

Network Pharmacology, Molecular Docking, Simulation Studies of Urolithin A by Inhibiting AKT and Caspase Pathways against Colorectal Cancer

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Background: Colorectal cancer (CRC) ranks as the second most common gastrointestinal malignancy globally, surpassed only by stomach cancer in incidence. Colorectal cancer (CRC) is recognized as the second most prevalent gastrointestinal cancer worldwide, with stomach cancer being the only cancer that surpasses it in terms of incidence.

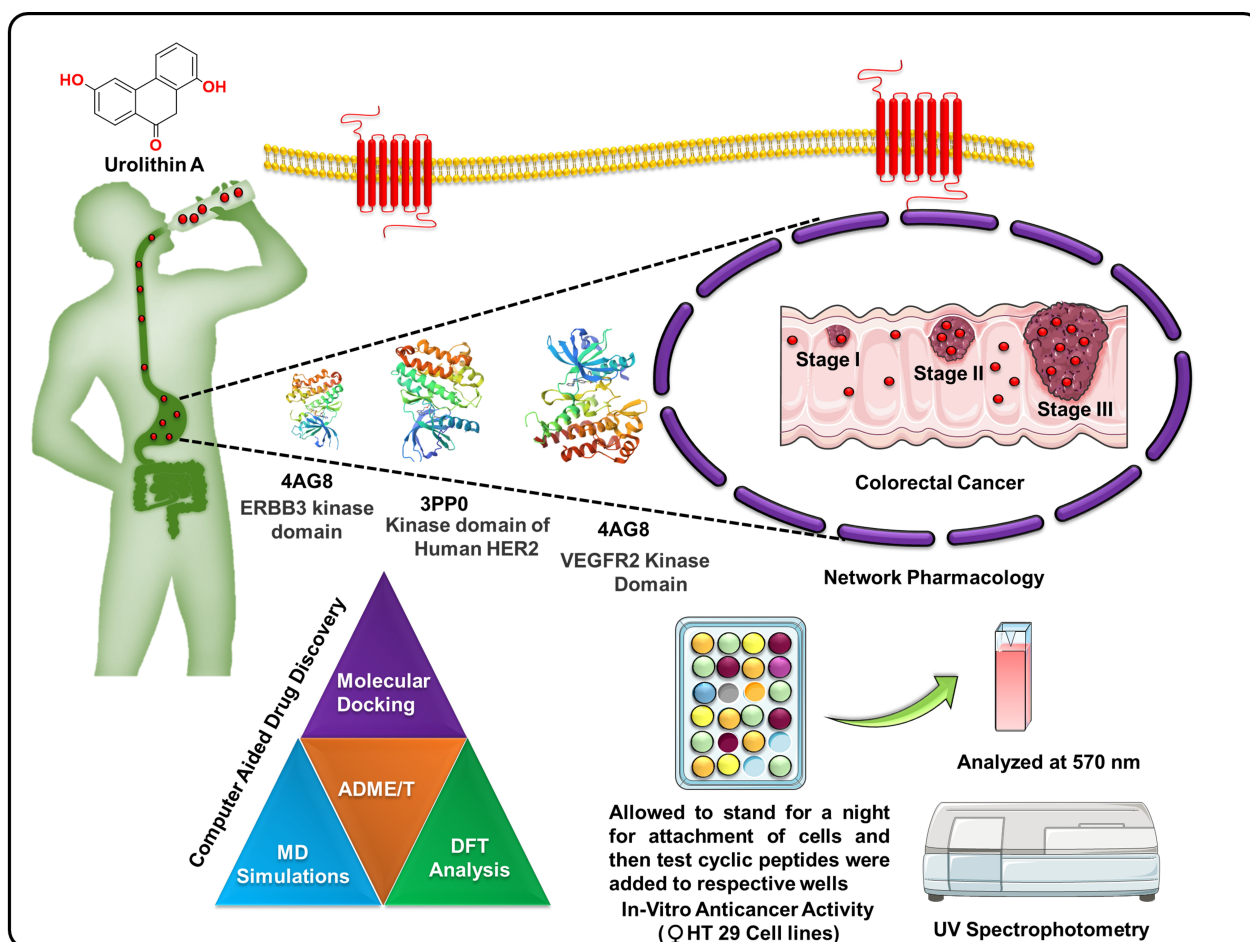
Purpose: This research aims to examine the possible anti-carcinogenic effects of Urolithins A, B, C, and D, which are metabolites present in pomegranate juice. These metabolites will be compared to established drugs such as folinic acid, doxorubicin, and 5-fluorouracil (5-FU), often used in treating CRC.

Method: In this study, we investigated the interactions between the epidermal growth factor receptor (EGFR) and three proteins, namely human epidermal growth factor receptor HER3 (Protein Data Bank (PDB) code: 3LMG), HER2 (PDB code: 3PP0), and HER1 (PDB code: 4AG8), utilizing the Protein Data Bank (PDB) principles. The findings of our study demonstrate the presence of persistent and reliable interactions within the protein-ligand complexes, specifically in the cases of 3PP0, 3LMG, and 4AG8, as indicated by the root mean square deviation (RMSD) values.

Results: The 3PP0 complex demonstrated RMSD values spanning from 1.17 to 3.23 Å. The 3LMG complex exhibited root RMSD values ranging from 1.16 to 2.93 Å, with a standard deviation of 0.25. Similarly, the 4AG8 complex showed root mean square deviation of RMSD values ranging from 1.27 to 3.35 Å, accompanied by a standard deviation of 0.42. The root mean square fluctuations (RMSFs) observed in these complexes exhibited a range spanning from 0.42 to 7.65 Å, providing more evidence of the stability of the interactions. The endurance of Urolithin A (Uro A) was established through an investigation using density functional theory (DFT), yielding an E value of 4.2700 eV, energy of the highest occupied molecular orbital (E_{HOMO}) of -5.8560 eV, and energy of the lowest unoccupied molecular orbital (E_{LUMO}) of -1.5860 eV. Uro A's *in vitro* anticancer potential has been evaluated through human colon adenocarcinoma cell line (HT-29) cell lines. The results of anticancer potential revealed that Uro A exhibited an half maximal inhibitory concentration (IC_{50}) value of 120.4087 ± 1.113 , whereas doxorubicin showed a slightly lower $\text{IC}_{50} = 117.89 \pm 0.537$. Uro A has demonstrated promising therapeutic effectiveness by engaging many pathways, such as inducing apoptosis, increasing the expression of pro-apoptotic genes (p53 and p21), inhibiting B-cell lymphoma 2 (Bcl-2), activating caspase and inhibiting protein kinase B (AKT) 1 and 2, and reducing levels of reactive oxygen species (ROS) in CRC cells. Our study in the field of network pharmacology has revealed encouraging possibilities for the treatment of CRC. This study offers a complete analysis of the mechanisms contributing to the possible anti-CRC effects of urolithin polyphenol complexes. Absorption, Distribution, Metabolism, Elimination, and Toxicity (ADMET) analysis reveals the promising potential of Uro A required for future drug development.

Conclusions: Our findings offer valuable insights for future CRC research and therapeutic strategy development, particularly in inhibiting AKT 1 and 2 and activating caspases to promote apoptosis, thereby contributing to effective CRC management.

Keywords: colorectal cancer; molecular modelling; epidermal growth factor receptor; urolithins; apoptosis



Graphical Abstract.

Introduction

In developing countries, colorectal cancer (CRC) is slowly becoming more common. It is already the third most common type of cancer in the world [1]. Colon cancer, or CRC, usually starts in the glandular epithelial cells of the large intestine. It is also called colon adenocarcinoma. Some epithelial cells get cancer when they get a set of genetic or epigenetic changes that give them an edge over other cells [2]. In the past, the incidence was highest in wealthy Western countries. However, as economies grow in many other parts of the world, the incidence is now rising quickly. Different mutations and reasons can lead to different types of CRCs. Because not all CRCs have the same driving mutations, it has been hard to make a molecular treatment that works for all of them. In the early stages, surgery is usually the best way to treat cancer. However, it doesn't work for about 25% of people whose cancer has spread, which makes managing complex cases harder [3].

Different countries have very different rates of colorectal cancer (CRC), which can be up to ten times higher or lower than the global average (Fig. 1). There is a clear trend that shows that the rate of CRC tends to rise as a country's economy does well. Age-adjusted rates of CRC are highest in wealthier countries, like Australia and some Eu-

ropean countries. In 2022, there were about 40 new cases of CRC for every 100,000 people in these countries, including women and men. Taking age into account, the rate of occurrence is about 50% higher in men than in women in most countries [4].

In some very developed countries, the number of cases of CRC has leveled off or even started to go down. In contrast, as the economies of most low and middle-income countries grow, the number of cases of CRC is rising quickly. Also, the overall worldwide incidence of CRC is expected to rise significantly over the next few decades. This is because the world's population is growing and people are becoming older. In particular, the number of new cases of CRC is expected to rise by 79%, from about 1.4 million cases in 2022 to over 2.4 million cases by 2035. The main reason for this rise is changes in the demographics of the population [5].

In developed countries, the average age of diagnosis for CRC is around 70 years old. CRC primarily affects people in their later years. CRC is influenced by various established risk factors, beyond age. These include gender, with a higher prevalence among males, as well as factors such as smoking, excessive alcohol consumption, a sedentary lifestyle, consumption of a diet rich in red and processed

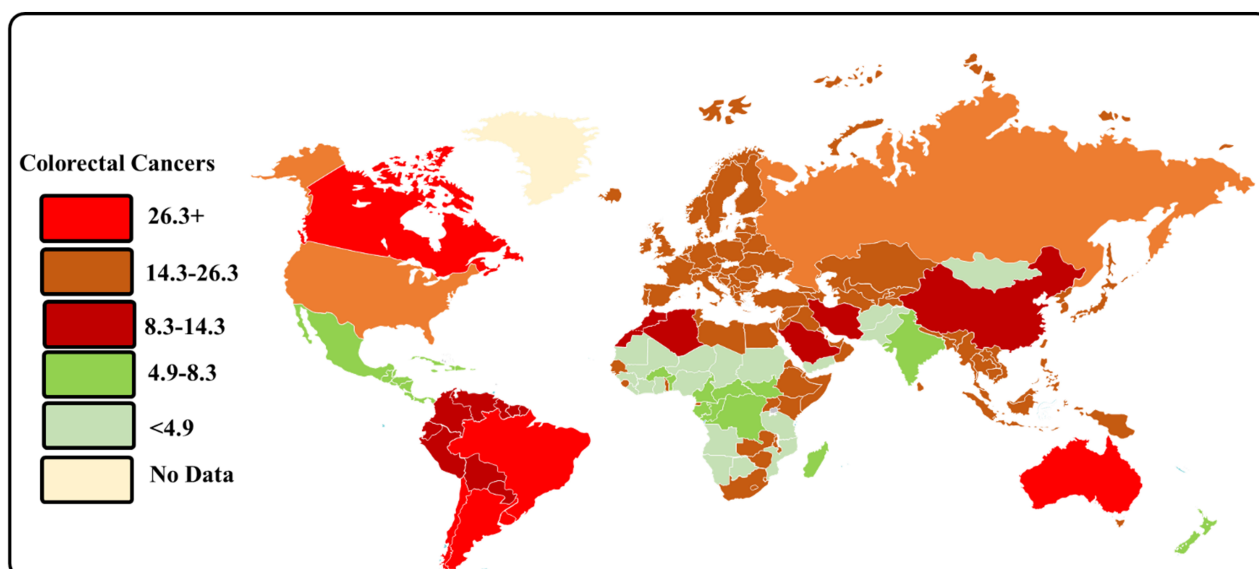


Fig. 1. The estimated rate of colon cancer worldwide in 2022. (The figure drawn in Power point, Microsoft office 2019, New Delhi, India).

meats, being overweight, and having a family history of the disease. In the vast majority of instances, surgery is the primary therapeutic route, and there has been a recent uptick in the utilisation of less invasive surgical techniques. In the management of stages II and III rectal cancer, neoadjuvant radiation therapy is commonly employed alongside surgical procedures. Additionally, for high-risk stage II and stage III colon cancer patients, adjuvant chemotherapy is routinely integrated into the treatment approach. The five-year relative survival rate for colorectal cancer (CRC) has demonstrated a consistent increase in the past few decades, surpassing 65 percent in affluent nations like the United States. However, survival rates persistently remain lower in numerous regions globally, including several European countries [6].

Many herbs that have great promise against CRC can be found in nature. An important group of herbs includes *Zingiber officinale*, *Curcuma longa*, *Allium sativum*, *Trigonella foenum graecum*, *Sesamum indicum*, *Linum usitatissimum*, and many more [7]. Urolithins (Uro) constitute a group of compounds produced by gut bacteria through the transformation of dietary polyphenols like ellagic acid (EA), ellagitannins (ET), and punicalagin. Among these compounds, Urolithin A (Uro A) and Urolithin B (Uro B) are the primary metabolites present in the gut, with Uro A identified as the most biologically active and possessing higher lipophilicity than EA. This characteristic opens avenues for potential drug development. Abundant quantities of urolithins have been observed in various human organs, including the breast, colon, and prostate. Circulating through the blood, urolithins have demonstrated potential in addressing various health conditions, including obesity, infections, inflammation, and cancer [8–10].

Fig. 2 illustrates the proposed hypothesis of the study, outlining molecular docking simulation investigations and Network Pharmacology involving of four urolithins i.e. Uro A, Uro B, Uro C, and Uro D and their anticancer potential. The selection of the most promising compound was based on its docking scores across three receptors—human epidermal growth factor receptor (HER)3, HER2, and HER1—belonging to the erythroblastic oncogene B (ERBB) family. The best docked compound, Uro A, underwent density functional theory (DFT) (E_{HUMO} , E_{LUMO}) analysis, and its anticancer potential was evaluated using HT-29 cell lines (♀ represent female), with doxorubicin serving as the standard. Network pharmacology has also been employed to elucidate the precise mechanisms by which Uro A exert its effect, as well as to investigate other biological processes occurring at the genetic and molecular levels.

Materials and Methods

Molecular Docking Studies

This study explored into the molecular docking and dynamics of peptides targeting the HER1, HER2, and HER3 receptors, specifically chosen for their roles in various diseases, including colorectal cancer. By exploring the interactions between peptides and these receptors, the research aimed to provide insights into potential therapeutic avenues, especially in the context of diseases where HER1, HER2, and HER3 play significant roles, such as colorectal cancer. The investigation sought to contribute to a deeper understanding of the molecular mechanisms involved, with the ultimate goal of informing the development of targeted therapies for colorectal cancer and other relevant diseases.

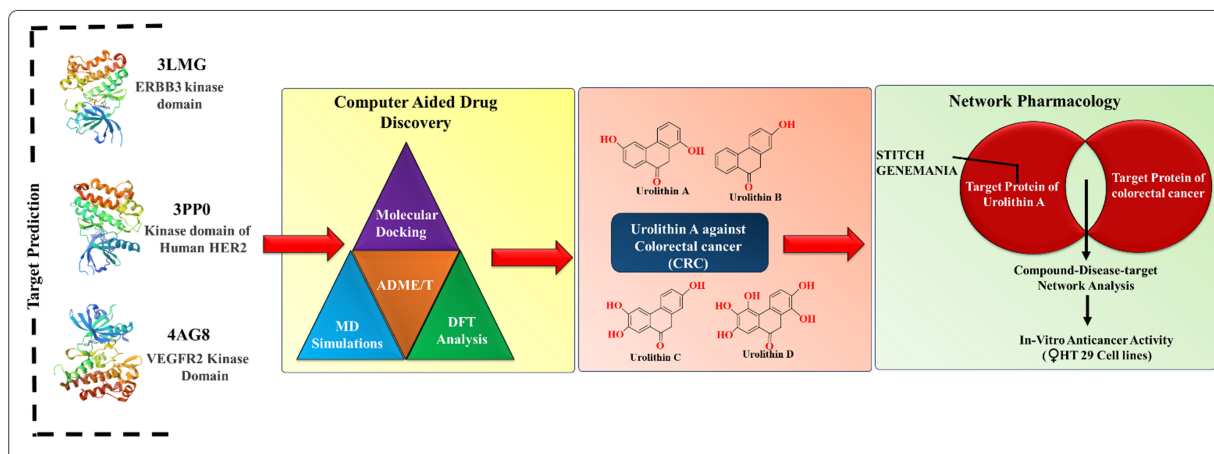


Fig. 2. Study layout of present work. (The figure drawn in Power point, Microsoft office 2019, New Delhi, India and 3D Protein Images taken from Protein Data Bank (PDB)). CRC, colorectal cancer; MD, molecular dynamics; ADMET, Absorption, Distribution, Metabolism, Elimination, and Toxicity; DFT, density functional theory.

Protein Preparation

Several protein kinase domain crystal structures were used in this research. These included the Human HER2 (ERBB2) kinase domain (Protein Data Bank (PDB) ID: 3PP0), the ERBB3 kinase domain (PDB ID: 3LMG) in complex with AMP-PNP, and the vascular endothelial growth factor receptors (VEGFR2) kinase domain (HER1) in compound with Axitinib (AG-013736). The proteins were autodocked using the Autodock Wizard before further investigation. Kollman charges were assigned, distributed uniformly across all atoms, and checked for any missing atoms in the residues after water molecules were removed, bonded heteroatoms/ligands were removed, polar hydrogens were added, and so on. After these procedures were completed, the PDB files were transformed into the Protein Data Bank, Partial Charge (Q), & Atom Type (T) (PDBQT) format for further study [11].

Ligand Preparation

The structure data files (SDF) of the ligands were downloaded from PubChem. MMFF94 is used to optimize ligands, making them as small as possible while maintaining a stable and energetically favorable structure. It is often employed for the optimization of molecular geometries, which involves adjusting the positions of atoms in a molecule to minimize its energy and achieve a stable conformation. Openbabel in the PyRx tool used to convert the ligands optimized by MMFF94 to the PDBQT format. PDBQT is a modified PDB format that includes information about atomic charges and atom types. This format is often used in molecular docking studies, where the interaction between ligands and proteins is simulated [11].

Grid Generation

For carrying out docking between prepared receptors and ligands, the grid was generated by taking the center on the attached ligand. The grid dimensions for PDB ID: 3LMG was number of points as 12.1246, -31.8049, and 46.6210 in X, Y, and Z directions 20.3975, 20.3975, 20.3975 respectively with default spacing. The grid dimensions for PDB ID: 3pp0 were the number of points as 16.3447, 14.6588, and 27.4367 in X, Y, and Z directions 20.0365, 20.0365, 20.0365 respectively with default spacing. The grid dimensions for PDB ID: 4ag8 were the number of points as 20.0876, 25.8236, and 40.1668 in X, Y, and Z directions 20.0726, 20.0726, and 20.0726 respectively with default spacing [11].

Docking and Visualization of Results

Docking has been performed to obtain possible conformations and orientations for the ligand at the binding site. The PyRx, Auto Dock Vina option was used to analyze the binding sites and docking runs of the target protein with the ligand, Simplified Molecular Input Line Entry System specific types of bonds or interactions (e.g., hydrogen bonds, hydrophobic interactions) [11].

Absorption, Distribution, Metabolism, Elimination, and Toxicity (ADMET)

It is very important to understand the features of Absorption, Distribution, Metabolism, Elimination, and Toxicity (ADMET) when judging the safety and efficiency of possible drug candidates. The objective of this study is to provide a comprehensive understanding of the pharmacokinetics of pharmaceuticals in the human body, which is crucial for both preclinical drug development and clinical trials. The Swiss ADME server was utilized to evaluate certain compounds after conducting molecular docking exper-

iments (Swiss ADME server, <http://www.swissadme.ch/>, accessed on August 12, 2023). The utilization of this web-based tool enables the assessment of diverse pharmacokinetic parameters and drug solubility through the importation of compounds' molecular structures in Simplified Molecular Input Line Entry System (SMILES) format. Subsequently, these factors are utilised to guide the selection of compounds for further phases of inquiry and experimentation.

Molecular Dynamics Study

The ligand-protein complex was subjected to a molecular dynamics simulation analysis using the Desmond tool developed by Schrödinger. Urolithins A, the most optimal docked molecule, was chosen for molecular dynamics (MD) simulations based on its hydrogen bond count, binding energy, and glide score characteristics. The simulation computation was conducted using a three-step process, namely system construction, energy minimization, and molecular dynamics. The solvated system was created by utilizing the SPC solvent model and the 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) membrane, which was enclosed within an orthorhombic box form. The buffer was chosen as the reference point for determining the size of the box, with a distance of 10.0 Å. The simulation box was brought to a physiological state by neutralizing its charge and adjusting the salt concentration to 0.15 M of Na⁺ and Cl⁻ ions. To minimize the volume of the simulation box, the solute's major axes were aligned either along the box vectors or along the diagonal. To minimize the energy of the ligand-protein complex, a low-temperature Brownian motion molecular dynamics (MD) simulation was conducted for a duration of 100 ps. This simulation was performed in the Number of particles, Volume, and Temperature (NVT) ensemble with the aim of resolving steric conflicts within the complex. The ligand protein complex that had undergone pre-processing was retrieved from the MD tab's workspace. Subsequently, an NPT ensemble was established with a temperature of 300 K and a pressure of 1.01325 bar. Following the relaxation of the model system, a simulation lasting 100 ns was conducted, during which the trajectory of the ligand-protein complex was recorded at intervals of 4.8 ps. The conformational behavior and stability of the complex were assessed during the 100 ns simulation by analyzing energy, ligand-protein root mean square deviation (RMSD), root mean square fluctuation (RMSF), protein-ligand interactions, and ligand properties [11].

DFT Study

The optimization of Urolithin A's geometry using the Becke, 3-parameter, Lee-Yang-Parr (B3LYP) functional and a 6-31 basis set was conducted through density functional theory (DFT) employing the Optimization, Reactivity, and Chemical Analysis (ORCA) software (version

5.0.3, Max Planck Institute for Coal Research, Mülheim an der Ruhr, Germany). The Avogadro visualization software (Avogadro 2, developed by Marcus D. Hanwell and Geoff Hutchison, Clifton Park, New York, NY, USA) facilitated the subsequent analysis and visualization of the optimized geometry. This study utilized DFT analysis to explore the reactivity properties of phytochemicals, focusing on their efficacy when interacting with the HR1 domain. The assessment involved examining the energies associated with highest occupied molecular orbital (HOMO) and lowest unoccupied molecular orbital (LUMO). Band energy gap determination was achieved through a provided equation. These descriptors, rooted in quantum physics principles and their computational methods, offer insights into the reactivity of phytochemicals. Avogadro software was utilized for constructing input files for a chemical analysis tool, while all energy calculations were executed using the ORCA program. The calculations specifically employed the B3LYP exchange-correlation functional, characterized as a hybrid exchange-correlation functional within the framework of density functional theory (DFT) [11].

Network Pharmacology

The present study focused on elucidating the specific mechanism of action on prospective molecular targets, to understand the underlying mode of action. The approaches utilised for this objective involved the application of Search Tool for Interactions of Chemicals (STITCH, <http://stitch.embl.de/>) and GeneMania (<https://genemania.org/>).

To establish a correlation between the predictions made by Swiss Target and the gene interaction data provided by Gene MANIA, we acquired the list of probable target genes recommended by Swiss Target for Urolithin A. Swiss Target employs similarity search methods and documented ligand-target interactions to identify prospective target genes. The aforementioned collection of target genes was then utilised as input for Gene MANIA, a comprehensive database that integrates many sources of functional genomics knowledge to predict gene interactions and functional linkages. The input for Gene MANIA consisted of the target gene list obtained from Swiss Target, with a specific organism or species specified for analysis. The provided input resulted in the creation of a network or interaction map using Gene MANIA. This map revealed probable functional relationships, including physical interactions, co-expression patterns, shared pathways, and genetic interactions among the genes provided as input. A complete understanding of potential functional networks and interactions among the anticipated target genes was achieved by combining the target gene predictions from Swiss Target with the gene interaction insights provided by Gene MANIA. The utilisation of this synergistic strategy enabled the elucidation of putative modes of action, molecular interactions, and biological pathways associated with the molecule or drug under investigation [12].

Co-Immunoprecipitation Assays

Cheng F *et al.* [13], reported Immunoprecipitation (IP) assays of Urolithin A through western blotting analysis. They have explored the possible mechanism of Urolithin A through mouse double minute 2 homolog (MDM2) regulation. Recently Uro A interacts with p53-MDM2 and triggers MDM2 by suppressing p53. Uro A expresses the distinct alterations in p53-null H1299 cells through MDM2 interaction. They reported that p53 and MDM2 interaction was observed to be significantly suppressed by Uro A.

Anti-Cancer Activity

The anticancer activity was performed at CSIR-Indian Institute of Chemical Technology (IICT), Hyderabad, India. The human female HT-29 cell line was authenticated through STR profiling, through CSIR-IICT, Hyderabad, India, and cross-contamination among cells was identified through mycoplasma testing (MT). Further, MT and bacterial infections were regularly examined through an inverted microscope. Cells were analyzed every 3–4 days and fresh culture medium was used three times a week.

The chemicals purchased for the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay were MTT from Sigma-Aldrich, Delhi, India with catalog number M5655, Dimethyl sulfoxide (DMSO) from Sigma-Aldrich, Delhi, India (with catalog number D2650, and phosphate-buffered saline (PBS)) from Thermo Fisher Scientific, India with catalog number 10010049. These reagents were utilized in the cell viability assay conducted as part of the *in vitro* anti-cancer experiment.

These cells were grown in a flask with a volume of 25 cm² using dulbecco's modified eagle medium (DMEM) as the medium. The medium was augmented with 10% fetal bovine serum (FBS, 12103C, Sigma Aldrich, St. Louis, MO, USA), L-glutamine, NaHCO₃, penicillin (100 U/mL), and streptomycin (100 µg/mL), and it was kept in an incubator with CO₂ at 37 degrees Celsius and 5% relative humidity. To conduct the anti-cancer test, two-day-old confluent monolayers of cells were trypsinized and then put into 96-well flat bottom tissue culture plates at a cell suspension volume of 100 µL, with 5×10^4 cells per well. These plates were then incubated at 37 °C. After that, the cells were placed in an incubator containing 37 degrees Celsius, humidified carbon dioxide, and a constant supply of CO₂. After 24 hours, the cell viability was examined under an inverted microscope. Fig. 3 provides a graphical illustration of the process that is followed during the *in vitro* anticancer experiment [14].

Growth inhibition percentage has been determined by viability determination may be calculated as

$$\% \text{ Viability} = \frac{A_{\text{Treated}}}{A_{\text{Control}}} \times 100$$

Where A_{test} is absorbance of test and A_{control} is absorbance of control.

Statistical Analysis

GraphPad Prism version 8.1.0 (GraphPad Software, Inc., San Diego, CA, USA) has been explored and data were presented in mean $\pm$ standard error of the mean (SEM). One-way analysis of variance has been used to explore the significance of data followed by Dunnett's multiple comparison test was performed. $p < 0.05$ means there is a significant difference, $p < 0.01$ means there is highly significant difference and $p < 0.001$ means extremely significant [12].

Results

Molecular Docking Study

UA metabolites namely A, B, C, D have been docked on 3 different PDBs revealed the significant binding and compared with standard drug doxorubicin and 5-fluorouracil (5-FU) as shown in Figs. 4,5,6 and Table 1.

The docking studies show Uro A exhibited the greatest docking score compared to all of the other proteins. In addition, Cheng *et al.* [13] have reported that, among the Uros, only Uro A, as well as the representative mixture mimicking the urolithin metabotype A (UM A), can selectively accelerate cellular senescence against CRC cell lines following long-term exposure. As a result, Uro A will serve as the subject of the molecular modeling investigations [13].

Results of ADMET

The results of ADMET are shown in Table 2.

The Table 2 presents a comprehensive overview of four polyphenol metabolites, Uro A, Uro B, Uro C, and Uro D, providing key chemical and pharmacological characteristics. Uro C and Uro D have the highest number of hydrogen bond acceptors. Uro C and Uro D have more hydrogen bond donors compared to Uro A and Uro B. Negative Log S values suggest good water solubility. Uro B has the highest solubility, while Uro D has the lowest. Uro B has the highest lipophilicity. All four metabolites exhibit high gastrointestinal absorption, suggesting they are efficiently taken up in the digestive tract. The negative Log Kp values indicate poor skin permeability, meaning these metabolites are unlikely to be absorbed through the skin. Uro A and Uro B can cross the blood brain barrier (BBB), while Uro C and Uro D cannot. None of the metabolites are substrates for P-glycoprotein, a transporter that can affect drug absorption. The Abbott bioavailability values are the same (0.55) for all four metabolites, indicating similar potential for oral absorption. Uro C is classified as active for hepatotoxicity, suggesting potential liver toxicity, while the others are inactive in this regard. Uro D is the only metabolite classified as active for potential carcinogenicity. Uro C shows activity for immunotoxicity, while the others are inactive. All four metabolites are inactive for mutagenicity and cytotoxicity. Uro B falls into toxicity class 6, indicating lower predicted toxicity, while the others are in class 4.

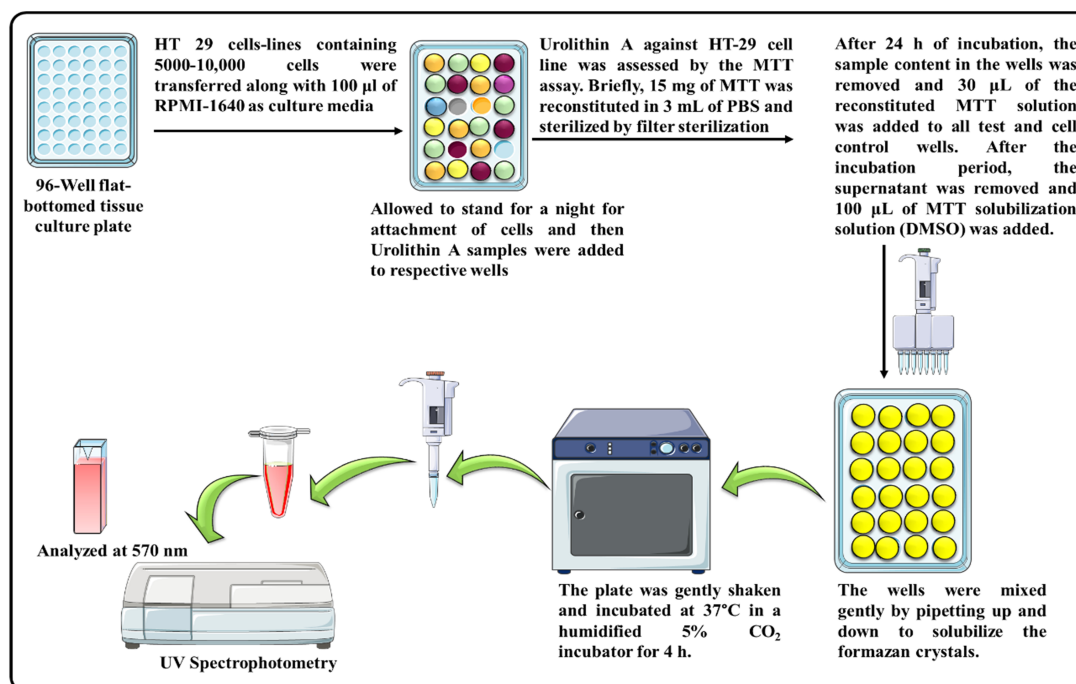


Fig. 3. Procedure of anticancer activity using HT 29 cell lines. (The figure drawn in Power point, Microsoft office 2019, New Delhi, India). MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

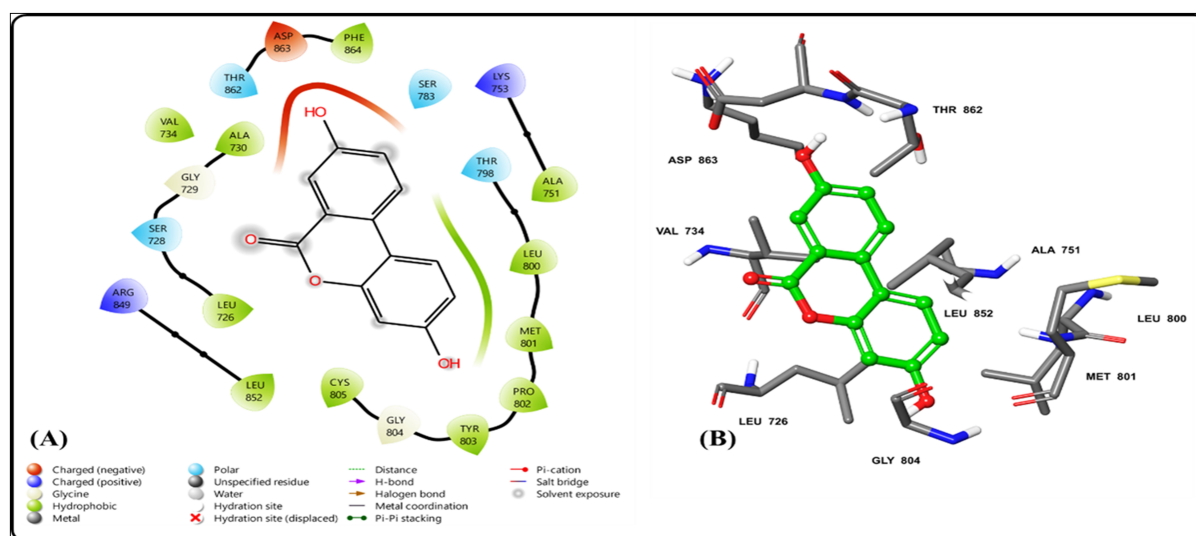


Fig. 4. Binding pattern of Urolithins A with HER3 using Maestro Visualizer (Schrödinger suite) software (version 12.3, New York, NY, USA). (A) 2-Dimensional. (B) 3-Dimensional. HER, human epidermal growth factor receptor.

Revankar AG *et al.* [15], 2023 reported the *in-vitro* and anti-inflammatory potential of Uro A. They have further reported the ADMET profile of Uro A, they revealed that Uro A exceeds all necessary parameters for designing it into an effective drug for the management of various disorders. Numerous conjugates of Uro A and its formulations have been synthesized after analyzing pharmacokinetic properties in a biological system [16].

US9394269B2 Patent has designed Uro A in salt form after analyzing the ADMET parameters in biological systems. Uro A is well-absorbed in the gastrointestinal tract due to its small molecular size and lipophilic nature, possibly via passive diffusion or active transport. Once absorbed, its lipophilic properties facilitate distribution throughout the body via the bloodstream, allowing it to reach various tissues and organs. Extensive metabolism

Table 1. Binding affinity of compounds by molecular docking study.

Compounds	PubChem ID	Source	Mechanism of action	Docking score (Kcal/mole)		
				3LMG	3PP0	4AG8
Folinic acid	135398742	Found in various leafy vegetables	Folinic acid is a form of vitamin B9 (folate) that can be converted into active forms of folate in the body.	-8.8	-9.3	-9.4
Uro A	44250707	Metabolites of ellagic acid	Urolithins A are derived from ellagic acid through the action of gut bacteria. They may have antioxidant and anti-inflammatory properties.	-7.8	-9.8	-9.6
Uro B	119783864	Metabolites of ellagic acid	Urolithins B are metabolites of ellagic acid and have antioxidant properties.	-7.7	-9	-9.5
Uro C	102227539	Metabolites of ellagic acid	Urolithins C are metabolites of ellagic acid and may have antioxidant properties.	-7.9	-9	-9.4
Uro D	104968197	Metabolites of ellagic acid	Urolithins D are metabolites of ellagic acid and may have antioxidant properties.	-8	-8.9	-8.8
5-FU	3385	Synthetic compound	5-FU interferes with DNA synthesis and repair, inhibiting the growth of cancer cells.	-4.9	-5.9	-5.2
Doxorubicin	31703	Isolated from Streptomyces species	Doxorubicin inhibits DNA replication and is cytotoxic to cancer cells.	-9.2	-8.1	-7.0

5-FU, 5-fluorouracil; Uro, urolithin.

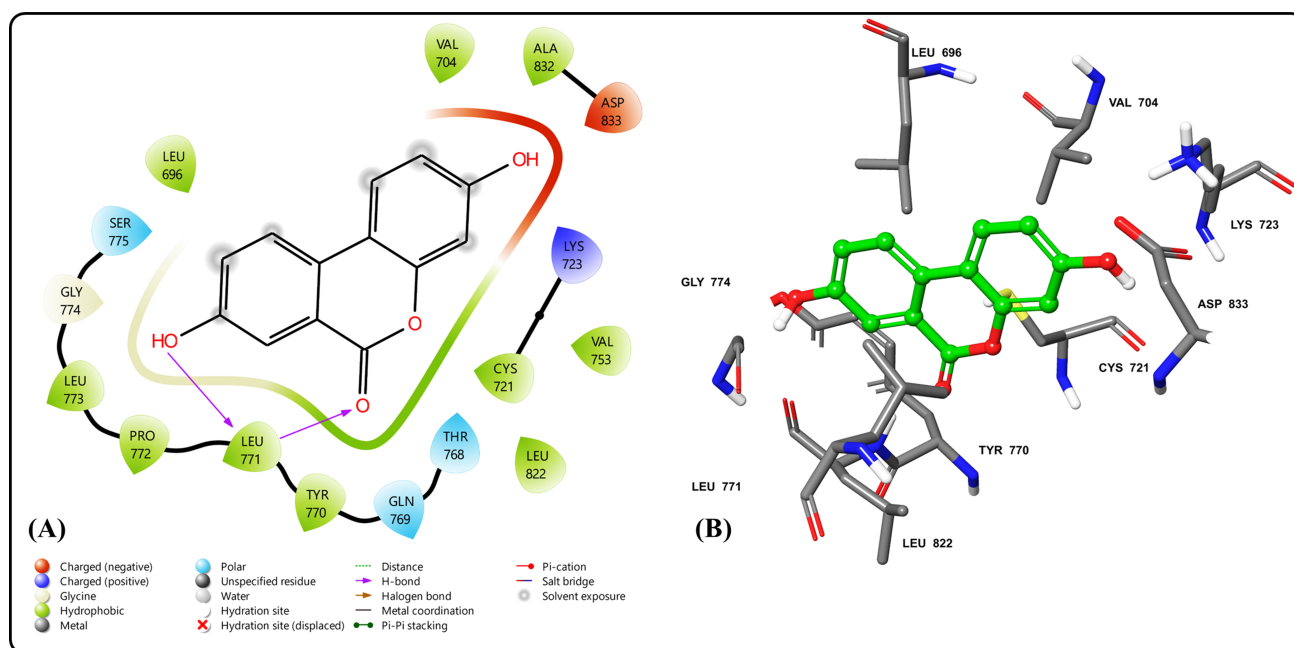


Fig. 5. Binding style of Urolithins A with human HER2 using Maestro Visualizer (Schrödinger suite) software (version 12.3, New York, NY, USA). (A) 2-Dimensional. (B) 3-Dimensional.

occurs in the liver and intestines, involving both phase I and phase II reactions to enhance water solubility. Metabolites, along with any unmetabolized Uro A, are excreted primarily via the kidneys through urine, with potential biliary excretion into feces. Uro A is generally considered safe, with low toxicity reported in preclinical studies, though further

research may be needed for long-term safety assessment, especially at higher doses or with chronic exposure [17].

Results of Molecular Dynamics

The results of molecular dynamics on protein HER2, HER3 and HER1 are shown in Figs. 7,8,9,10,11,12,13.

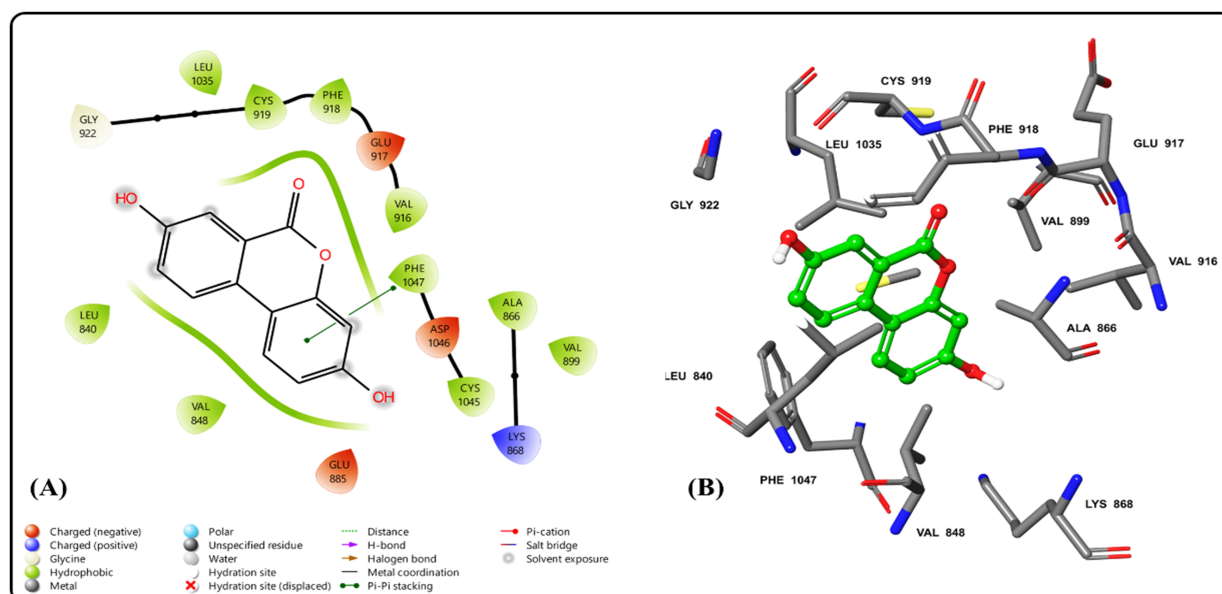


Fig. 6. Binding style of Uro A with HER1 using Maestro Visualizer (Schrödinger suite) software (version 12.3, New York, NY, USA). (A) 2-Dimensional. (B) 3-Dimensional.

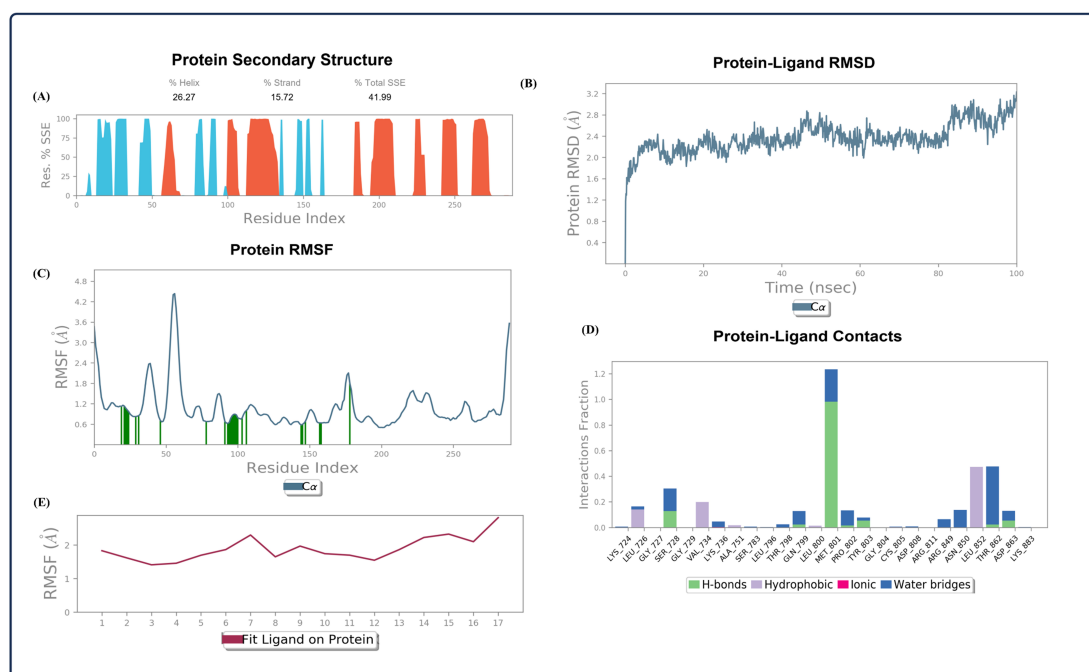


Fig. 7. Molecular simulation of Urolithins A with human HER2. (A) Protein secondary structure. (B) Protein-ligand RMSD. (C) Protein RMSF. (D) Protein-ligand contacts (amino acids involved in protein-ligand). (E) Ligand RMSF using Desmond Software (Schrödinger suite, version 2021.2, New York, NY, USA). RMSD, root mean square deviation; RMSF, root mean square fluctuation.

RMSD and RMSF analyses were performed to evaluate the trajectory resulting from simulations involving the Human HER2 protein complex, as illustrated in Figs. 7,8. In the context of the protein-ligand complex, the RMSD

exhibited a range spanning from a minimum of 0.08 Å to a maximum of 0.37 Å, with an average value of 0.18 Å throughout 100 ns, accompanied by a standard deviation of 0.05. The radial gyration (rGyr) displayed variations,

Table 2. Results of Absorption, Distribution, Metabolism, Elimination, and Toxicity (ADMET).

	Uro A	Uro B	Uro C	Uro D
Chemical formula	C ₁₃ H ₈ O ₄	C ₁₃ H ₈ O ₃	C ₁₃ H ₈ O ₅	C ₁₃ H ₈ O ₆
Molecular weight	228.2	212.2	244.2	260.2
Hydrogen bond acceptors	4	3	5	6
Hydrogen bond donors	2	1	3	4
Water solubility (Log S)	-3.33	-3.49	-3.17	-3.02
Lipophilicity (Log P)	1.47	1.81	1.37	0.58
Gastrointestinal absorption	High	High	High	High
Skin permeation (Log Kp (cm/s))	-2.777	-2.642	-2.735	-2.735
BBB permeation	Yes	Yes	No	No
P-glycoprotein substrate	No	No	No	No
Bioavailability	0.55	0.55	0.55	0.55
Hepatotoxicity	No activity	No activity	Active	No activity
Mutagenicity	No activity	No activity	No activity	No activity
Cytotoxicity	No activity	No activity	No activity	No activity
Carcinogenicity	No activity	No activity	No activity	Active
Immunotoxicity	No activity	No activity	Active	No activity
Predicted toxicity class	4	6	4	4
Predicted lethal dose ₅₀ (mg/kg)	2.71 mg/kg	1.512 mg/kg	2.627 mg/kg	2.458 mg/kg

LD₅₀, lethal dose 50; BBB, blood brain barrier.

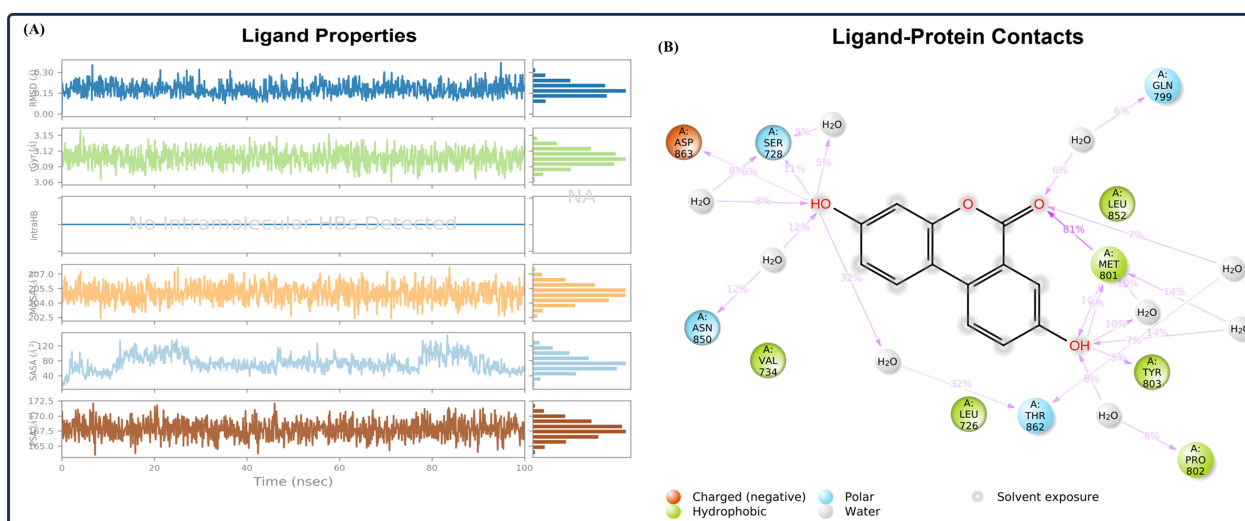


Fig. 8. Molecular simulation of Urolithins A with human HER2. (A) Ligand properties. (B) Protein amino acids interacted with ligands using Desmond Software (Schrödinger suite, version 2021.2, New York, NY, USA).

reaching a minimum of 3.06 Å and a maximum of 3.16 Å, while maintaining an average of 3.11 Å, and a standard deviation of 0.02. RMSF calculations, designed to gauge residue mobility and conformational alterations in the context of complex-ligand interactions, were also conducted.

Regarding molecular surface properties, the Van der Waals surface area (MolSA) exhibited a range from a minimum of 202.38 Å² to a maximum of 207.74 Å², with an average value of 204.99 Å² and a standard deviation of 0.89. The solvent accessible surface area (SASA) showcased variations from a minimum of 10.60 Å² to a maxi-

um of 148.73 Å², yielding an average of 73.58 Å², accompanied by a standard deviation of 2.54. Similarly, the molecular polar surface area (PSA) demonstrated fluctuations ranging from a minimum of 163.54 Å² to a maximum of 172.12 Å², resulting in an average value of 167.80 Å², with a standard deviation of 1.33. In addition, the B factor, indicative of atomic thermal motion, displayed an average value of 40.20, coupled with a standard deviation of 1.57.

The assessment of RMSD and RMSF was also conducted for simulations involving the protein HER3 and protein HER1 complexes (Figs. 9,10,11,12,13). Notably, the

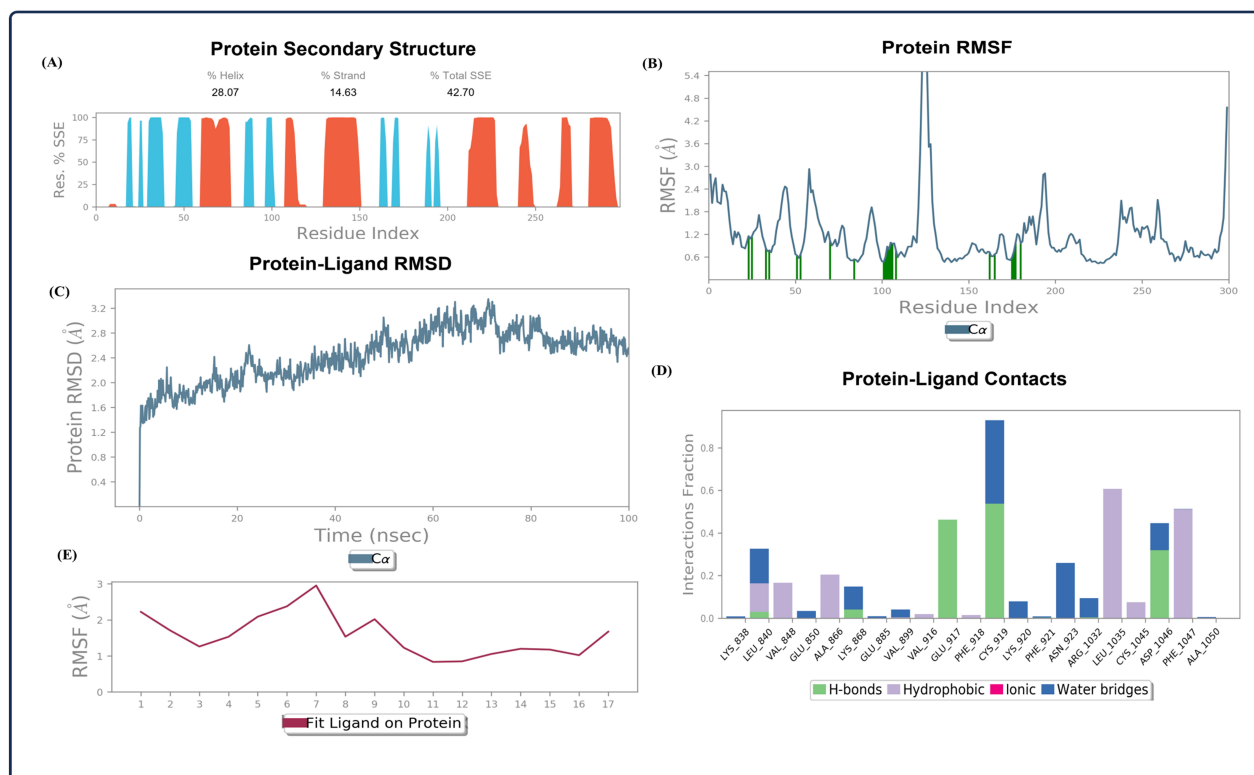


Fig. 9. Molecular simulation of Urolithins A with HER3 domain. (A) Protein secondary structure. (B) Protein-ligand RMSD. (C) Protein RMSF. (D) Protein-ligand using Desmond Software (Schrödinger suite, version 2021.2, New York, NY, USA) Contacts (Amino acids involved in Protein-Ligand). (E) Ligand RMSF.

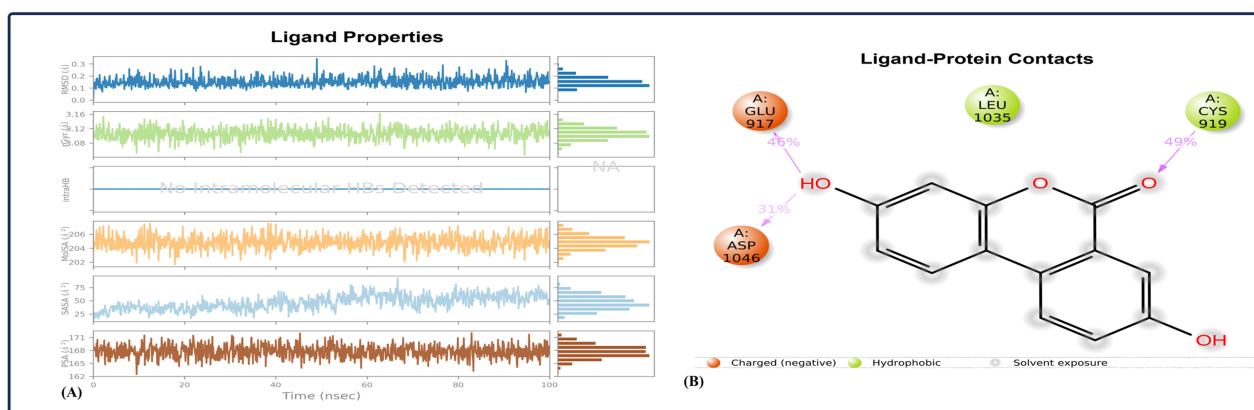


Fig. 10. Molecular simulation of Urolithins A with HER3 domain. (A) Ligand properties; (B) Protein amino acids interacted with ligands using Desmond Software (Schrödinger suite, version 2021.2, New York, NY, USA).

RMSD values for these complexes exhibited similar trends, achieving minimum values of 0.08 Å and maximum values of 0.33 Å and 0.34 Å, respectively, over a 100 ns trajectory.

Based on the aforementioned analyses, the protein-ligand complex resulting from the docking of Human HER3 with Uro A demonstrated stability, thereby validating the docking outcome. Analysis of the protein secondary structure revealed proportions of 26.27% helical, 15.72% strand,

and 41.99% protein secondary structure elements (SSE), as illustrated in Figs. 9,10. The interaction landscape exhibited polar interactions with ASN850; GLN 799; SER 728, and charged interactions with ASP863. Additionally, hydrophobic interactions were observed with MET 801, LEU 852, VAL734; LEU726; TYR803, and PRO802. Approximately 81% of the interactions were characterized by hydrogen bonding with MET 801, while no intramolecular hy-

drogen bond interactions were identified. The torsion profile of the ligands further delineated the mobility of specific chemical bonds, with notable characteristics exhibited in Fig. 14, revealing rotational capacities around specific bonds. Notably, both hydroxyl (OH) groups exhibited rotational potential of up to 180°, while maintaining a linear alignment with each other [11].

Results from DFT Analysis

Table 3 and Fig. 15 present comprehensive insights into the energy gap (ΔE) existing between the E_{HOMO} (eV) and the E_{LUMO} (eV) for Urolithins A. The other parameters included are Chemical/Global Hardness (n); Chemical Potential (μ); Chemical Softness (σ); electron affinity (A); Electronegativity (χ) (eV); Electrophilicity (ω); Ionization Potential (I); Maximum Charge Transfer Δn_{max} and Nucleophilicity (N) and shown in Table 3. The depicted free molecular orbital diagram visually encapsulates the intricate electronic interactions within Urolithins A, providing a detailed representation of its orbital energy levels and their relative positions in relation to the HOMO and LUMO [11].

Table 3. DFT study of Urolithin A.

S. No.	Compound Name	Urolithins A
(1)	E_{HOMO} (eV)	-5.8560
(2)	E_{LUMO} (eV)	-1.5860
(3)	ΔE (eV)	4.2700
(4)	μ (eV)	-3.7210
(5)	n (eV)	2.1350
(6)	σ (eV)	0.4684
(7)	I (eV)	5.8560
(8)	A (eV)	1.5860
(9)	χ (eV)	3.7210
(10)	ω (eV)	3.2426
(11)	Δn_{max} (eV)	1.7429
(12)	N (eV)	3.5126
(13)	Number of canonical Structures	278

DFT, density functional theory; LUMO, lowest unoccupied molecular orbital; HOMO, highest occupied molecular orbital. ΔE , energy gap; n , Chemical/Global Hardness; μ , Chemical Potential; σ , Chemical Softness; A , electron affinity; χ , Electronegativity; ω , Electrophilicity; I , Ionization Potential; Δn_{max} , Maximum Charge Transfer; N , Nucleophilicity.

The absorption of electrons occurs during molecule interactions at both the lowest unoccupied molecular orbital (LUMO) and the highest occupied molecular orbital (HOMO). The energy level of the lowest unoccupied molecular orbital (LUMO) exhibits a direct relationship with the electron affinity (A) of the molecule, indicating its inclination to take electrons. Conversely, the energy level of the highest occupied molecular orbital (HOMO) is associated with the ionization potential (IP), which signifies

the energy necessary for electron removal. The HOMO-LUMO energy gap, which refers to the energy difference between the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO), holds great importance in providing insights into the electrical transport characteristics of molecules. The energy gap mentioned offers a valuable understanding of the underlying mechanisms of charge transfer occurring within the molecule structure. A significant disparity in energy levels between the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO) indicates that the process of introducing an electron to the energetically higher LUMO and removing electrons from the energetically lower HOMO necessitates a substantial input of energy. As a result, the observed difference in energy levels makes these operations unfavorable from an energetic standpoint. The aforementioned condition leads to a decrease in chemical reactivity and an increase in the kinetic stability of the molecule. Compounds that possess a significant difference in energy between the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO), as shown by urolithins with $E_{\text{HOMO}} = -5.8560$ eV, $E_{\text{LUMO}} = -1.5860$ eV, and $\Delta E = 4.2700$ eV, have an increased capacity to resist chemical transformations owing to their inherent stability. Urolithin A can be classified as a group of compounds that exhibit enhanced stiffness and stability, characteristics that are attributed to their significant energy gap between the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO).

Results of Anticancer Activity

Uro A significantly suppresses the growth of HT-29 cells, *in-vitro* anticancer analysis showed the half maximal inhibitory concentration (IC_{50}) value of 120.4087 ± 1.113 in comparison to doxorubicin (standard) 117.89 ± 0.537 . The p -value for the difference between the treated and untreated samples was <0.0001 , which indicates that the difference was highly significant. These results suggest the use of Uro A may a potential chemotherapeutic agent against HT-29 cells. Fig. 16 depicts the results of *In-vitro* anticancer activity. The statistical analysis shows the $p^{***} < 0.0001$, which shows that all the results are extremely significant and the symbol ♀ represents females.

Results of Network Pharmacology

Fig. 17 depicts the gene-gene interaction of Urolithins evaluated through network pharmacology and shows the interaction of Urolithins A with many genes namely mammalian target of rapamycin (mTOR), phosphatase and tensin homolog (PTEN), Bcl2-like 11 (BCL2L11), ras-related C3 botulinum toxin substrate 1 (RAC1), caspase-3 (CASP3), caspase-8 (CASP8), T-cell leukemia/lymphoma protein 1A (TCL1A), Bcl-2-modifying factor (BMF), protein phosphatase 2 catalytic subunit alpha (PPP2CA),

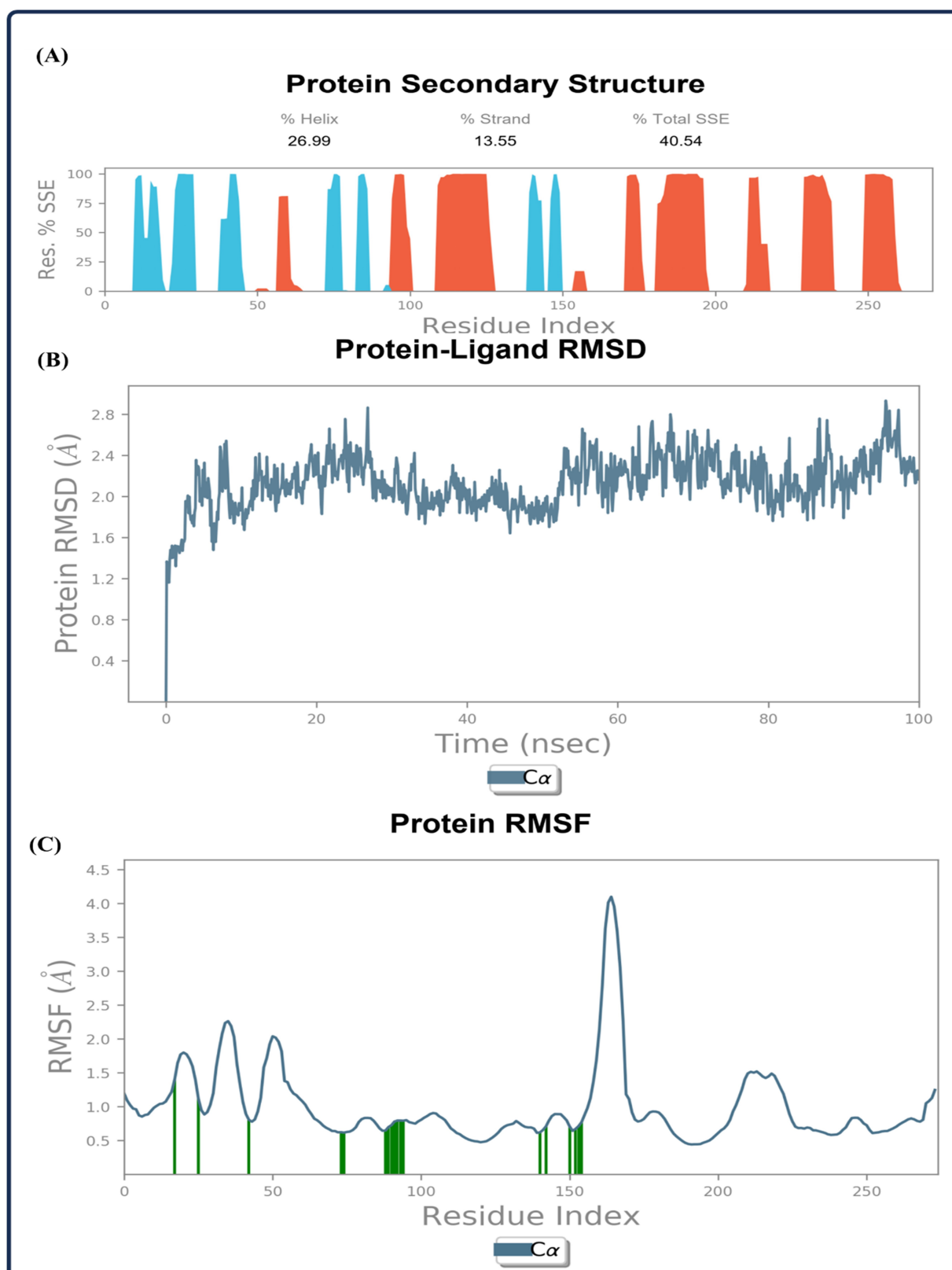


Fig. 11. Molecular simulation of Urolithins A with HER1. (A) Protein secondary structure. (B) Protein-ligand RMSD. (C) Protein RMSF using Desmond Software (Schrödinger suite, version 2021.2, New York, NY, USA).

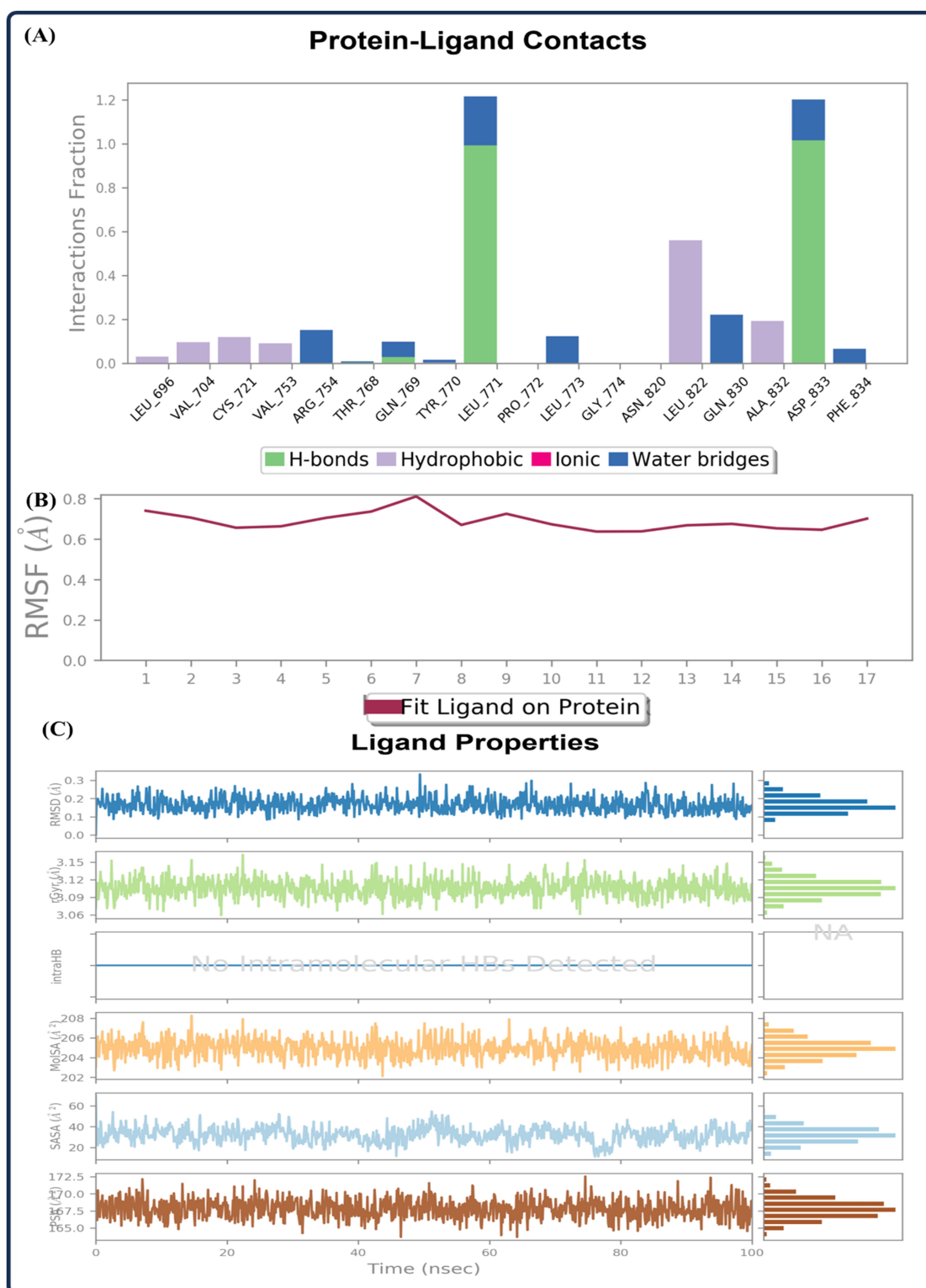


Fig. 12. Molecular simulation of Urolithins A with HER1. (A) Protein-ligand contacts (amino acids involved in Protein-Ligand). (B) Ligand RMSF. (C) Ligand properties using Desmond Software (Schrödinger suite, version 2021.2, New York, NY, USA).

growth factor receptor-bound protein 10 (GRB10), Phosphoinositide-3-kinase regulatory subunit 1 (PIK3R1), mouse double minute 2 homolog (MDM2), protein phos-

phatase, Mg^{2+}/Mn^{2+} dependent 1A (PPM1A), protein kinase B alpha (AKT1), ribosomal protein S6 kinase beta-1 (RPS6KB1), mitogen-activated protein kinase associated

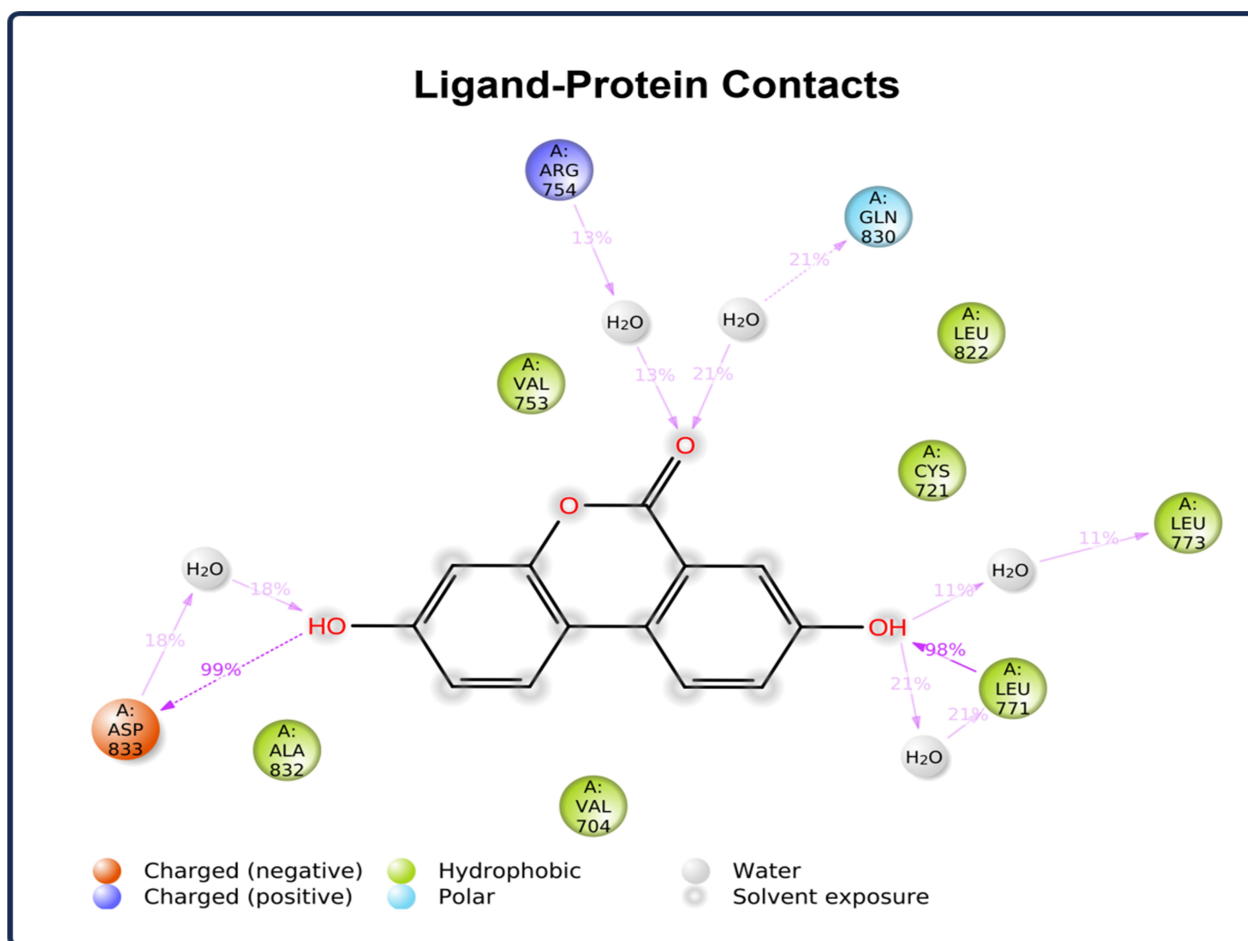


Fig. 13. Molecular simulation of Urolithins A with HER1. Protein amino acids interacted with ligands using Desmond Software (Schrödinger suite, version 2021.2, New York, NY, USA).

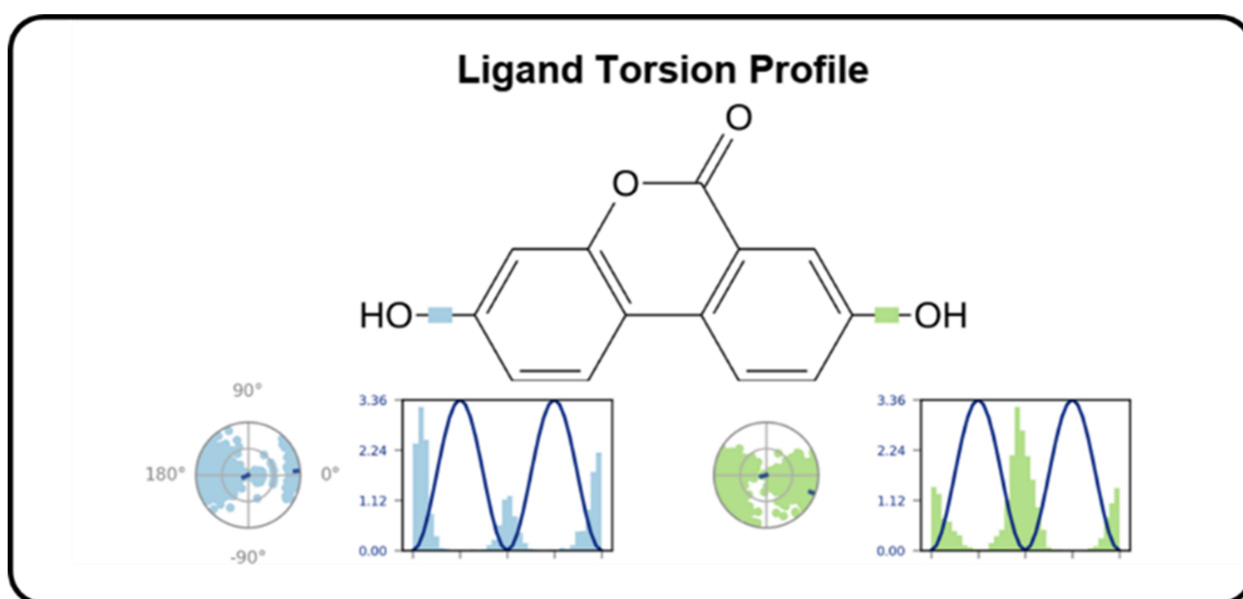


Fig. 14. The torsion profile of the Urolithin A using Desmond Software (Schrödinger suite, version 2021.2, New York, NY, USA).

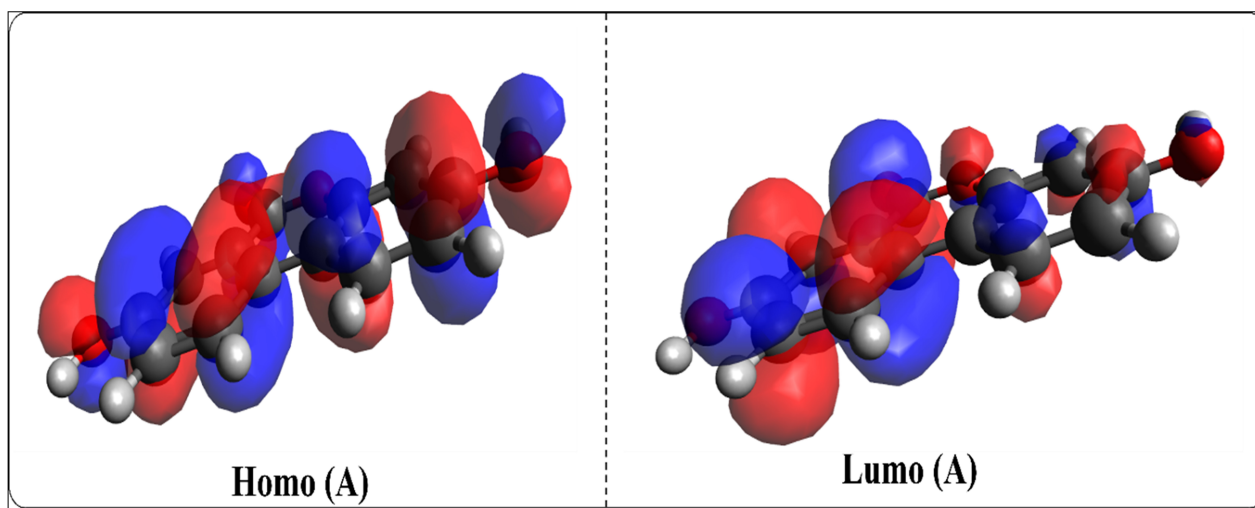


Fig. 15. DFT studies of Urolithins A obtained through the geometry optimization using Optimization, Reactivity, and Chemical Analysis (ORCA) software (version 5.0.3, Max Planck Institute for Coal Research, Mülheim an der Ruhr, Germany).

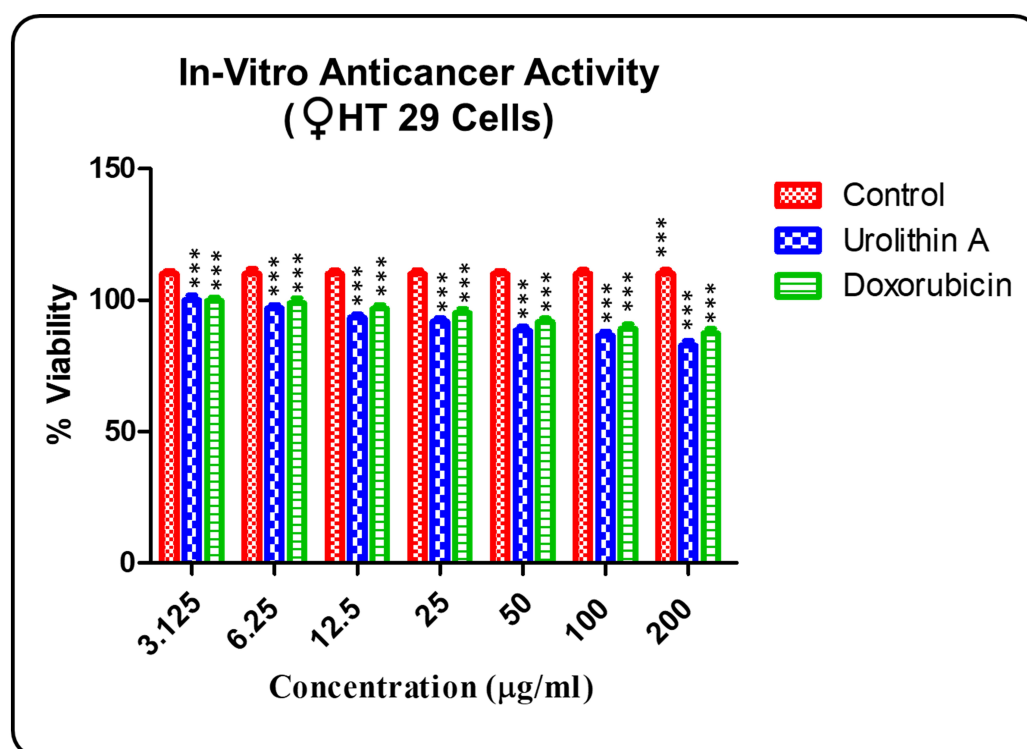


Fig. 16. *In-vitro* Anticancer activity assay of Uro A against q HT 29 Cell lines. *** < 0.0001, which shows that all the results are extremely significant. q represent female.

protein 1 (MAPKAP1), tuberin 2 (TSC2), eukaryotic translation initiation factor (EIF), heme-binding protein 1 (HEBP1), adaptor protein, phosphotyrosine interaction (APPL1), PH domain, and leucine zipper containing 1 (ESR1), estrogen receptor 1, 3-phosphoinositide-dependent protein kinase 1 (PDPK1), etc.

In response to upstream signals, the mTOR controls cell viability, metabolism, development, and protein syn-

thesis in both healthy physiological and pathological situations, particularly in cancer (Tian T). The most significant inhibitor of the phosphoinositide-3-kinase regulatory subunit 1 (PI3K) signaling pathway is phosphatase and tensin homolog (PTEN) may work as a tumor inhibitor [12,15].

The B-cell lymphoma 2 (Bcl-2) protein family plays a vital role in the management of carcinogenesis, apoptosis and cellular reactions to anticancer therapy have been

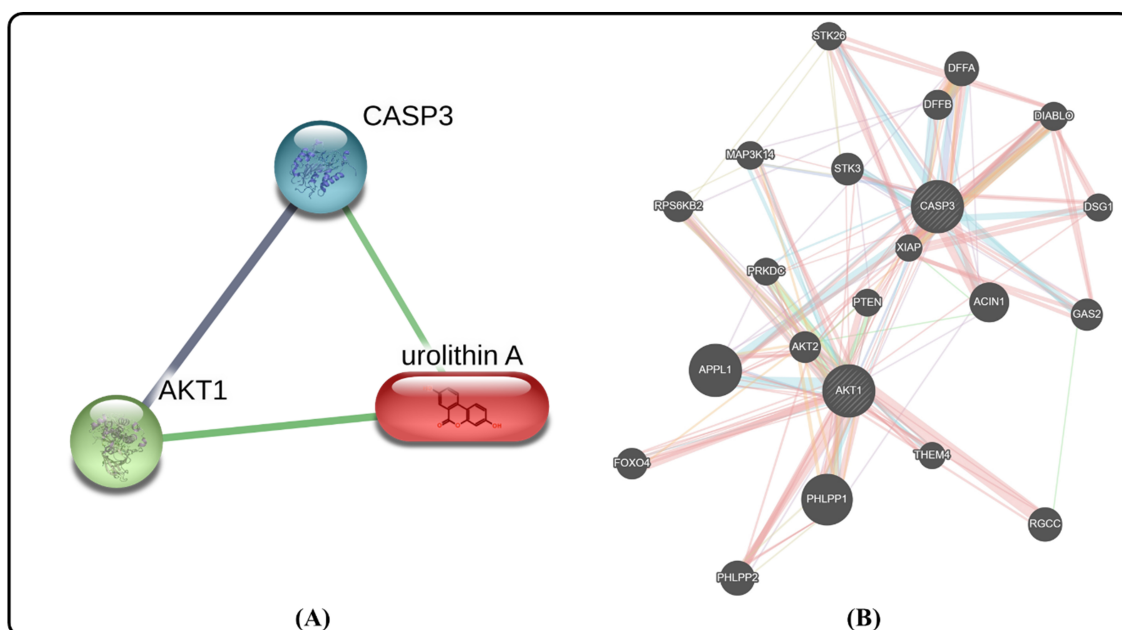


Fig. 17. Gene-gene interaction of Uro A. (A) Gene interaction of Urolithin A Search Tool for Interactions of Chemicals (STITCH, <http://stitch.embl.de/>); (B) Gene interaction from GeneMania Software (version 3.5.1, Toronto, Ontario, Canada). CASP3, caspase-3; AKT1, protein kinase B alpha; AKT, protein kinase B; RPS6KB2, ribosomal protein S6 kinase beta-2; PTEN, phosphatase and tensin homolog; APPL1, adaptor protein, phosphotyrosine interaction.

reported. Ras-related C3 botulinum toxin substrate 1 (RAC1), Matrix metalloproteinases (MMPs) regulate angiogenesis whereas the caspase genes regulate apoptosis, gene therapy methods along independent cell death. However, it has been suggested that these genes may contribute to tumor development when exposed to chemicals or radiation [18,19].

MDM2 gene activation is negatively associated with distant metastasis in CRC and associated with disease progression [16,20]. The risk and prognosis of CRC may also be influenced by MAPK-signaling [17]. GSK-3 β is required by CRC for maintaining mitosis via translocated promoter region (TPR)-dynein interaction; suppression of the same may be a novel target for CRC therapy [21].

Discussion

The findings of the study suggest a considerable and persistent binding towards all three PDB, and docking analysis demonstrated a higher binding score compared to that of the reference medication that was employed. This strongly indicates that the drug may have a promising function in the treatment of CRC. The epidermal growth factor receptor (EGFR) family is comprised of four distinct tyrosine kinases, namely known as HER1, HER2, HER3, and HER4. Although these genes are located on four distinct chromosomes, they exhibit shared traits such as

being lipophilic and transmembrane, having a carboxy-terminal domain, and an intercellular domain. Alterations in the ERBB family have been linked to the formation and advancement of various forms of human malignancies, including prostate, melanoma, lung, breast, colorectal, laryngeal, esophageal, and ovarian cancers, respectively. The process of interaction between growth factors and ligands initiates transphosphorylation, which then activates many signaling pathways, including rat sarcoma rapidly accelerated fibrosarcoma/mitogen-activated protein kinase/extracellular signal-regulated kinase/phosphoinositide 3-kinase/protein kinase B/mammalian target of rapamycin (RAS/RAF/MEK/ERK, PI3K/AKT/mTOR), Src kinases, and STAT transcription factors. In particular, the HER2 protein does not engage in direct interactions, but instead induces a specific conformational change that leads to the activation of a ligand activating state, ultimately resulting in dimerization. The activation of HER2 receptors results in the formation of dimers, specifically the HER2/HER3 dimer. This dimerization process initiates complex signal transduction pathways, which in turn have significant effects on various aspects of cancer cell behaviour, including survival, progression, translocation, and invasiveness. While the HER3 receptor is often considered to have weaker potential compared to other receptors, it nonetheless plays a crucial role in cell survival and promotion [22].

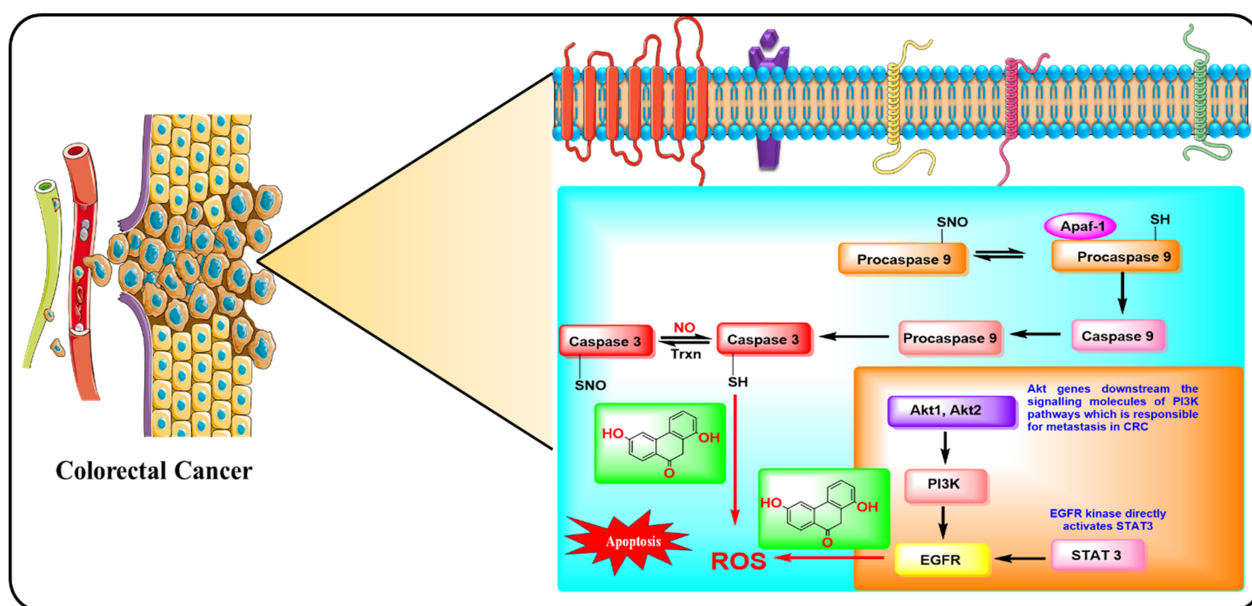


Fig. 18. Depicts the gene-gene interaction of Urolithins evaluated through network pharmacology shows the interaction of Urolithins A with many gene namely Protein kinase B and caspase-3 (The figure drawn in Power point, Microsoft office 2019, New Delhi, India and ChemDraw Software, PerkinElmer Informatics, Waltham, MA, USA). ROS, reactive oxygen species; PI3K, phosphoinositide 3-kinase; EGFR, epidermal growth factor receptor; STAT3, signal transducer and activator of transcription 3; SNO, S-nitrosothiols; SH, sulfhydryl group.

AKT serves as the pivotal protein in a prominent cellular signalling pathway that plays a critical role in various cellular processes, including cell survival, metastasis, drug resistance, metabolism, and radiation resistance. Although blocking AKT can hinder crucial cell-survival processes, it can also trigger the activation of other proteins further down the line, such as receptor tyrosine kinases (e.g., HER3, IGF-1R, and insulin receptor), which allows cancer cells to stay alive. The existence of three AKT isoforms, namely AKT1, AKT2, and AKT3, is attributed to their expression from distinct genes, despite their significant similarities. Their expression is contingent upon the kind of tissue and they are postulated to possess distinct functions [20,21].

AKT 1 and AKT 2 play a significant role in supporting malignancy in humans. Numerous AKT2 genes trigger cancer cells as well as their invasion may lead to associated cellular consequences. Therefore, overexpression of AKT and its isoforms in cancer reveals that AKT inhibitors will effectively therapy against colon cancer. Urolithins have been showed to interact with these genes. It has been also previously reported that *AKT* isoform mRNA expression significantly improved in *AKT1* and *AKT2* mRNA expression in CRC in comparison with other normal cells [23,24].

The inhibition of AKT genes will in turn down stream the signaling molecules of PI3K pathways which are responsible for metastasis in CRC. The inhibition of PI3K pathways leads to turn off of the EGFR pathways suggesting that will be beneficial in the management of CRC as

well as may prolong the duration of as well as the progression of CRC from one stage to another will enhance the chances of successful treatment of CRC [25,26].

Urolithins also interact with caspases that carry out apoptosis, namely caspase-3, 6, and 7 respectively. Numerous proteins both functional and structural play vital roles in cell survival and are regulated through these genes. The most noteworthy executioner caspase-3, activates both mechanisms (internal and external). They also trigger mitochondrial function lead to energy generation and further promote normal cell functioning. Therefore, caspase-3 inhibitors play a major role in CRC management. Fig. 18 shows the conversion of procaspase into different caspases namely 3, 6, 7, 9, etc. The caspase inhibition will lead to apoptosis absolutely required in CRC therapy which will not only control cell growth but also proliferation in CRC [27].

Additionally, it investigates the involvement of the PI3K/AKT and WNT/ β -catenin signaling pathways in this process. The growth factor receptors (x) of NF- κ B (ix). Fig. 18 illustrates many cellular processes such as tumor development, apoptosis induction, senescence, transcriptional regulation of oncogenes and tumor suppression genes, and cell cycle arrest. The observation that UA-A exhibits greater resistance to glucuronidation by Caco-2 cells compared to Iso-A implies that UA-A possesses a more potent anti-proliferative effect. The stimulation of cellular senescence, a mechanism that diminishes the proliferative capac-

ity of cancer cells, is a promising avenue for cancer therapy. The p53 gene, recognized as a tumor suppressor gene, exhibits interaction with p21 and serves as a pivotal gene in the context of tumorigenesis [28,29]. CRC represents the mutation in this gene which promotes cancer progression through interaction with HER1, HER2, and HER3. The altered p53 gene leads to a progression of CRC and enhanced tumorigenicity and drug resistance. Besides this, Mutated p53 also triggers the CD44, Lgr5, and ALDH expression, markers of CRC, further leading to more aggressive cancer [30].

Cellular senescence is the permanent halting of cell division in response to stress. This can be a tumor-suppressive mechanism, as it prevents cancer cells from proliferating. Senescent cells exhibit several molecular signatures, including increased β -galactosidase activity and upregulation of p21 and p16 gene expression. Urolithins have been shown to promote senescence in colon cancer cell lines [28–30].

Birk *et al.* [31], 2021 revealed the Uro A as promising antioxidant and anti-inflammatory. They have selected 40 patients and after giving Uro A rich food, the quantity of urinary Uro A has been evaluated. Therefore, further more studies are required in order to establish the drug at market for large population size.

Conclusions

Addressing CRC, a significant global health issue, necessitates the exploration of natural compounds like Uro A for potential therapeutic uses. Uro A, a stable metabolite derived from pomegranate, exhibits superior absorption compared to its precursors. Docking simulations involving protein-ligand complexes such as human epidermal growth factor receptor 3 (HER3), human epidermal growth factor receptor 2 (HER2), and epidermal growth factor receptor (HER1) have yielded promising results, comparable to established gold-standard drugs like folinic acid, doxorubicin, and 5-FU. Density functional theory (DFT) analysis has been performed to confirm Uro A stability, and the results showed that Uro A is viable for therapeutic usage due to its favorable energy values and electronic properties. The proposed mechanism of action for Uro A involves some pathways, including apoptosis induction, enhancement of pro-apoptotic proteins p53 and p21, downregulation of anti-apoptotic B-cell lymphoma 2 (Bcl-2) protein, activation of caspase enzymes and release of cytochrome c, as well as suppression of reactive oxygen species (ROS) within CRC cells. These results show that Uro A affects CRC cells in many different ways, which makes it an interesting area to study further. Although the study provides valuable insights into the potential benefits of Uro A from pomegranate, further research for formulation development, evaluation, and clinical studies are required for the development of targeted drug delivery at the colon and

rectum site, to provide the effective treatment against CRC and demographic evaluation for different races, genders, age groups, etc is required for the management of CRC.

Availability of Data and Materials

Data will be made available from the corresponding authors on reasonable request.

Author Contributions

Conceptualization, AT, VT and AS; methodology, AT; software, AT, and VT; validation, AT, ALM and AA; formal analysis, AA, ALM, and FAN; investigation, AT; resources, VT and MAZ; data curation, MK, SV, GK, AAQ, FAN, MM, MAZ, AS, ALM and AA; writing—original draft preparation, AT, VT; writing—review and editing, AT, VT, MK, SV, GK, AAQ, FAN, MM, MAZ and AA; visualization, AT; supervision, AT and VT; project administration, AT; funding acquisition, MAZ and FAN. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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