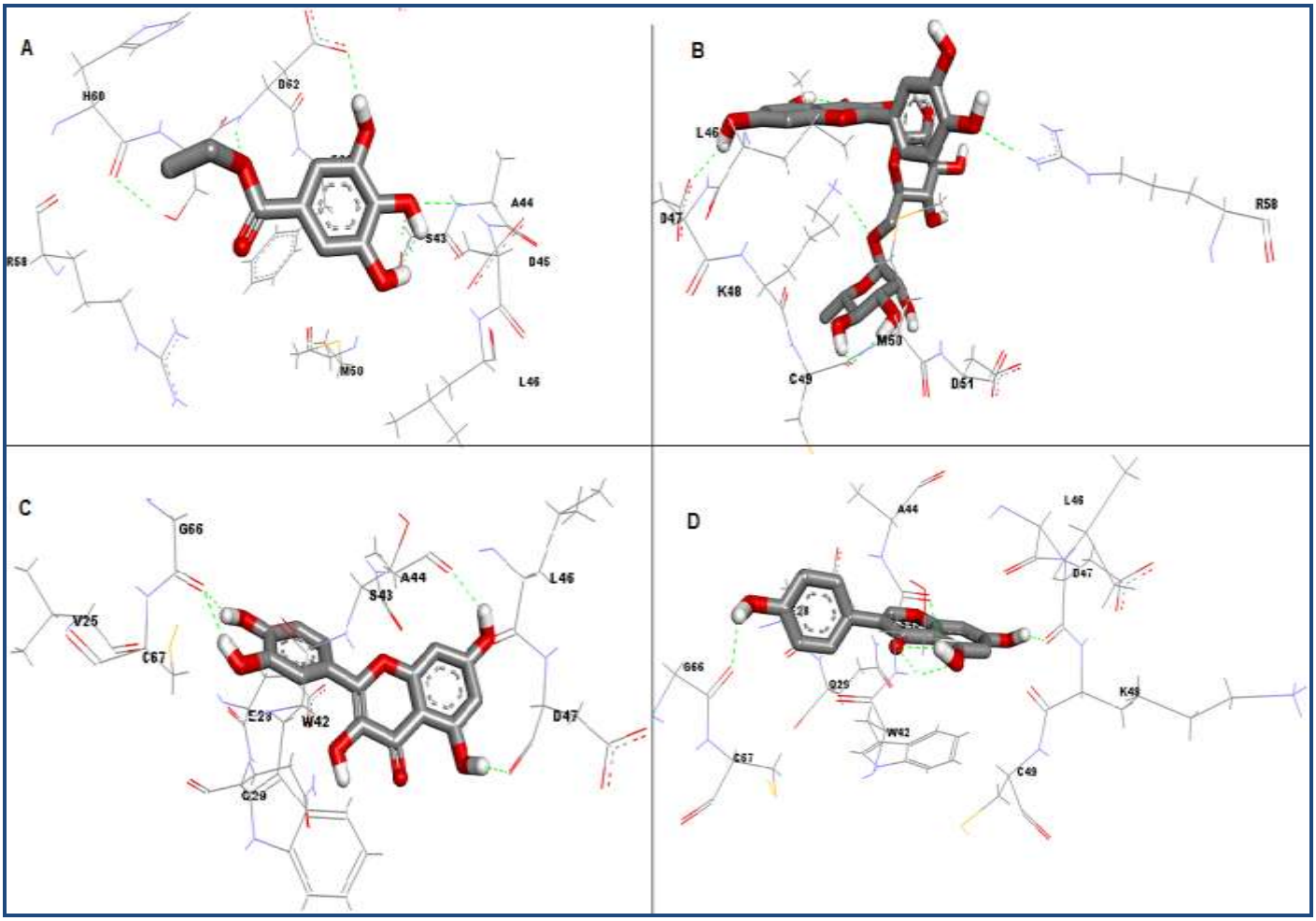

Figure 2: *Best docked conformer of* **(A)** *Gallaic acid ethay ester (CID: 13250);* **B)** *Rutin (CID: 5280805);* **C)** *Quercetin (CID: 5280343) and* **(D)** *Genistein (CID: 5280961) with TNFRSF12Am -TWEAK interacting sites.*

Conclusions:

This study has been the first to develop a homology model of human Fn14 protein, because no crystallographic structure for human Fn14 is available so far. Further, based on the binding properties of tannins and flavonoids, this study predicted that Genestein, Rutin and Gallic acid are effective in preventing harmful effects of radiation by preventing Fn14-TWEAK interaction and therefore their uncontrolled expression. This study has application in development of targeted radiation countermeasures as well as improving radiotherapy treatment by utilizing non-toxic flavonoids and tannins as may be present in plants like *Hippophae rhamnoides*.

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Supplementary material:

Table 1: Structure validation scores generated by various validation algorithms after modeling by Server TNFRSF12A and TNFRSF12Am after minimization

Ramachandran Plot statistics	TFNR12A	TFNR12Am
% Amino acid in most favored regions	77.1%	81.9%
% Amino acid in additional allowed regions	12.4%	15.2%
% Amino acids in generously allowed regions	6.7%	1.9%
% Amino acids in disallowed regions	3.8%	1.0%
Errat score	23.967	41.538
Verify_3D score	70.00	83.08
Overall G-factor score	-0.64	-0.71

Table 2: Comparative docking result of best conformer of each inhibitor at TWEAK interacting site of TNFRSF12A m.

TFNR12A inhibitors	Binding Energy KJ/mol	No. of Hydrogen Bonds	H-Bonding Residues
Gallic acid Ethayal ester	-3.10	4	Ser43, Asp45, Asp47 & Asp62
Rutin	-4.40	5	Asp47, Lys48, Met50 &Arg58,
Quercetin	-4.00	3	Ala44, Asp47&Gly 66
Genistein	-4.32	4	Ala 40, Asp43, Asp47, Lys48 & Gly 66
Ellagic Acid	Not binding	Not binding	Not binding