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Original Article

AN *IN SILICO* AND *IN VITRO* EVALUATION OF CYTOTOXICITY, APOPTOTIC ACTIVITY AND GENE EXPRESSION MODULATION OF SARSASAPOGENIN IN HUMAN COLORECTAL CANCER CELL LINE HT-29

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ABSTRACT

Objective: Search for natural drugs against Colo Rectal Cancer (CRC) is ever-growing. Sarsasapogenin is a steroidal sapogenin known for various biological activities. The current study intends to investigate it's anticancer activity in vitro against the Human Adenocarcinoma CRC cell line (HT-29). Additionally, the *in silico* interaction between sarsasapogenin and selected anticancer drug-protein targets was investigated.

Methods: To evaluate cell viability, HT-29 cells were subjected to several concentrations of sarsasapogenin. Flow cytometry was used to study apoptosis. The expression of the genes Epidermal Growth Factor Receptor Tyrosine Kinase (EGFR-TK) and Kirsten Rat Sarcoma oncogene homolog (KRAS) was elucidated by real-time Polymerase Chain reaction. Molecular docking was used in conjunction with Molecular Dynamics (MD) simulation to comprehend the Sarsasapogenin's interaction with EGFR-TK and KRAS.

Results: Sarsasapogenin affected the viability of HT-29 cells dose-dependently. In HT-29 cells, sarsasapogenin treatment decreased the levels of KRAS and EGFR and caused apoptosis. In silico study demonstrated the interaction of sarsasapogenin in the Adenosine triphosphate binding site of EGFR-TK and the switch I/switch II site of KRAS. Post-MD analysis determined the stable binding of sarsasapogenin with these proteins. The binding energy with EGFR-TK and KRAS was found to be-46.0 ± 1.5 kcal/mol and-28.8 ± 6.3kcal/mol.

Conclusion: Altogether, Sarsasapogenin, through modulation of EGFR and KRAS has shown promising anticancer effect against HT-29 cells.

Keywords: Sarsasapogenin, HT 29 cells, In silico, Apoptosis

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INTRODUCTION

Colo Rectal Cancer (CRC) occurs frequently and in developing countries, the mortality rate is found to be high [1, 2]. Although chemotherapy, radiation therapy and surgery are available options for the treatment of CRC, these strategies have various drawbacks such as the high cost of the drug, the development of resistance to available drugs, intra-tumour heterogeneity and the evolution of the tumour [3]. Hence, the identification of natural-based compounds which are cost-effective and have minimal side effects is preferred. Accordingly, research is being carried out on the potential chemotherapeutic benefits of medicinal plants and their active constituents against cancer [4, 5]. In the past decade, two different proteins Epidermal Growth Factor Receptor Tyrosine Kinase (EGFR-TK) and Kirsten Rat Sarcoma oncogene homolog (KRAS) have been targeted for the identification of drugs against CRC.

Ligands like transforming growth factor-alpha and Epidermal growth factor bind to their targets, activating downstream signaling pathways that set off a series of molecular events (Phospho Inositide 3 Kinase (PI3K)/Akt/mammalian Target of Rapamycin (mTOR) pathway) leading to the protection of cancer cells from apoptosis, facilitation of invasion and promotion of angiogenesis [6]. Thus, EGFR has been associated with the development of several cancers, including those of the bladder, lung, head and neck, breast, and gastrointestinal tract. In the case of CRC, about 25% to 82% overexpression of EGFR is detected in the cancer tissue [7, 8]. Due to this, EGFR is considered an effective drug target for the treatment of CRC. Nevertheless, the response rate of patients to anti-EGFR therapy like Cetuximab or Panitumumab, is rather low [9]. With anti-EGFR therapy, almost 50% of individuals experience adverse drug reactions [10]. In clinical practice, Regorafenib, an EGFR-TK inhibitor, is used as a third-line therapeutic option for CRC [11]. Research done *in vivo* and *in vitro* has shown the way that Regorafenib inhibits tumour cell proliferation and triggers apoptosis in colorectal cancer [12]. Hence, the search for novel, effective small molecule inhibitors, in particular, natural compounds against EGFR-TK is ever-growing [13].

KRAS is one of the most frequently mutated oncogenes and about 40% of CRC patients were found to harbor mutant protein [14]. KRAS helps to transduce upstream cellular signals, including EGFR to downstream effectors (PI3K/AKT/mTOR pathways) that lead to cancer cell survival and metastasis. Aberrant activation of KRAS due to mutation in turn affects the upstream signal transduction, thereby, resistance is developed against EGFR drugs in cancer [14]. The specific molecular structure characteristic of KRAS made it an undruggable target for a long time.

However, active research on KRAS led to the identification of a hypervariable loop, an allosteric lobe and an effector domain (two pockets on the surface of KRAS, namely, switch-I and switch-II formed by the effector domain); hence the KRAS is druggable [15]. Although most of the drugs are identified to target various KRAS mutant proteins, studies have shown that the presence of the wild-type KRAS allele in the KRAS mutant lung adenocarcinoma has tumour suppressor function, increased mitogen-activated protein kinase inhibitor resistance and effect on treatment response [16, 17]. This is achieved through dimerization of wild-type KRAS with mutant KRAS [16]. Hence, it is necessary to target wild-type KRAS for effective treatment of cancer.

Sarsasapogenin is a steroidal sapogenin possessing a wide range of biological actions like anti-inflammatory, antioxidative, antidiabetic, antibacterial, neuroprotective, anti-atherosclerosis, anti-arthritis, memory-improvement properties and prevention of lipopolysaccharide-induced bone loss [18-22]. Also, it has hindered the secondary complications of diabetes, like diabetic nephropathy, diabetic encephalopathy and diabetes-associated memory impairment [21-24]. Further, it has been studied for its effect against cancers, including hepatocellular carcinoma and cervical carcinoma [25, 26]. Its derivatives have been shown to exhibit anticancer efficacy against a range of cell lines [27, 28]. However, its effect on CRC has not been reported. Keeping these views, its anticancer potential against the HT-29 cell line was studied. Analysis was done to determine how it affected the expression of the EGFR and KRAS

genes and comprehensive in silico investigations were carried out to determine the binding to the EGFR-TK and KRAS proteins. Consequently, this study aims to evaluate the anticancer potential of sarsasapogenin in causing cytotoxicity, apoptosis, and modulation of gene expression.

MATERIALS AND METHODS

Materials

Doxorubicin and sarsasapogenin were procured from Sigma-Aldrich, USA. Himedia Pvt limited, Mumbai, India supplied Dulbecco's Modified Eagle's Medium (DMEM), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) and Foetal Bovine Serum (FBS). Propidium iodide and Annexin V were acquired from BD Biosciences in San Jose, California. RNeasy Mini kit was obtained from Qiagen in the USA. The IScript complementary DNA synthesis kit was procured from Bio-Rad, USA.

Cell culture and treatments

The HT-29 cell line was acquired from the National Centre for Cell Science, Pune, India. It was cultured at 37 °C, 5% CO2 atmosphere in DMEM containing 10 % FBS.

Determination of cell viability using MTT assay

Approximately 20,000 cells were placed in a 96-well plate and cultured for 24 h to ascertain the impact of sarsasapogenin on the viability of HT-29 cells. After that the cells were exposed to its different doses [6.25, 12.5, 25, 50 and 100 µg/ml] for 24 h. Cell viability was assessed at the end of treatment by exposing the cells to MTT (0.5 mg/ml in DMEM) for one hour at 37 °C in a CO2 incubator. After removing the MTT, the dye crystals were dissolved in 100 µl of Di-Methyl Sulf Oxide (DMSO) and quantified with an ELISA reader at 570 nm [29, 30].

Determination of apoptosis by flow cytometry

For a period of 24 h, the HT-29 cells were exposed to 35 µg/ml of sarsasapogenin and 1 µM of positive control Doxorubicin. The media was taken out at the end of the treatment. Trypsinization was used to obtain the cells, and 1x Phosphate-Buffered Saline (PBS; pH 7.4) was used to wash them. Two treatments were applied to the cells Annexin-V and Propidium iodide. Using flow cytometry, the cells were examined [31].

Gene expression analysis

The sequence of forward and reverse primer for KRAS, F: CAGTAGACACAAAACAGGCTCAG and R: TGTCGGATCTCCCTCACCAATG; EGFR, F: AACACCCTGGTCTGGAAGTACG, R: TCGTTGGACAGCCTTCAAGACC; beta-actin, F: CAAGATCATTGCTCCTCCTG and R: ATCCACATCTGCTGGAAGG. The relative quantification of the genes [EGFR and KRAS] expression was done using the SYBR Green Chemistry in QuantStudio3 system (Thermo Fisher, USA). An internal control was employed, namely the beta-actin gene. The relative mRNA levels of the genes were determined using the Comparative Ct (ΔΔCt) technique [32].

In silico analysis

Molecular docking study

The three-dimensional structure of Sarsasapogenin was obtained from the PubChem database. The protein data store provided the three-dimensional protein framework of EGFR and KRAS, with IDs 1M17 and 8AZV, respectively [33, 34]. The ligand and proteins were prepared and docked using the DOCK6 software package [35].

Molecular dynamics study

The Optimized Potentials for liquid Simulations (OPLS) force field included in the Desmond routine of the Schrödinger software package was used for MD simulation. Using the Transferable Intermolecular Potential with 3 Points (TIP3P) water association system as a buffer, the orthorhombic periodic boxes with 10 Å3 dimensions were created. An isotopically organised arrangement of charged ions (Na^+/Cl^-) was used to offset the Ewald charge summation. Steepest descent and conjugated gradient techniques were used to minimise the system, and canonical ensemble was maintained while heating the system from 0 to 300K at 200 picoseconds.

Finally, the MD production was carried out up to 100 nano seconds (ns). The complex systems were run at constant temperature (300 K), constant pressure (1 bar), and an isothermal-isobaric ensemble in steps of two femtoseconds. Post-MD analysis of Root mean square deviation (RMSD) and Root mean square fluctuation (RMSF) were shown to be predictive of the degree of intermolecular interactions and conformational change. Using Bio-3D R-package software, Detailed Cross-Correlation Map (DCCM) and Principal Component Analysis (PCA plots) determined the stable binding of SSP with these proteins [36, 37]. PyMOL software and Discovery Studio Visualizer were used to analyse the complexes' intermolecular interactions. Using the Prime application included in the Schrodinger software package, the binding free energy of the complex structures was computed from the fractions acquired using MD simulation trajectories [38-40].

Statistical analysis

Three duplicates of each experiment were conducted. A one-way ANOVA with Tukey's post-hoc was used to determine the significance of difference.

RESULTS

Effect on cell viability

Administering different sarsasapogenin concentrations to HT-29 cells significantly lowered their viability (fig. 1A). The half-maximal inhibitory concentration (IC50) against HT-29 was found to be 35 µg/ml. The morphology of cells from various treatment groups observed in a bright field microscope is given in fig. 1B. The cells are healthy in control cells. However, treatment with sarsasapogenin in a dose-related manner has disturbed the morphology, in particular at 100 µg/ml concentration most of the cells were rounded up, indicating apoptosis.

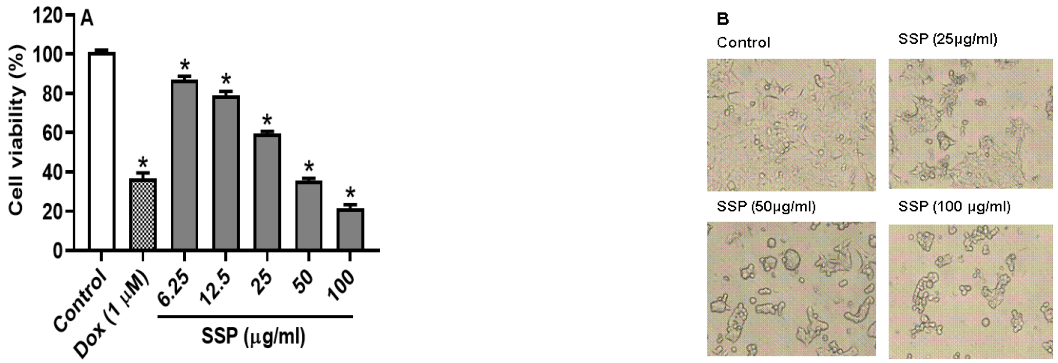

Fig. 1: (A) Effect of sarsasapogenin (SSP) on the viability of HT-29 cells. (B) Cells viewed under bright field microscope from various groups. Control cells were healthy, whereas the treatment has affected the morphology of the cells. n = 3; value are measured in mean±SD. *P<0.05 vs. control cells

Effect on cells apoptosis

The HT-29 cells were exposed to the IC50 concentration that is 35 µg/ml to determine the apoptotic-inducing effect. In addition, the positive control used was Doxorubicin (1 µM). The representative histogram of the cells is given in fig. 2A. Treatment of sarsasapogenin has decreased the percentage of live cells. A significant percentage of the cells were found to be in late apoptotic stage. In comparison to late apoptotic cells, the dead cells were found to be less, indicating that only a few cells have undergone necrosis (fig. 2B). The apoptotic induction at 35 µg/ml (85 µM) concentration was found to be equivalent to positive control at 1 µM concentration.

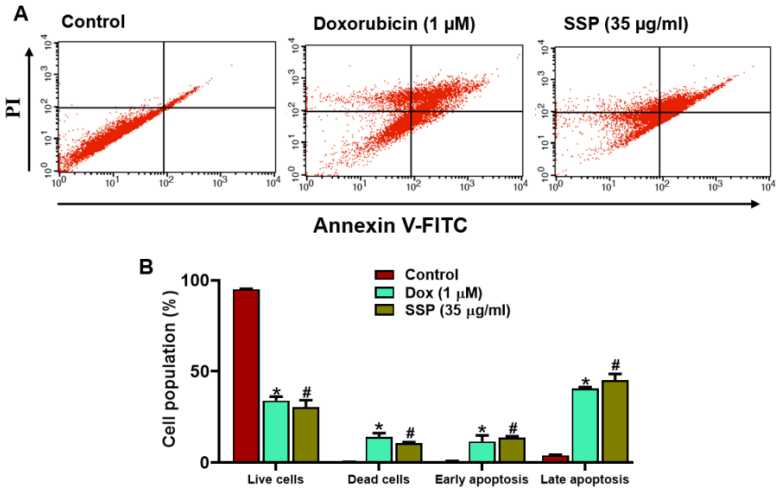

Fig. 2: (A) Representative histogram obtained using flow cytometry of apoptosis assay from various groups are shown. Quadrant: Upper left (Dead cells); Upper Right (Late apoptosis); lower left (Live cells); lower Right (Early apoptosis). (B) Percentage of viable, dead, early apoptotic and late apoptotic cells in control and compounds treated groups. n = 3; value are measured in mean±SD.*P<0.05 vs. control cells

Effect on expression of EGFR and KRAS

After treating HT-29 cells for 24 h with a 35 µg/ml dose of Sars sapogenin, EGFR and KRAS mRNA levels were markedly reduced (fig. 3).

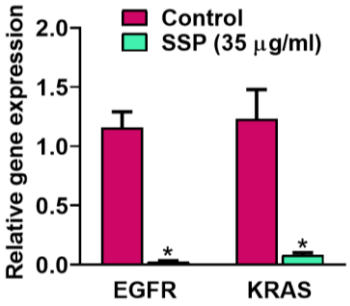

Fig. 3: mRNA levels of EGFR and KRAS in sarsasapogenin (35 µg/ml) treated HT-29 cells. n = 3; value are measured in mean±SD, *P<0.05 vs. control cells

***In silico* analysis of the interaction with EGFR-TK and KRAS**

EGFR-TK

It was found that sarsasapogenin and EGFR-TK had a docking score of-32.5 kcal/mol. The interaction of EGFR-TK during docking and MD simulation is shown in fig. 4. The final result of the MD simulation and the shown docking structure demonstrate the stable binding to the ATP binding site, which is the location of interest in EGFR-TK. The details of amino acids from EGFR-TK forming hydrogen bonds and hydrophobic interactions are given in table 1. During docking, Sarsasapogenin formed hydrogen bonding with amino acids leu694, Cys773, Asp776, Asn818 and Asp831; and hydrophobic interaction with leu694 and Cys773. Its orientation had whirled inside the binding site by the time the MD simulation had ended. leu694, an amino acid, was involved in a comparable interaction to docking near the conclusion of the MD simulation. New hydrogen bonding contacts were generated with the amino acids Cys751, Thr766, Gln767, Met769, and Pro770. After the MD simulation, hydrophobic contacts were found to be higher than during docking. It had formed hydrophobic interactions with amino acids, leu694, Phe699, Val702, Ala719, lys721, Cys751and leu820.

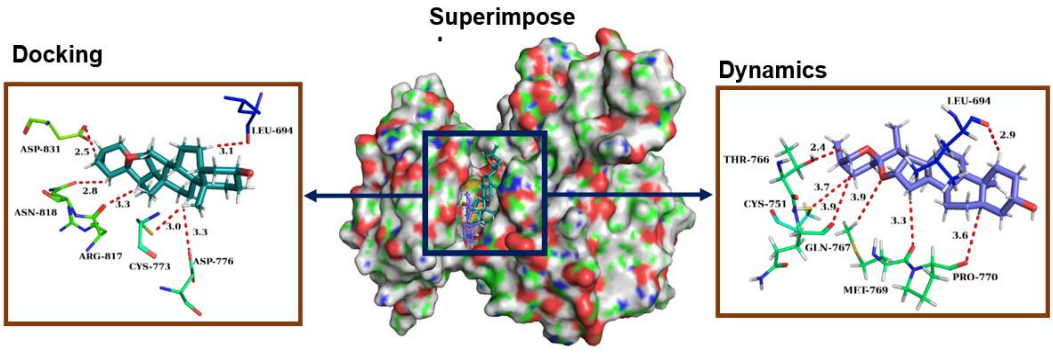

Fig. 4: Interaction of sarsasapogenin with EGFR-TK during docking and at the end of 100 ns MD simulation

Table 1: Intermolecular interactions between sarsasapogenin and amino acids of different proteins (EGFR-TK and KRAS) during docking and 100 ns MD simulation

Protein/ligand complex	Docking/MD	Interacting amino acid residues (Distance Å)	
		Hydrogen bond	Hydrophobic interaction
EGFR-TK/Sarsasapogenin	Docking	Leu694 (3.1), Cys773 (3.0), Asp776 (3.3), Asn818 (2.5), Asp831 (2.8)	Leu694 (5.2), Cys773 (4.2)
	MD	Leu694 (2.7), Cys751 (3.7), Thr766 (2.4), Gln767 (3.9), Met769 (3.3), Pro770 (3.6)	Leu694 (4.7), Phe699 (5.0), Val702 (3.7), Ala719 (5.0), Lys721 (4.2), Cys751 (5.3), Leu820 (4.9)
KRAS/Sarsasapogenin	Docking	Pro34 (3.1), Gly60 (2.9), Glu63 (2.3), Asp92 (2.7), His95 (3.9), Tyr96 (3.0)	-
	MD	Gln25 (3.3), His27 (3.3), Phe29 (2.9)	Ile21 (5.2), Val23 (5.3)

The RMSD of the sarsasapogenin/EGFR-TK complex is given in fig. 5A. The protein backbone deviation was first discovered to be unstable for 10 ns. However, after 10 ns to 100 ns of MD simulation, the RMSD stabilised and was kept within 1 Å value. The RMSF of the sarsasapogenin/EGFR-TK complex is shown in fig. 5B. The fluctuation was found to be high in the N-and C-terminal sequences of EGFR-TK. In addition, the RMSF was found to be high in the region Asp892 to lys905. However, the RMSF in the ligand binding site as well as other regions was found to be below 4 Å value. The DCCM plots indicate the presence of a pairwise correlation between sarsasapogenin and EGFR-TK (fig. 5C). In the SSP/EGFR-TK complex PCA graph (fig. 5D), the eigenvalues were plotted against the eigenvector indices. Higher eigenvalues, or dominant movement, were seen in the first five eigenvectors, which accounted for 13.3% to 54.5% of the variation overall. The eigenfraction reached a static elbow point with no appreciable fluctuations after the sixth eigenvector. Comparable variability was displayed by PCs 1 and 2, which accounted for 13.25% and 10.68% of the variation overall. The corresponding PC3 value is 7.84 % which denotes the low mobility and high stability of the complex structure. The binding free energy calculated by Molecular Mechanics/Generalized Born Surface Area (MM/GBSA) was given in table 2 and it was found to be-46.0 ±1.5 kcal/mol. The Van der Waals (vdW) and lipophilic (hydrophobic) energy were found to contribute to the binding of sarsasapogenin with EGFR-TK. In addition, electrostatic interactions (coulomb) have also contributed to the interaction with EGFR-TK.

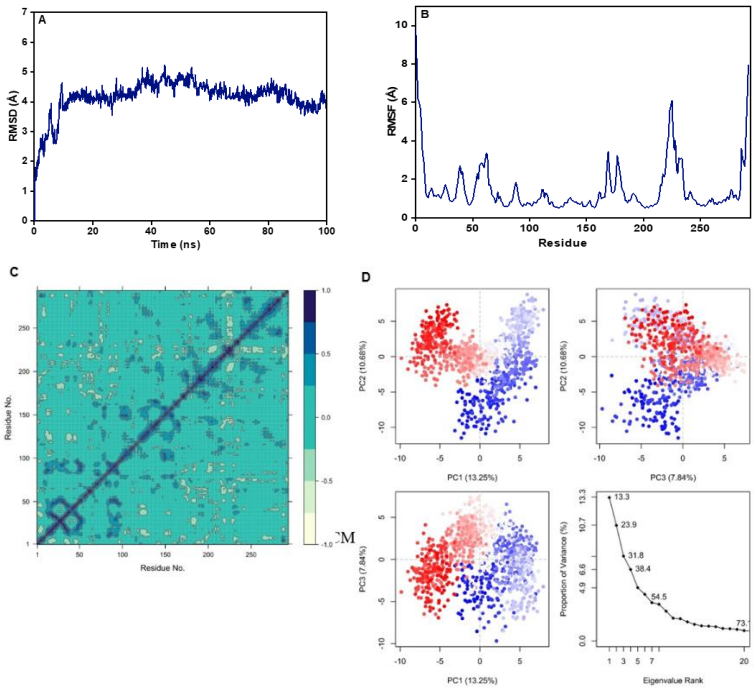

Fig. 5: (A) RMSD; (B) RMSF; (C) DCCM and (D) PCA plots of sarsasapogenin/EGFR-TK complex during MD simulation

Table 2: MM/GBSA binding free energy (in kcal/mol) of various protein/ligand complexes

Protein/ligand complex	ΔG Bind (Kcal/mol)	Coulomb	Covalent	H_bond	Lipo	vdW
EGFR-TK/Sarsasapogenin	-46.0 ±1.5	-2.8±0.1	1.1±0.8	0	-19.0±2.4	-40.7±1.1
KRAS/Sarsasapogenin	-28.8±6.3	-3.1±0.7	1.8±0.5	-0.3±0.2	-14.2±3.8	-27.8±1.6

Value are measured in mean±SD

KRAS

In molecular docking, the docking score of sarsasapogenin was found to be-21.2 kcal/mol for KRAS. It formed strong interactions with switch II residues of the KRAS at the end of docking (fig. 6). It formed hydrogen bond interactions with amino acids Pro34, Gly60, Glu63, Asp92, His95 and Tyr96. Molecular docking structures were superimposed, and after 100 ns of MD simulation, it was shown that sarsasapogenin had moved away from the docking site. The trajectory files had shown that the molecule moved after 20 ns of MD simulation

and formed a stable interaction in new sight, as shown in fig. 6. It had formed hydrogen bonds with amino acids present in the switch I site, namely, Gln25, His27 and Phe29. Hydrophobic interactions were formed with amino acids Ile21 and Val23 (table 1).

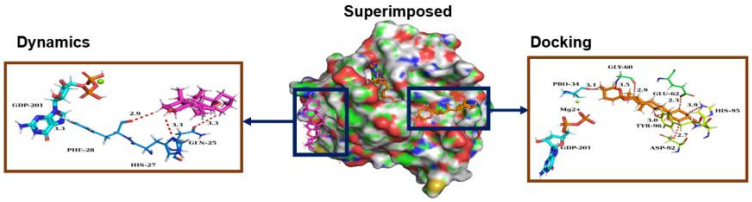

Fig. 6: Interaction of sarsasapogenin with KRAS during docking and at the end of 100 ns MD simulation

The stability of the sarsasapogenin/KRAS complex was confirmed by the discovery that the RMSD of its protein backbone was within 0.6 Å. (fig. 7A). According to the RMSF, the protein's N-terminal region and the amino acids in the ligand binding site were shifted to accommodate the ligand more favourably (fig. 7B). The DCCM plot reveals the distribution of pairwise cross-correlating residues in the sarsasapogenin/KRAS complex (fig. 7C). PC1 and PC2 displayed 34.35% and 20.31% of the total variance in the PCA plot, respectively. The PC3 value of the complex is 8.67% denoted the high stability of the system during MD simulation (fig. 7D). The MM/GBSA calculation yielded a binding free energy of-28.8 ± 6.3 kcal/mol. The vdW and hydrophobic energy contributed to the SSP binding with KRAS.

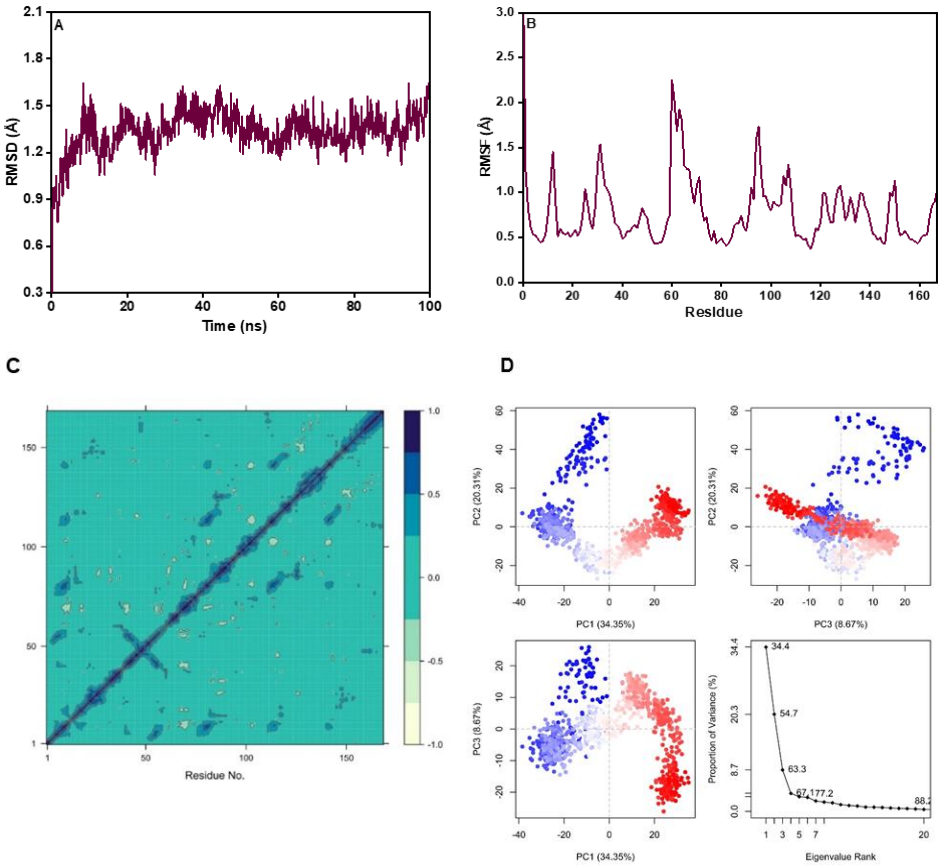

Fig. 7: (A) RMSD; (B) RMSF; (C) DCCM and (D) PCA plots of sarsasapogenin/KRAS complex during MD simulation

DISCUSSION

The effect of naturally occurring compounds such as Epigallocatechin gallate, Resveratrol, Curcumin, Kaempferol, Silibinin, Baicalin, Delphinidin, Raddeanin A, alanolactone, Oiperlongumine and organosulfur compounds from garlic against CRC are largely studied. Either these compounds are studied directly or in combination with chemotherapeutic agents to understand the possibility of therapeutic effect of these compounds [4, 5]. likewise, the present study evaluates the effect of sarsasapogenin, another naturally occurring substance, on colorectal cancer *in vitro*. Its impact on different cancer cell lines, including HepG2 and HeLa cell lines has been studied in the past. The IC50 value against HT-29 was found to be 35 µg/ml (85 µM), which is less than that of against HepG2 (IC50 = 42.4 µg/ml after 48 h treatment) [41]. On the other hand, in the HeLa cells, the presence of 60 µM of sarsasapogenin has induced cell death [26]. Sarsasapogenin-induced apoptosis in HT-29 cells thereby affected the cell viability and has been reported to trigger the endoplasmic reticulum stress in HeLa cells [26]. It is a known fact that Reactive Oxygen Species (ROS) would mediate intracellular signaling cascades that are involved in mitochondrial membrane disintegration, thereby initiating the mitochondria-dependent apoptotic event [26]. Overwhelming ROS generation in cancer cells would cause oxidative stress (that is imbalance between oxidants and endogenous antioxidants) that leads to cellular dysfunction and, ultimately apoptosis. Similar to these reports, in HT-29 cells also, sarsasapogenin treatment could have enhanced the ROS generation, thereby leading to apoptosis as observed in the present study.

Sarsasapogenin has downregulated the expression of EGFR in HT-29 cells. EGFR level is frequently elevated in up to 80% of CRC cases [42]. Hence, for the anticancer effect, it is imperative to decrease the level of EGFR in HT-29. Other natural compounds such as epigallocatechin gallate, dihydrotansinone, tetrandrine and curcumin were reported to decrease the level of EGFR in CRC cell lines [43-46]. In addition to EGFR, the level of KRAS has been decreased in the HT-29 cells. Other compounds such as eugenol, combined ginger, quercetin, luteolin, Gelam honey, and crocin have previously been reported to decrease the level of KRAS in CRC cell lines [47-50]. Both EGFR and KRAS are critical regulators of CRC progression, hence, downregulation of expression of these genes gives a hint on further possible progress of therapeutic usage against CRC. However, in vivo studies are warranted before such inferences.

Among various therapeutic possibilities for the treatment of cancer, improvement in survival has been significantly contributed by the addition of molecular-targeted drugs to chemotherapy [6, 11]. Hence, the possibility to target the proteins of interest in the present study, EGFR and KRAS has been determined. For targeting EGFR, the inhibition of the EGFR-TK domain is prominently studied. Small molecules that bind in the ATP-binding site of EGFR-TK are vastly searched for therapeutic efficiency against various cancers [13]. It is highly conserved and consists of a hydrophobic pocket; hence the sarsasapogenin, which is a lipophilic steroidal moiety was able to stably orient in this site of EGFR-TK through the formation of hydrophobic interaction with amino acids present in this site [50]. The significant contribution of vdW and lipophilic (hydrophobic) energy in the binding further confirms this notion. Other natural compounds, epigallocatechin gallate, Honokiol, capsaicin, oxymatrine and tetrandrine, are reported as EGFR-TK inhibitors. In addition, triterpenoid saponins such as 20(S)-ginsenoside Rh2 and 20(R, S)-protopanaxatriol are found to inhibit EGFR [51, 52].

Sarsasapogenin bound in the switch II site (residues 57-72) of the KRAS protein during docking. Nevertheless, the molecule aligned close to the protein's switch I site (residues 25–40) after the MD simulation. The effector proteins functioning as regulators for associating with guanosine exchange factors in the modulation of the KRAS protein bind to the switch regions [53, 54]. Additionally, these adjacent switch areas are known to contribute to the stabilisation of the protein framework [55]. Hence, both these sites are considered important for the functioning of the KRAS, this site might influence the functioning of the KRAS protein, which needs further *in vitro* evaluation. In addition to binding in KRAS protein, sarsasapogenin has reduced the expression of KRAS in the HT-29 cells which might have a positive effect towards the inhibition of HT-29 cell proliferation. Altogether, in silico analysis clearly indicated the stable association with the ATP-binding site of EGFR-TK and interaction with the switch I/II site of KRAS. The molecular mechanism that would have induced cell death in HT-29 cells may have involved inhibition of EGFR and KRAS activity as well as a decrease in the number of genes that express these proteins.

CONCLUSION

Apoptosis driven on by sarsasapogenin reduced the viability of the cells. Sarsasapogenin reduces the expression of the EGFR and KRAS genes in HT-29 cells. The interaction in the ATP-binding site of EGFR-TK was discovered using in silico study. Additionally, sarsasapogenin was positioned at the KRAS protein's switch I/switch II site. MD analysis further elaborated the structural modifications in these proteins, which helped in the binding of sarsasapogenin. Further research is necessary to fully comprehend how sarsasapogenin prevents CRC. Overall, the present study described that by altering the EGFR and KRAS, sarsasapogenin acted as an anticancer agent against CRC cells.

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Nil

AUTHORS CONTRIBUTIONS

All authors contributed equally to this work. The authors confirm contribution to the paper as follows: study conception and design by TABREEZ AHAMED; Data analysis and interpretation of results by KAVITHA RAMASAMY; Draft Manuscript guidance and preparation by RAMYA. S. All authors reviewed the results and approved the final version of the manuscript.

CONFLICTS OF INTERESTS

The authors declare that there are no conflicts of interest.

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