

Deciphering the Molecular Mechanisms of Gentisic Acid in Breast Cancer: Combining Network Pharmacology, Molecular Modelling, and Experimental Approaches

Rohit Singh Negi

Indian Institute of Technology (Banaras Hindu University)

Rakesh Kumar Gupta

Banaras Hindu University

Nitish Kumar

Indian Institute of Technology (Banaras Hindu University)

Pawan Kumar Dubey

Banaras Hindu University

Sunil Kumar Mishra

`skmishra.phe@iitbhu.ac.in`

Indian Institute of Technology (Banaras Hindu University)

Research Article

Keywords: Gentisic acid, Breast cancer, System pharmacology, EGFR signalling, Molecular dynamics, MM-PBSA

Posted Date: October 16th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-11239015/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Additional Declarations: No competing interests reported.

Deciphering the Molecular Mechanisms of Gentisic Acid in Breast Cancer: Combining Network Pharmacology, Molecular Modelling, and Experimental Approaches

Rohit Singh Negi^a, Rakesh Kumar Gupta^b, Nitish Kumar^a, Pawan Kumar Dubey^b, Sunil Kumar Mishra^{a*}

#first two authors contributed equally

^aDepartment of Pharmaceutical Engineering and Technology, Indian Institute of Technology (Banaras Hindu University), Varanasi, 221005, Uttar Pradesh, India

^bCentre for Genetic Disorders, Institute of Science, Banaras Hindu University, Varanasi-221005, India

***Corresponding author:**

Dr. Pawan Kumar Dubey

Assistant Professor

Centre for Genetic Disorders, Institute of Science, Banaras Hindu University, Varanasi, 221005, Uttar Pradesh, India

E-mail: pkdubey@bhu.ac.in

ORCID ID: 0000-0002-1438-3773

Dr. Sunil Kumar Mishra

Associate Professor

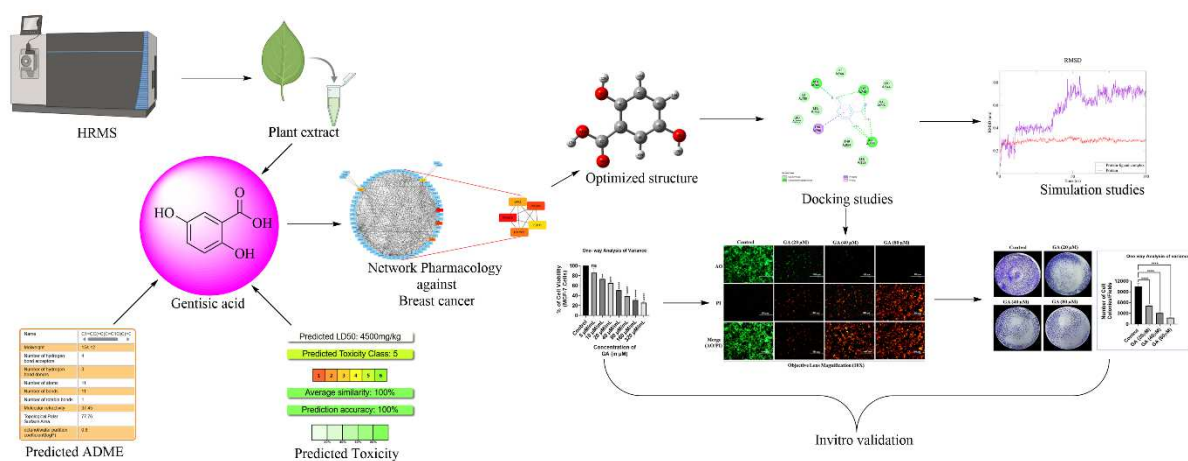
Department of Pharmaceutical Engineering and Technology

Indian Institute of Technology (Banaras Hindu University), Varanasi, 221005, Uttar Pradesh, India.

E-mail: skmishra.phe@iitbhu.ac.in

ORCID ID: 0000-0003-3749-5935

Graphical abstract



Abstract

Gentisic acid (GA), a naturally occurring phenolic compound identified in *Nyctanthes arbor-tristis*, has demonstrated diverse pharmacological activities; however, its molecular mechanisms against breast cancer (BC) remain poorly understood. This study investigated the anti-BC potential of GA using an integrated systems pharmacology, computational, and experimental approach. The presence of GA in the ethanolic leaf extract of *N. arbor-tristis* was confirmed by high-resolution mass spectrometry. Network pharmacology identified 604 overlapping genes between GA-predicted targets and BC-associated genes, with PIK3CA, PIK3R1, PTPN11, JAK2, and EGFR emerging as key hub genes. Functional enrichment highlighted EGFR- and SHP2-associated signalling as important regulatory pathways. Molecular docking demonstrated interactions of GA with the identified hub proteins, while molecular dynamics and MM-PBSA analyses supported the stability of the GA–EGFR complex. In vitro studies using MCF-7 cells showed that GA inhibited cell viability in a concentration-dependent manner, with an IC₅₀ of 40 µM, and suppressed cell migration and clonogenic survival. GA treatment also increased intracellular reactive oxygen species, reduced mitochondrial membrane potential, and promoted apoptosis, accompanied by increased Bax and caspase-3 and decreased Bcl-2 expression. Collectively, these findings suggest that GA exerts anti-BC effects through modulation of EGFR-associated signalling, oxidative stress, mitochondrial dysfunction, and apoptotic pathways, providing mechanistic support for further investigation of GA as a potential natural anti-cancer candidate.

Keywords

Gentisic acid; Breast cancer; System pharmacology; EGFR signalling; Molecular dynamics; MM-PBSA

Introduction

It is well established that BC is the second leading contributor to cancer mortality in women. Because BC is a complex disease, single-gene abnormalities account for less than 10% of all cases (monogenic) [1]. Multiple cell types are involved in the multi-step process of BC development, and preventing the disease remains difficult globally [2]. According to epidemiological research, there will be more than 1.9 million BC cases worldwide by 2030 [3]. With around 1.7 million incident cases and 521,900 deaths reported globally in 2012, the most

prevalent cancer in women and the second most common cancer overall was breast cancer [4]. Between 1965 and 1985, the incidence in India rose dramatically by about 50% [5]. The incidence of BC in India is predicted to increase by roughly 5.6% annually, or by 0.05 million new cases [6]. With an expected 2,21,757 cases reported in 2022, BC is one of the most prevalent cancers in India and accounts for over a quarter (22.8%) of cancer cases in women [7]. Environmental, behavioural, hormonal, and genetic variables are thought to play a role in the development of BC [8]. BRCA1 and BRCA2 play critical roles in maintaining genomic stability by regulating transcription, cell cycle progression, and DNA repair in response to DNA damage [9]. Several endogenous and external variables can exacerbate BC pathology. Over 30% of patients with inflammatory BC (IBC), a more aggressive subtype of BC, had overexpressed EGFR. While EGFR gene amplification and activating mutations are uncommon in BC, polysomy can increase EGFR expression, and greater EGFR expression in primary tumours is associated with enhanced metastatic potential and reduced survival in patients with triple-negative breast cancer (TNBC) [10]. These findings highlight the EGFR signalling pathway as a viable target for the development of novel therapeutic strategies.

Phenolic acids have been shown in numerous scientific investigations to have positive therapeutic benefits. (GA), A naturally occurring phenolic acid found in various fruits and vegetables has been related to multiple beneficial health effects [11]. GA, chemically 2,5-dihydroxybenzoic acid (2,5-DHBA), is a benzoic acid-derived diphenolic compound with a pKa of 2.95. Its molecular formula is $C_6H_3(OH)_2COOH$ [12]. It is naturally occurring in various plants as a secondary metabolite, including *Olea europaea*, *Gentiana* species, *Citrus* species, *Helianthus tuberosus*, *Vitis vinifera*, *Hibiscus rosa-sinensis*, *Pterocarpus santalinus*, and *Sesamum indicum* [13]. Apart from plants, mushrooms like *Polyporus tumulosus* and *Penicillium patulum* also produce GA [13]. GA is a metabolite of aspirin that is also naturally produced by roots of the species *Gentiana* [14]. Antiparkinsonian, Antioxidant, anti-rheumatic, anti-inflammatory, and anti-diabetic properties are among its positive impacts on human health [15-19]. Evidence suggests that it possesses antineoplastic properties across multiple cancer types, including glial, breast, brain, and skin cancers, and may also prevent progression from cardiac hypertrophy to heart failure [16, 20, 21]. In the present study, an integrated strategy encompassing network pharmacology, in silico modelling, and in vitro assays was employed to systematically characterize the anticancer potential and underlying molecular mechanisms of gentisic acid.

2. Materials and Methods

2.1 Chemicals and Reagents

GA was procured from Yucca Enterprises (Mumbai, India), while all additional chemicals and reagents employed in the study were of analytical grade and sourced from commercial vendors.

2.2 Plant Collection and Authentication

We collected the plant material from the Department of Chemistry, IIT (BHU), Varanasi, India. The collected specimen was subjected to taxonomic identification and authentication by

experts at the Centre of Advanced Study in Botany, Institute of Science, Banaras Hindu University (BHU), Varanasi.

2.3 Preparation of ethanolic leaves extract of *Nyctanthes arbor-tristis* (ELENA)

The leaves of *Nyctanthes arbor-tristis* were shade-dried and finely powdered. Approximately 5 g of the powdered dried leaf was subjected to cold maceration for extraction. The powdered material was initially macerated with n-hexane, then with chloroform and ethyl acetate, and finally extracted with ethanol as the polar solvent. A rotary evaporator (IKA® India Private Limited, India) was used to concentrate the resulting ethanolic liquid extract, which was then dried in a lyophilizer (Christ-Alpha 1-2 LDplus). We performed HR/MS analysis of the extract.

2.4 HRMS Identification

Data acquisition was performed using a Thermo Scientific Orbitrap Eclipse Tribrid mass spectrometer operated in small-molecule mode with a heated electrospray ionization (H-ESI) source. The analysis used a mobile phase composition of 0.1% formic acid in water (A), acetonitrile (B), and methanol (C), with spray voltages of +3.5 kV and −2.5 kV. A comprehensive MS/ddMS² (Orbitrap HCD) acquisition method was employed to enhance the identification of ELENA. An ion transfer tube temperature of 325 °C was employed during operation, with a vaporizer temperature of 350 °C, an auxiliary gas flow of 10 arb, a sheath gas flow of 50 arb, and a sweep gas flow of 1 arb. These additional factors were pertinent determinants of the current investigation. We determined that the predominant charge state was 1. MS² spectra of glycosylated ELENA molecules were acquired using stepped collision energy mode under HCD conditions at collision energies of 30%, 45%, and 60%. Data were acquired and analyzed using Xcalibur 4.1 software provided by Thermo Fisher Scientific.

2.5 ADMET and Toxicity Prediction

The pharmacokinetic properties and toxicity profile of GA were predicted using in silico ADMET prediction tools, as mentioned in Table 1. ADME parameters were predicted using SwissADME based on GA's canonical SMILES. Key properties, including physicochemical parameters, lipophilicity, water solubility, gastrointestinal absorption, blood-brain barrier permeability, drug-likeness, and Lipinski's rule compliance, were assessed. Toxicity prediction was performed using ProTox-3 to estimate toxicity class, LD₅₀ value, and potential toxicological endpoints [22]. We conducted these analyses to evaluate the drug-likeness and safety profile of GA before experimental validation.

Table 1. Database/Software used in the study.

Database/Software list	Website
SwissADME	https://www.swissadme.ch/
Protox	https://tox.charite.de/protox3/
Genecard	https://www.genecards.org/
DisGeNET	https://disgenet.com/
OMIM	https://www.omim.org/
SEA	https://sea.bkslab.org/

Swiss Target Prediction	https://www.swisstargetprediction.ch/
TargetNET	http://targetnet.scbdd.com/
SuperPRED	https://prediction.charite.de/
Cytoscape 3.10.4	https://cytoscape.org/
Venny 2.1.0	https://bioinfogp.cnb.csic.es/tools/venny/
String v12.0	https://string-db.org/
Metascape	https://metascape.org/gp/index.html#/main/step1

2.6 Network Pharmacology Analysis

2.6.1 Gene Collection for BC & Prediction of GA-Associated Target Gene

BC-associated target genes were collected from multiple publicly available databases, including GeneCards, DisGeNET, and OMIM, as listed in Table 1. In contrast, target genes of GA were predicted using multiple databases, including SwissTargetPrediction, SuperPred, TargetNet, and SEA (Table 1). To ensure data reliability, we filtered targets based on relevance and association scores provided by the respective platforms, retaining only *Homo sapiens* genes [23]. We compiled predicted targets from all databases, standardized them using UniProt nomenclature, and removed duplicate genes to generate a consolidated list of potential GA-associated genes for subsequent intersection analysis.

2.6.2 Identification of Overlapping genes and Protein–Protein Interaction (PPI) Network Development

The overlapping target genes between BC-associated genes and GA-predicted targets were identified using Venn diagram analysis with the Venny web server, as shown in Table 1. These protein–protein interaction common genes were subsequently imported into the STRING for PPI analysis, with *Homo sapiens* as the organism. We applied the highest-confidence (0.9) interaction parameters to construct the PPI network, which we then used for topological and functional analyses to identify key regulatory proteins involved in GA's pharmacological action [24].

2.6.3 Hub Gene Identification by Network Topological Analysis

After constructing the PPI network, the hub gene was identified via the CytoHubba plugin in Cytoscape. We analyzed the PPI network imported from STRING to identify key nodes using network topological parameters. Centrality measures, consisting of degree, betweenness, and closeness, were taken to find the relative importance of each node within the network. Genes exhibiting the highest connectivity and central roles were identified as hub genes, as they are likely to play critical regulatory functions in BC progression and represent potential molecular targets mediating the pharmacological effects of GA [25].

2.6.4 Enrichment Analysis of Hub Genes Using Metascape

To clarify the biological importance of the hub genes, we performed functional enrichment analysis of the top 5 hub genes using the Metascape database. We uploaded the top 5 selected hub genes to Metascape, restricting the species to *Homo sapiens*. We identified enriched terms with statistically significant p-values to reveal key biological functions and signalling pathways

associated with BC, and to provide mechanistic insights into the molecular processes potentially regulated by GA [26].

2.7 Density Functional Theory (DFT) Calculations

We calculate DFT properties to investigate the electronic features of GA. The molecular structure was constructed in GaussView 5.0.8 and subsequently optimized using Gaussian 09W at the B3LYP/6-311G level of theory [27]. FMO energies, including HOMO and LUMO, along with dipole moment, were computed, and the energy gap ($\Delta E = E_{\text{LUMO}} - E_{\text{HOMO}}$) was also determined to evaluate chemical reactivity. Additionally, we generated a molecular electrostatic potential (MEP) surface to identify reactive sites and to map potential electrophilic and nucleophilic regions [28].

2.8 Molecular Docking

To examine the binding relationships between GA and certain target proteins, we ran docking calculations using AutoDock 1.4.5. We obtained the target proteins' three-dimensional crystal structures from the RCSB's Protein Data Bank. During protein preparation, we removed all crystallographic water molecules and heteroatoms, as well as the co-crystallised ligand, to obtain a clean protein structure. We then added polar hydrogen atoms, assigned Kollman charges, and assigned Gasteiger charges to the protein. The grid parameters are discussed in Table 2. The Lamarckian Genetic Algorithm (LGA) was used for docking. Discovery Studio v25.1.0 was used to see 2D interactions between 1XKK and GA, including H-bonds, hydrophobic interactions, and π - π contacts [29].

Table 2. Grid box parameters used for molecular docking of target proteins

Gene	PDB ID	Grid centers	Grid size
EGFR	1XKK	X = 15.453; y = 34.399 and z = 25.662	X = 40; y = 36 and z = 48
JAK2	2B7A	X = 115.868; y = 65.728 and z = 8.298	X = 54; y = 76 and z = 66
PIK3CA	7K6M	X = 18.4768; y = 10.7265 and z = 3.5792	X = 36.03; y = 13.3787 and z = 13.1228
PIK3R1	5XGI	X = 12.095; y = 31.363 and z = 27.419	X = 60; y = 60 and z = 60
PTPN11	3MOW	X = 29.65; y = -15.18 and z = 31.16	X = 21.75; y = 21.75 and z = 21.75

2.9 Molecular Dynamics Simulation

MD simulation was conducted using GROMACS 2020 and the CHARMM36 force field. System solvation was performed using NaCl as the ionic solvent, with ion placement via Monte Carlo to maintain charge neutrality and physiological conditions ($\text{Na}^+ = 45$, $\text{Cl}^- = 43$). We fixed the system pH to body pH. The simulation was performed under the NPT ensemble at 310.5

K, with constant pressure and temperature throughout the run. We subsequently performed trajectory analysis using the QTGrace software to calculate RMSD and RMSF profiles, providing essential insights into the structural stability and binding persistence of the GA-1XKK complex [30].

2.10 MM-PBSA Calculations for estimation of binding free energy

To quantitatively evaluate the thermodynamic stability and binding affinity of the protein-ligand complex, the Molecular Mechanics Poisson-Boltzmann Surface Area (MM-PBSA) calculations were performed. Calculations were performed with the gmx_MMPBSA tool of the GROMACS simulation suite, following established best practices for free energy analysis.

The binding free energy (ΔG_{bind}) was obtained by the following equation:

$$\Delta G_{\text{bind}} = G_{\text{complex}} - (G_{\text{receptor}} + G_{\text{ligand}})$$

The free energy term (G) was calculated as:

$$G = E_{\text{MM}} + G_{\text{polar}} + G_{\text{nonpolar}} - TS$$

Where:

- E_{MM} : Molecular mechanics energy including electrostatic and van der Waals interactions
- G_{polar} : Polar solvation energy calculated using the Poisson-Boltzmann equation.
- G_{nonpolar} : Non-polar solvation energy, estimated from solvent accessible surface area (SASA)
- TS: entropic contribution

Explicit water molecules and counterions were removed before energy calculations to prevent artefacts. Only the stable region of the molecular dynamics trajectory was used for the calculations, meaning that calculations were performed on equilibrated conformations. More specifically, we systematically extracted 500 snapshots from the production trajectory in the interval 500-1000 frames with a 5-frame spacing. All the MM-PBSA analyses were performed with the following set of parameters: General settings: startframe=200, endframe=300, interval=1, verbose=2, forcefields=oldff/leaprc.ff99SB,leaprc.gaff, Poisson-Boltzmann parameters: istrng=0.15, fillratio=4.0, inp=1. The binding free energy was calculated using the last 100 equilibrated frames and averaged over the 100 frames to obtain the final binding free energy, which gave a robust and statistically reliable estimate of the binding affinity of the complex.

2.11 Free radical scavenging activity

Each sample's radical-scavenging activity was evaluated using DPPH, according to the protocol described by Kumar and colleagues [31]. We prepared different concentrations of 10 to 200 μ L. The diluted samples were then mixed with 200 μ L of methanol (MeOH). This stage ensures consistency and homogeneity across all experimental conditions. The samples were properly mixed after the addition of MeOH, then incubated in the dark at 37 °C for 30 minutes. The drug and the radicals in the tests can react and interact optimally throughout this incubation period. We measured absorbance in a 96-well plate at 517 nm for DPPH using a microplate reader (SynergyTM HT, BioTek Instruments, Inc.). Ascorbic acid at 0.1 mg/mL was used as the reference standard to establish a baseline for comparison. This standardisation ensures precision and reliability in the assessment of antioxidant activity [31].

2.12 In Vitro Cytotoxicity Assay

2.12.1 Resources acquisition & Cell culture mntenance

MTT was procured from Himedia, India (Cat. No.: RM1131-1 G) dissolved into sterile 1X PBS (phosphate buffered saline) to prepare 5 mg/mL solution and then used according to mentioned in material and method section, Acridine orange (AO) procured from Himedia, India (Cat No.: GRM387 25G), Propidium iodide procured from SRL, India (Cat No.: 11,195), MCF-7 cells used in this study were procured from NCCS, Pune, India. Dimethyl sulfoxide was also obtained from Sigma-Aldrich Co. LLC (Cat. No. D4540), 10 % foetal bovine serum (FBS; Cat. No. F7524-500ML, Sigma-Aldrich) and 1% antibiotics (100 U/mL penicillin and 100 μ g/mL streptomycin; we added Cat. No. A5955-100ML, Sigma-Aldrich) were added to the Dulbecco's Modified Eagle Medium (DMEM, high-glucose; Cat. No. D6429-1L, Sigma-Aldrich) media. The MCF-7 cells were cultured under standard laboratory conditions using a high-glucose formulation of DMEM; Cat. No. D6429-1L, Sigma-Aldrich). The growth medium was enriched with 10% fetal bovine serum to support cellular proliferation. In addition, we added an antibiotic mixture containing penicillin (100 U/mL) and streptomycin (100 μ g/mL) to the medium to prevent microbial contamination.

2.12.2 MTT Assay

In order to facilitate cell adhesion, 96-well plates were seeded with a density of 10000 cells/well and incubated for 24 hours [32]. Following treatment with GA at various concentrations (5, 10, 20, 40, 80, 120, and 160 μ M), an additional 24 h incubation was carried out under standard CO₂ conditions. We prepared a 5 mg/mL stock solution of MTT in sterile PBS and diluted it with serum-free medium to obtain a working solution. We incubated the mixture for four hours after adding MTT solution to reach a final concentration of 0.5 mg/mL in each well. To detect the absorbance at 590 nm, a microplate reader was used after formazan was dissolved in 100 μ L DMSO. Nonlinear regression analysis performed in GraphPad Prism v5.0 was used to estimate IC₅₀ values [33]. Cell viability was calculated as a percentage of the untreated control group, with experiments conducted in triplicate, and calculated using the formula below.

$$\% \text{ of cell survival} = \frac{\text{Mean OD of the drug – treated well}}{\text{mean OD of the control well}} * 100$$

2.12.3 Acridine Orange /Propidium Iodide (AO/PI) Assay

MCF-7 cells were cultured under CO₂ for 24 hours, either with or without the ideal inhibitory dose of GA. After that, cell staining is performed using 100 µL of AO/PI solution, prepared by combining equal volumes of 100 µg/mL AO and PI in PBS [34]. Subsequently, the mixture was kept in the dark for 10 min. A fluorescence microscope was used to examine cellular morphology after a brief PBS wash (EVOS FL, Life Technologies). Viable cells exhibited green fluorescence, whereas apoptotic or dead cells showed orange/red fluorescence [34]. Images were captured at 10× magnification from multiple fields using an inverted fluorescence microscope. To guarantee reproducibility, every experiment was conducted in triplicate.

2.12.4 Analysis of ROS Level

After being seeded in 24-well plates and reaching 75–80% confluency, the cells were exposed to GA (20, 40, and 80 µM) for 24 hours. Following treatment at 37 °C, cells were incubated with 10 µM dichlorofluorescein diacetate (DCF-DA) for 30 minutes after being washed with sterile 1× PBS [22]. An inverted fluorescence microscope was used to monitor fluorescence at 20× magnification after excess dye was removed by washing three times with 1× PBS. Green emission from DCF was seen at an excitation wavelength of 488 nm. ImageJ (version 152-win-Java8) was used to quantify fluorescence intensity. Every experiment was carried out in triplicate, and we used GraphPad Prism v5.0 and Microsoft Excel for statistical analysis.

2.12.5 Cell migration assay

12-well plates (Cat. No. 980020, Tarsons, Korea) containing high-glucose DMEM along with 10% FBS and 1% antibiotic-antimycotic solution were seeded with MCF-7 cells (3×10^3 cells/well) and incubated for 24 hours [33]. When the confluence reached about 90%, we scratched cells with a sterile 200 µL pipette tip to form a linear wound, and debris was removed by rinsing with sterile PBS. Cells were then divided into control and GA-treated groups (20, 40, and 80 µM). An inverted microscope (Olympus, Japan) with a 10× objective was used to capture images at 0 hours. After that, we kept the plates in a humidified environment with 5% CO₂ at 37 °C. We acquired Additional images at 12 and 24 h. The extent of wound closure was analysed using ImageJ software. We used GraphPad Prism v5.0 for statistical analysis, and each experiment was performed in triplicate [33].

2.12.6 Colony Assay

We carried out our cell culture in 6-well plates (Cat. No. 980010, Tarsons, India) comprising DMEM enhanced with 1% antibiotic–antimycotic combination and 10% foetal bovine serum. We planted MCF-7 cells at a density of 500 cells per well [33]. Cells were incubated for 24 h at 37 °C in a humidified 5% CO₂ atmosphere before treatment with GA (20, 40, or 80 µM) or left untreated. Following a 24-hour treatment period, we added fresh complete media and cultured the cells for an additional 5 days to allow colony formation. After fixing and staining the colonies with the 0.5% crystal violet prepared in the 1:1 ratio of the methanol and sterile distilled water and incubated for 10 min at room temperature, we removed excess stain by washing them three times with a methanol: water solution (1:1). Colony images were captured with a smartphone camera (realme 5S, 48 MP), and colonies containing more than 50 cells were counted using ImageJ software. We used GraphPad Prism v5.0 for statistical analysis, and each experiment was conducted independently in triplicate [35].

2.12.7 Mitochondrial membrane potential assay

The JC-1 fluorescent probe (1 μ M) was used to measure the mitochondrial membrane potential of granulosa cells from the control and gentisic acid (GA)-treated groups, following the established protocol [36]. After 72 h of exposure to GA at 20, 40, and 80 μ M, After fixing the cells with 3.7 % formaldehyde, they were treated for ten minutes with JC-1 dye made in the proper staining buffer. An inverted fluorescence microscope was used to obtain fluorescence images. We quantified mitochondrial membrane potential by measuring fluorescence intensity and normalising it to cell area using the corrected total cell fluorescence (CTCF) method. The results were expressed as total red fluorescence intensity, calculated from 3 independent replicates.

2.12.8 Real-time PCR (qRT- PCR) Analysis

qRT-PCR was conducted to quantify the mRNA expression levels; Table 3 lists the primer sequences that were utilised. In MCF-7 cells treated with GA at 20, 40, and 80 μ M, we examined the expression profiles of pro-apoptotic genes (Bax and Caspase-3) and anti-apoptotic genes (Bcl-2), along with untreated control cells. Cells were trypsinised after 24 h of treatment and harvested into microcentrifuge tubes containing TRIzol reagent. TRIzol was used to isolate total RNA in accordance with the procedure, and residual genomic DNA was eliminated by DNase I treatment. RNA concentration and purity were assessed using a NanoDrop 2000 spectrophotometer. Subsequently, A RevertAid cDNA Synthesis Kit was used to create first-strand cDNA from purified RNA. The synthesised cDNA was quantified, and qRT-PCR was performed using SYBR Green chemistry on an Applied Biosystems QuantStudio 6 RT-PCR system, according to the manufacturer's instructions. The $2^{-\Delta\Delta CT}$ approach, in which ΔCT is the difference between the target gene and the internal control (GAPDH) and $\Delta\Delta CT$ is the difference between the treated and control groups. Each experiment was carried out in triplicate to guarantee reproducibility, and statistical analysis was done using GraphPad Prism 5 [33].

Table 3 List of primers used to amplify and quantify the expression of enlisted genes

S. No.	Gene	Primer Seq (5'-3')	Amplicon size
1	GADPH	Forward- GGGCATCCTGGGCTACACTGA	165
		Reverse- TCCACCACCCTGTTGCTGTAG	
2	Bcl-2	Forward- GTGGATGACTGAGTACCT	118
		Reverse- CCAGGAGAAATCAAACAGAG	

3	Caspase-3	Forward- GTAGATGGTTTGAGCCTGAG	106
		Reverse- CCAGTGCGTATGGAGAAATG	
4	Bax	Forward- GGTTGTCGCCCTTTTCTA	133
		Reverse- CGGAGGAAGTCCAATGTC	

2.12.7 Statistical Analysis

We used GraphPad Prism v5.0 and Microsoft Excel 2019 to analyse the study's data, which were presented as means $\pm$ SE (standard error) from triplicate experiments. To measure the intensity of fluorescence, we used ImageJ software (ver 1.44, NIH, USA), and the intensity of fluorescence is presented with its significance levels, i.e., $p < 0.01$ (*), $p < 0.001$ (**), or $p < 0.0001$ (***) in the histogram.

3. Results and discussion

3.1 Plant Authentication & HRMS Identification of GA from ELENA

The Center of Advanced Study in Botany at the Institute of Science, Banaras Hindu University (BHU), Varanasi, successfully verified the authenticity of the plant sample. The specimen was confirmed, and a voucher number (Olea. 2026/01) was assigned. This authenticated plant material was used for subsequent experimental analysis, ensuring the reliability and reproducibility of the study. HRMS analysis of the ethanolic extract confirmed the presence of GA in ELENA. The mass spectrum exhibited a prominent ion peak corresponding to GA, with an observed m/z value of 154.02621 that closely matched the theoretical mass, indicating high mass accuracy, as shown in Fig. 1. These results validate the successful identification of GA and confirm its presence as a bioactive constituent in the *Nyctanthes arbor-tristis*.

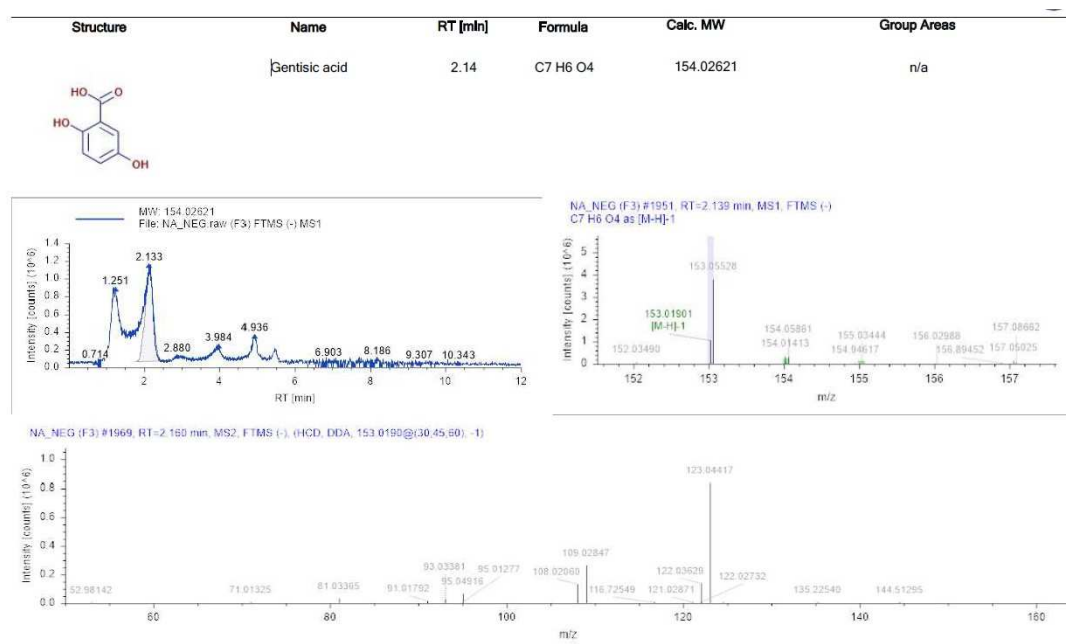


Fig. 1. High-resolution mass spectrometric (HRMS) spectrum of gentisic acid showing the characteristic $[M-H]^-$ ion peak at 154.02621, confirming its molecular identity

3.3 ADMET and Toxicity Prediction Results

GA exhibit a formula of $C_7H_6O_4$ with a molecular weight of 154.12 g/mol and 11 heavy atoms. The compound exhibited low structural flexibility, with one rotatable bond and a Csp³ fraction of 0, indicating a planar aromatic structure. The presence of 4 HBA and 3 HBD, along with a TPSA of 77.76 Å², suggests favourable polarity for oral bioavailability as highlighted in Table 4. The compound demonstrated high predicted gastrointestinal absorption and was not predicted to permeate the BBB. Importantly, no violations of Lipinski's rule of five were observed, and the predicted bioavailability score was 0.56, supporting the drug's likeness and oral pharmacokinetic suitability.

Table 4. Pharmacokinetic properties of GA calculated by SwissADME.

S. No.	Results
Formula	$C_7H_6O_4$
Molecular weight	154.12 g/mol
Heavy atoms	11
Fraction Csp3	0
Rotatable bonds	1
H-bond acceptors	4
H-bond donors	3
Molar refractivity	37.45
TPSA	77.76 Å ²
iLOGP	0.49
XLOGP3	1.74
WLOGP	0.8
MLOGP	0.4
Silicos-IT Log P	0.26

Consensus Log P	0.74
GI absorption	High
BBB permeation	No
P-gp substrate	No
CYP1A2 inhibitor	No
CYP2C19 inhibitor	No
CYP2C9 inhibitor	No
CYP2D6 inhibitor	No
CYP3A4 inhibitor	Yes
Lipinski violations	No
Bioavailability Score	0.56

The compound exhibited a predicted oral LD₅₀ of 4500 mg/kg, as reported in Table 5, corresponding to toxicity class 5, indicating relatively low acute toxicity. The web server predicted no hepatotoxic, neurotoxic, cardiotoxic, carcinogenic, mutagenic, immunotoxic, or cytotoxic liabilities. However, we observed potential nephrotoxicity and respiratory toxicity alerts. Overall, the predicted toxicity assessment suggests a favourable safety profile with low systemic toxicity, although the predicted organ-specific risks warrant further experimental validation.

Table 5. Toxicity data of GA, calculated by the Protox web-server

S. No.	Results
Predicted LD ₅₀	4500mg/kg
Predicted Toxicity Class	5
Hepatotoxicity	Inactive
Neurotoxicity	Inactive
Nephrotoxicity	Active
Respiratory toxicity	Active
Cardiotoxicity	Inactive
Carcinogenicity	Inactive
Immunotoxicity	Inactive
Mutagenicity	Inactive
Cytotoxicity	Inactive

3.4 Network Pharmacology Analysis Results

3.4.1 BC-Associated Target Genes & GA Target Gene Prediction

A total of 160,602 unique genes were identified as being associated with BC from the known databases. This extensive gene pool reflects the molecular heterogeneity and complex pathophysiology of BC and served as the foundational dataset for subsequent intersection analysis with GA-predicted targets. Similarly, after consolidating results and removing redundant entries, 799 unique genes were identified as potential GA targets across various databases.

3.4.2 Identification of Common Targets

Venn diagram analysis revealed 604 overlapping genes between the BC-associated target set and the GA-predicted targets (Fig. 2). These common genes were considered potential therapeutic targets mediating the compound's anti-BC effects. We subsequently uploaded the overlapping gene list to the STRING database to construct a PPI network, investigate functional associations, and identify key regulatory nodes.

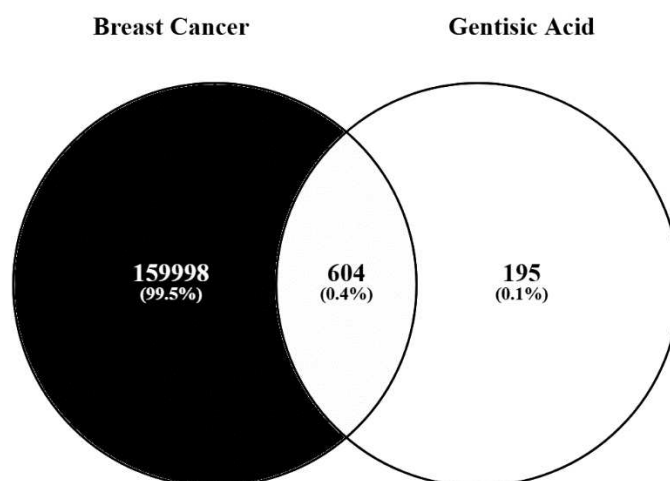


Fig. 2. Overlapping genes shared between BC-associated genes and GA-predicted targets

3.4.3 PPI Network Analysis

After applying the highest confidence of 0.9, 417 genes were retained for network construction from 604 overlapping genes. With an average node degree of 7.3, the final PPI network has 415 nodes and 1,514 edges indicating substantial interconnectivity among the targets (Fig. 3). The network exhibits an average local clustering coefficient of 0.543, reflecting a moderate degree of node interconnectivity. We observed a significantly greater number of interactions than expected: 449 edges, and the PPI enrichment p-value ($< 1.0 \times 10^{-16}$) confirmed that this network enrichment was not due to chance. These results suggest strong functional associations among the common targets and support their coordinated involvement in BC-related biological processes.

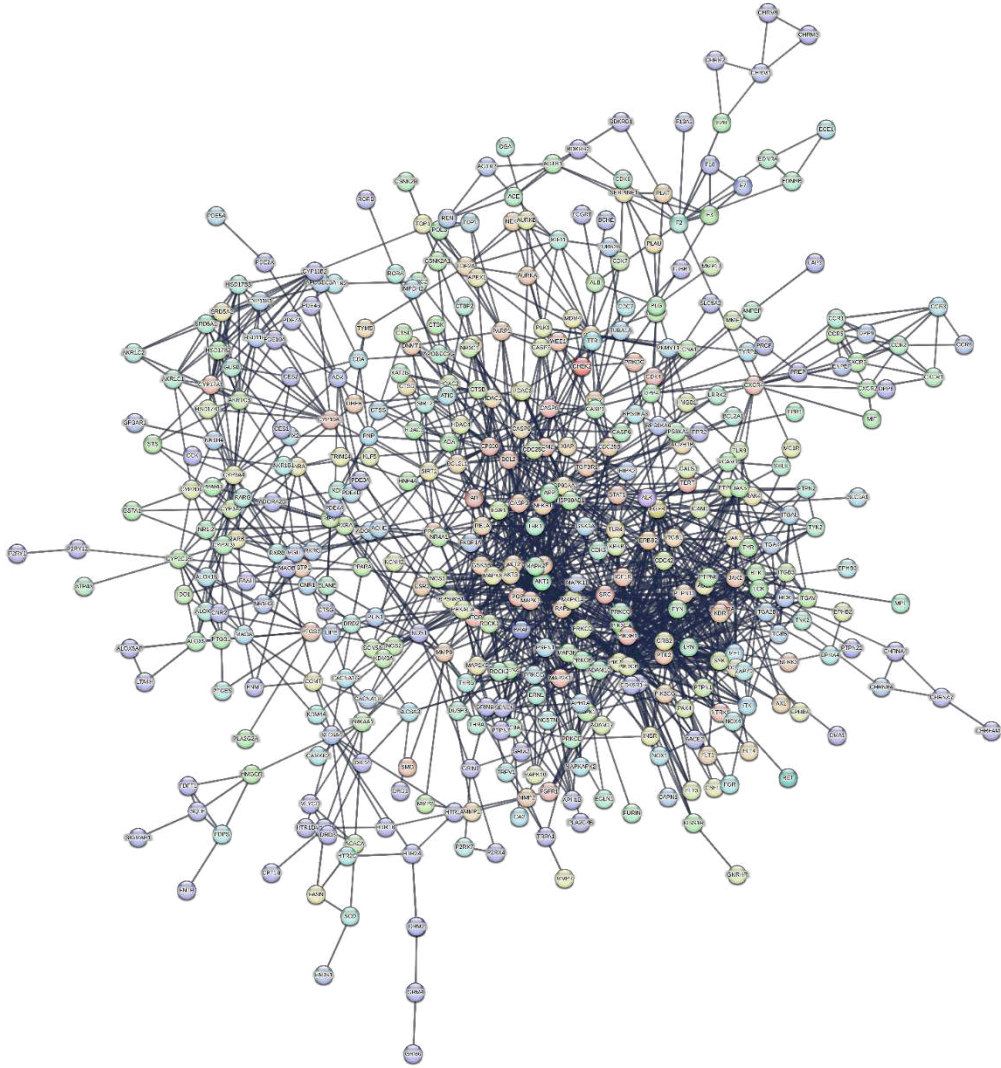


Fig. 3. PPI network of overlapping genes constructed using the STRING database (confidence score ≥ 0.9)

3.4.4 Hub Gene Identification

We used the CytoHubba plugin in Cytoscape to perform topological analysis and identify key hub targets in the PPI network. Cytoscape. The MCC algorithm was applied to rank nodes based on their network significance and connectivity strength. The top five hub genes identified were PIK3CA, PIK3R1, PTPN11, JAK2, and EGFR, as shown in Fig. 4. These genes exhibited the highest MCC scores, indicating their central roles within the interaction network. Notably, these hub genes may be critically involved in oncogenic signalling pathways regulating cell proliferation, survival, and apoptosis, suggesting that GA may exert its anti-BC effects by modulating these core molecular regulators.

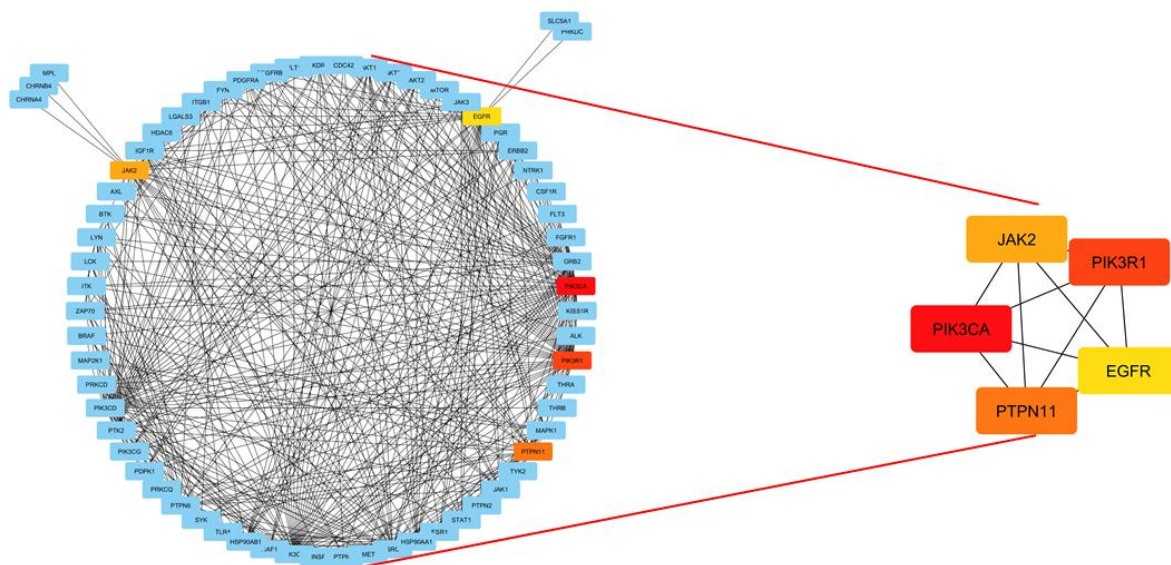


Fig. 4. Top 5 hub genes identified from the PPI network using Cytoscape based on the MCC algorithm

3.4.5 Functional Enrichment of Hub Genes

The enrichment network visualization revealed tightly connected clusters representing biologically related pathways. When colored by cluster, distinct functional modules were observed, primarily associated with receptor tyrosine kinase signalling, cytokine-mediated signalling, and phosphatidylinositol 3-kinase (PI3K) regulatory processes. Significant enrichment was observed in pathways including PID SHP2 (Fig. 5), PID IL5, GAB1 signalling, PID PTP1B, and EGFR signalling. In the epidermal growth factor receptor (EGFR) signalling pathway, Src homology phosphotyrosyl phosphatase 2 (SHP2) is a crucial transducer of mitogenic and cell survival signalling [37]. This indicates a possible mechanism by which the compound may regulate aberrant growth signalling in BC.

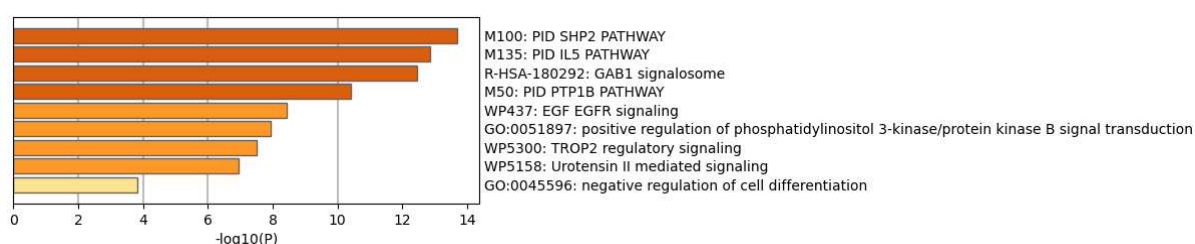


Fig. 5. Pathway enrichment analysis of hub genes was performed using Metascape, showing significantly enriched biological pathways.

These clusters indicate strong involvement of the identified hub genes in oncogenic growth factor signalling and intracellular kinase cascades, as highlighted in Fig. 6. Collectively, the Metascape analysis supports that GA-associated hub genes are predominantly involved in growth factor receptor signalling, cytokine response pathways, and PI3K-mediated survival signalling, reinforcing the mechanistic link between the predicted targets and BC progression.

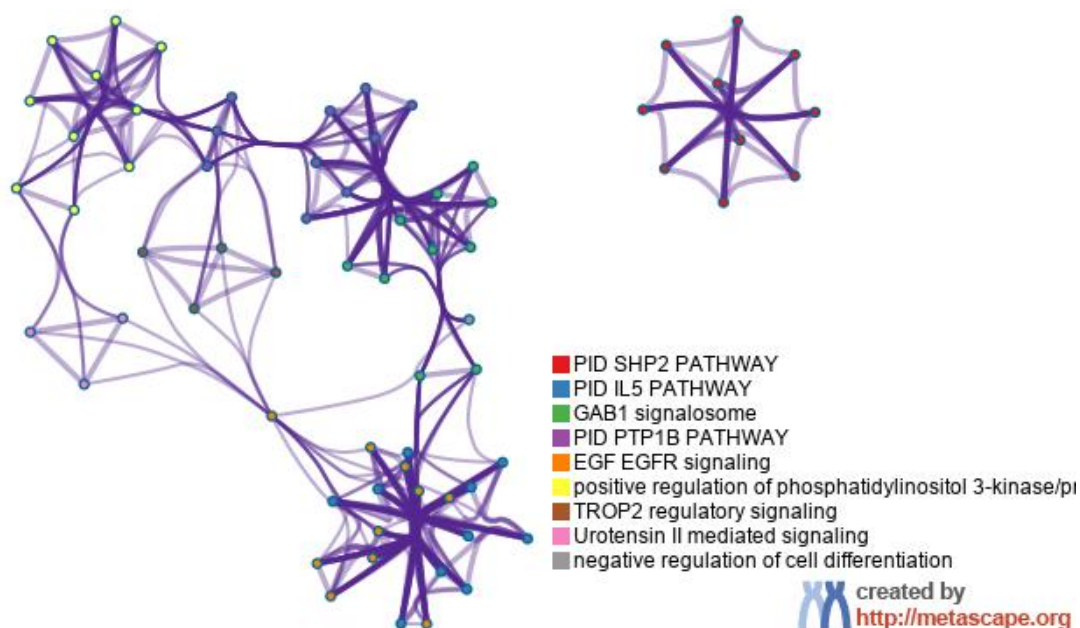


Fig. 6. Enrichment ontology network of hub genes based on colored by cluster

3.5 DFT Analysis

The optimized molecular structure of GA was obtained by geometry optimization prior to further calculations, as shown in Fig. 7. To confirm the structure of GA, IR spectrum is calculated by DFT as shown in S2.

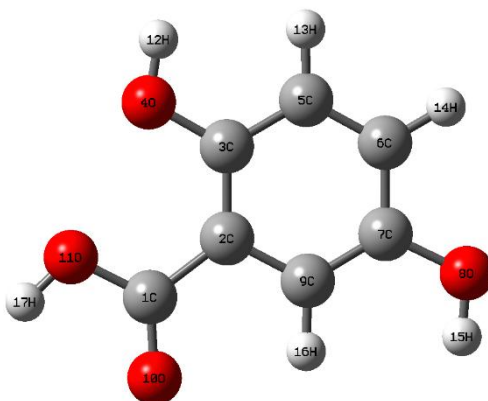


Fig. 7. Optimised molecular structure of gentisic acid obtained using density functional theory (DFT).

We performed Frontier molecular orbital (FMO) analysis of GA to assess its electronic characteristics and reactivity. The HOMO and LUMO energies were -0.22978eV and -0.06567eV , respectively, giving an energy gap of 0.16411 eV as highlighted in Fig. 8. The HOMO was primarily localised over the aromatic ring and oxygen-containing functional groups, indicating regions of high electron density and potential electrophilic attack sites. In contrast, the LUMO was distributed over the carboxyl and adjacent aromatic regions, suggesting favourable sites for nucleophilic interactions. Additionally, the calculated dipole

moment of 2.2492 Debye indicates a polar molecular nature, which may enhance intermolecular interactions with biological targets.

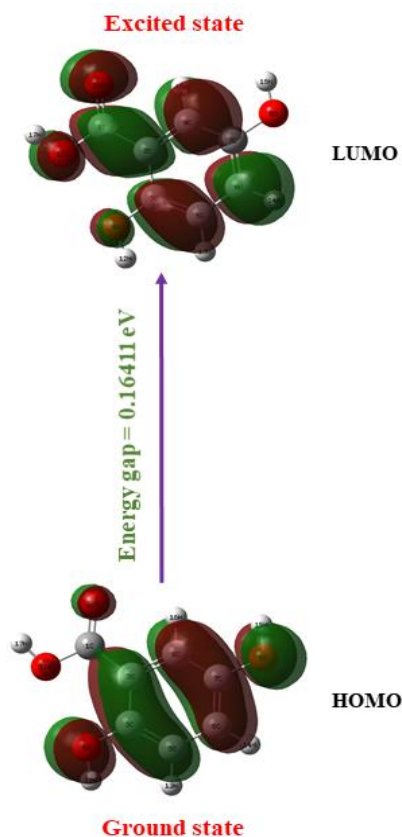


Fig. 8. FMO of Gentisic acid

A strong negative electrostatic potential was predominantly localised around the oxygen atoms in the MEP graph, particularly O₁₀ of the carboxyl group, O₁₁ of the hydroxyl group, and O₈ of the phenolic hydroxyl group, identifying these as primary nucleophilic centres with high electron density (Fig. 9). In contrast, positive electrostatic potential was mainly observed over hydrogen atoms, especially H₁₅ and H₁₇ attached to hydroxyl groups, indicating their role as electrophilic sites and potential hydrogen bond donors. This charge distribution suggests that the oxygen-containing functional groups are the principal sites for intermolecular interactions, supporting the compound's potential for hydrogen bonding and biological activity.

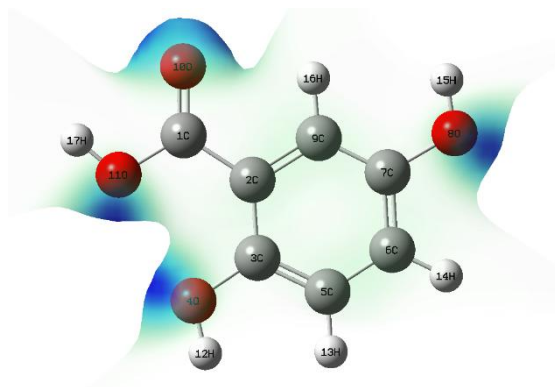
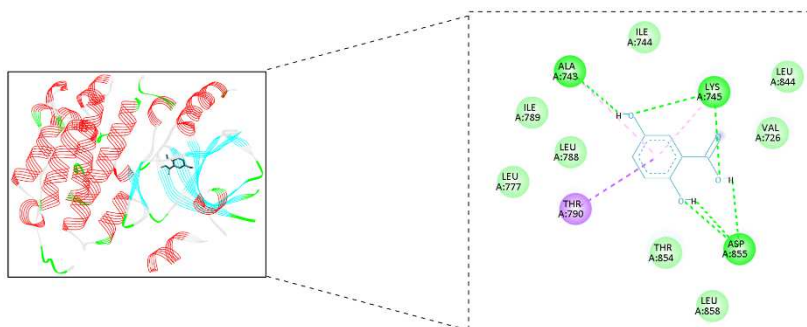


Fig. 9. MEP showing reactive sites of Gentisic acid

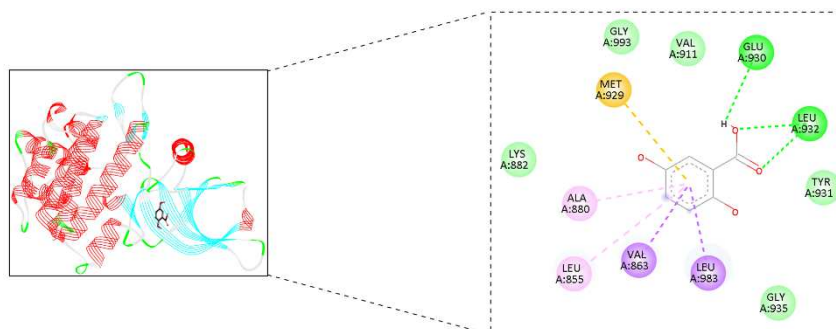
3.6 Molecular Docking

We performed molecular docking analysis to evaluate the binding affinity and interaction profile of GA with key hub gene-encoded proteins, including PIK3CA (PDB ID: 7K6M), PIK3R1 (PDB ID: 4JPS), JAK2 (PDB ID: 2B7A), EGFR (PDB ID: 1XKK), and PTPN11 (PDB ID: 3MOW). The docking scores were -5.8, -5.6, -5.9, -6.2, and -3.8 kcal/mol, respectively, as highlighted in Fig. 10. Among these targets, GA exhibited moderate binding affinity toward EGFR (-6.2 kcal/mol), although lower than the co-crystallised ligand of EGFR (-11.2 kcal/mol), shown in S1, indicating comparatively weaker but stable binding. In the EGFR binding pocket, the ligand interacted with key residues, including Lys745, Asp855, and Ala743, through hydrogen bonding and pi-sigma interactions with Thr790, and through van der Waals interactions with Ile744, Leu844, Val726, Thr854, Leu777, Leu788, and Ile789, thereby stabilising binding. Similarly, in JAK2, we observed hydrogen bond interactions with residues including Glu930 and Leu932, pi-sigma interactions with Leu983 and Val863, and van der Waals interactions with Gly935, Tyr931, Val911, Gly993, and Lys882, indicating favourable binding within the catalytic domain. For PIK3CA, GA formed hydrogen bonds with residues such as Gln597, Asn996, and Glu600, along with van der Waals contacts involving Glu563, Lys567, Arg537, Thr536, Thr536, Ser535, Pro539, Asn1000, Glu596, and Leu997, stabilizing the ligand within the binding cavity. In PIK3R1, GA forms hydrogen bond with Glu849, along with van der waal interaction with Ile848, Val850, Trp780, Phe930, Met922, Ser854, Ile800 and Met772. In the case of PTPN11, hydrogen interactions with residues such as Asn339, Glu379, and Gly381 were identified, whereas fewer van der Waals interactions were observed with Lys378, Tyr380, Asn336, and Gln335. These findings support its potential multi-target binding capability and its role in modulating key oncogenic signalling pathways.

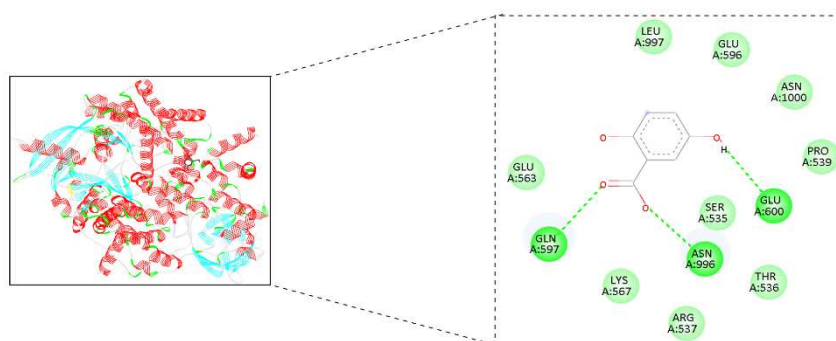
EGFR



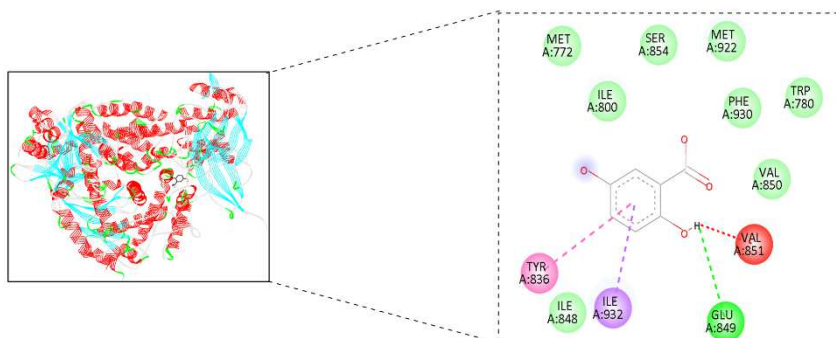
JAK2



PIK3CA



PIK3R1



PTPN11

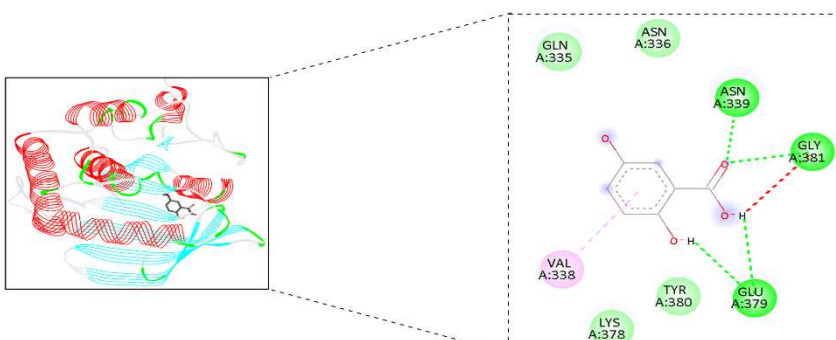


Fig. 10. Docking interaction of Gentisic acid with the hub genes

3.7 Molecular Dynamics Simulation & MM-PBSA

The RMSD profile of the apo protein (1XKK) exhibited an initial equilibration phase within the first ~10 ns, after which it stabilised with minor fluctuations around ~0.25-0.30 nm throughout the simulation, indicating maintenance of structural integrity. However, the GA-1XKK complex exhibited a higher RMSD trajectory than the protein alone. After an initial equilibration phase, the complex showed a gradual increase in RMSD, reaching from ~0.3-0.4 nm in the first 10 ns to ~0.7-0.8 nm between 40-60 ns, suggesting conformational adjustments upon ligand binding (Fig. 11). Following this phase, the RMSD values stabilised with moderate fluctuations, indicating that the system reached a moderately conformational state despite initial structural rearrangements.

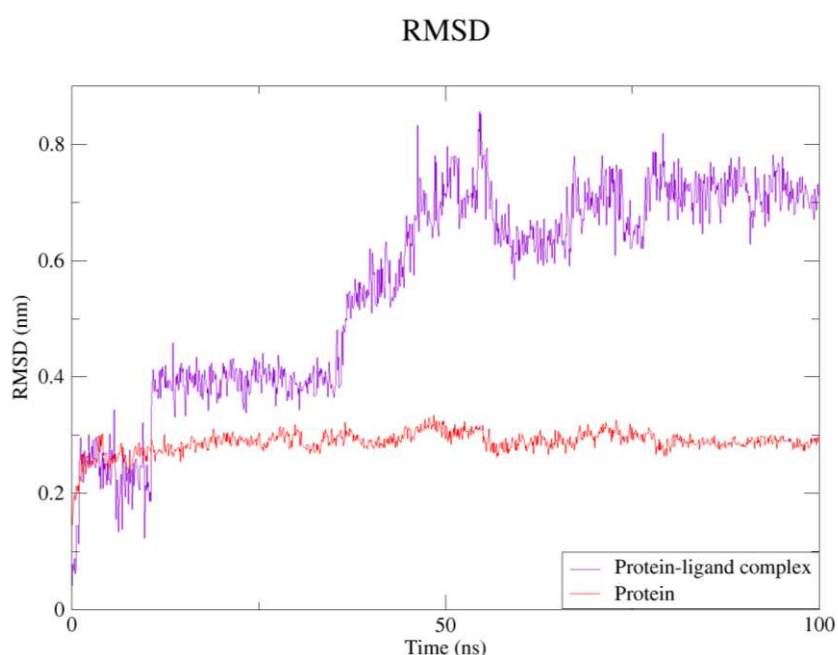


Fig. 11. RMSD of the 1XKK protein and the GA-1XKK complex.

Moderate fluctuations in the RMSF profile were observed in specific regions of the sequence near residue 870, where peaks reached ~0.30–0.33 nm, suggesting localised flexibility likely associated with loop regions or solvent-exposed segments. Notably, higher fluctuations were observed toward the C-terminal region, where RMSF values increased up to ~0.45-0.50 nm near residue number 1000, reflecting increased flexibility and conformational mobility of terminal residues (Fig. 12). This behaviour is typical, as terminal regions are generally less structurally constrained than the protein core. Overall, the RMSF analysis indicates that the protein maintains a stable core structure, with flexibility primarily localised to the loop and terminal regions, supporting the system's dynamic stability throughout the simulation. RMSF of ligand, SASA, Rg and h-Bond data are given in S3-S6.

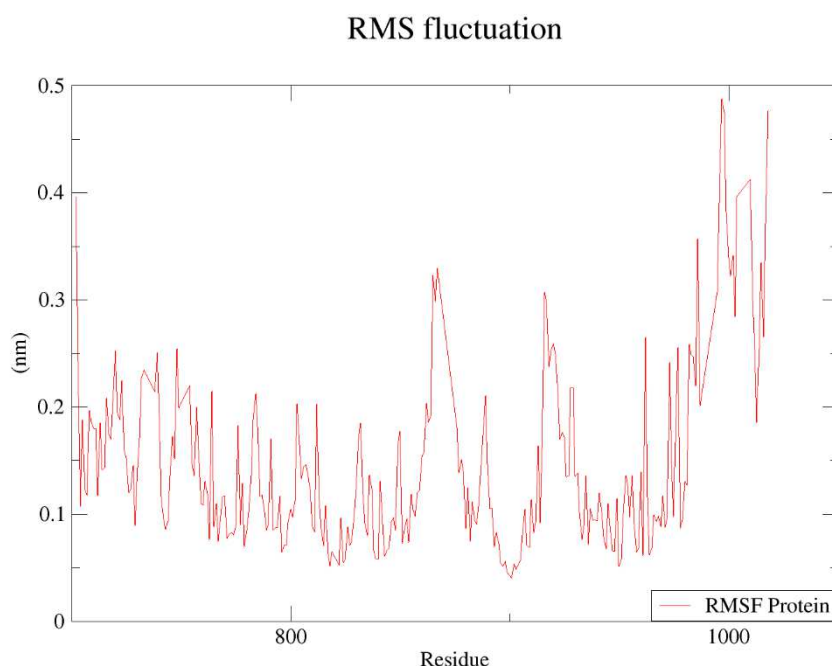


Fig. 12. RMSF of protein 1XKK

The overall binding free energy ($\Delta G_{\text{binding}}$) was found to be -4.18 ± 2.00 kcal/mol, indicating a weaker binding affinity as shown in Fig. 13. A thorough breakdown of individual energetic elements indicated that non-polar interactions were the key contributors to complex stability. Specifically, Van der Waals interactions (ΔE_{vdw}) contributed -17.55 ± 1.08 kcal/mol, representing the dominant driving force of binding, supplemented by a favourable electrostatic energy (ΔE_{elec}) of -70.96 ± 0.70 kcal/mol and non-polar solvation energy ($\Delta G_{\text{nonpolar}}$) of -3.20 ± 0.00 kcal/mol. Conversely, the polar solvation energy (ΔG_{polar}) presented an unfavourable penalty of 87.53 ± 1.38 kcal/mol, which is typical for biological macromolecular structures due to desolvation penalties.

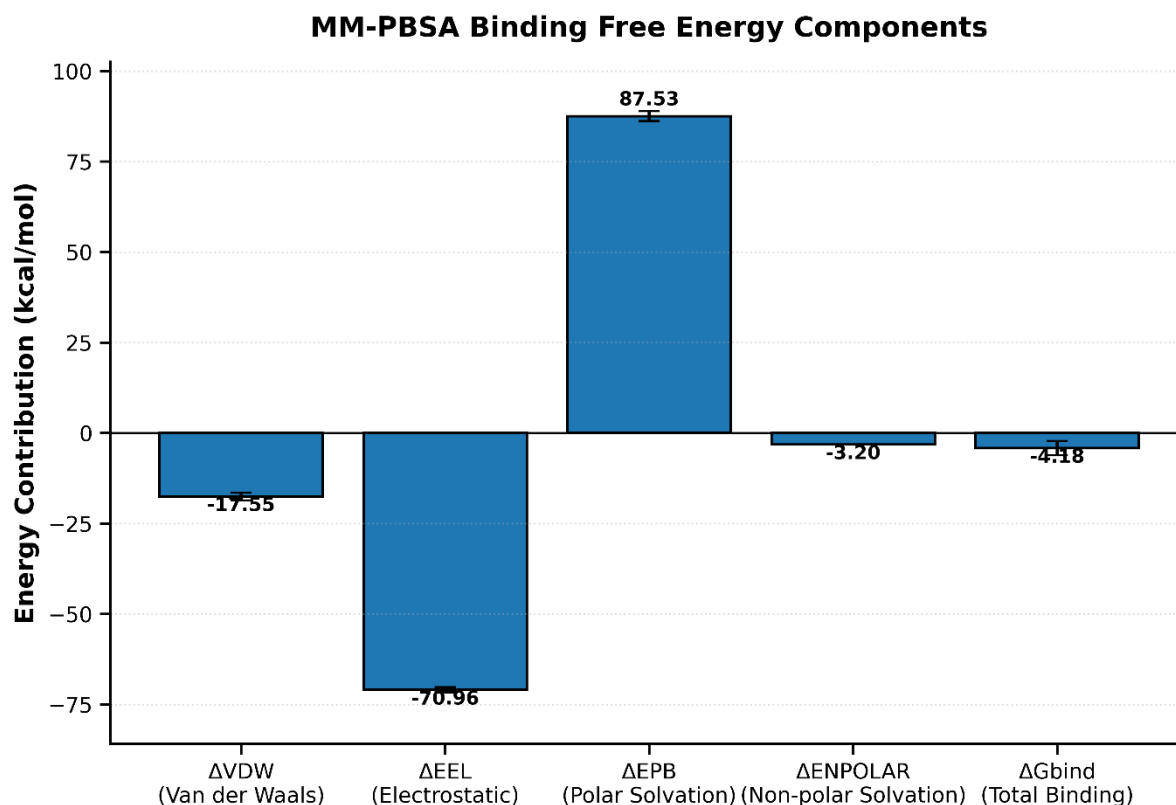


Figure 13. Binding energy of GA-Protein 1XKK complex

3.8 DPPH radical scavenging activity

The compound showed pronounced antioxidant activity in both radical scavenging assays, indicating strong antioxidant capacity. The DPPH assay showed a relatively higher IC_{50} value of $51.4 \pm 2.52 \mu\text{g/mL}$, indicating moderate scavenging activity against DPPH radicals.

3.9 In vitro assays

After GA treatment, a concentration-dependent decrease in cell viability was noted (Fig. 14). We optimised the dosing concentration and calculated the IC_{50} value to be $40 \mu\text{M}$ after one-way ANOVA, as mentioned in Fig. 14. Overall, these findings indicate that GA effectively suppresses breast cancer cell viability at a $40 \mu\text{M}$ concentration in vitro, highlighting its potential as an anti-cancer agent.

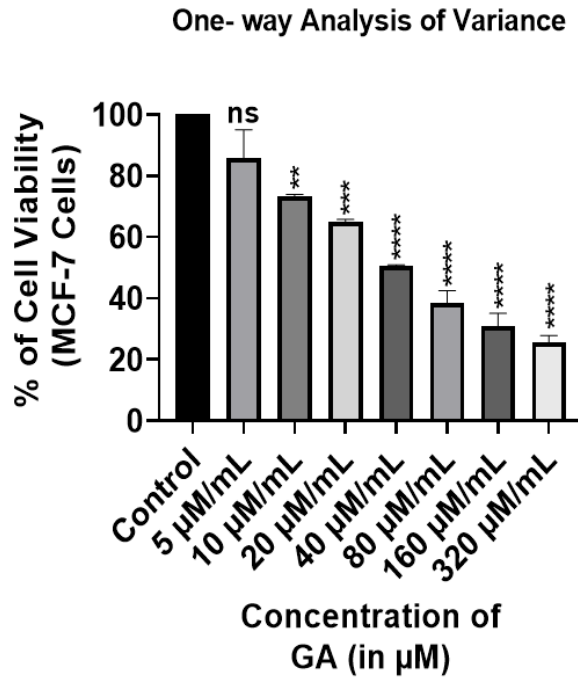


Fig. 14. Showing the IC_{50} values of GA in the MCF7 cells via the Cell Viability Assay.

AO/PI dual staining was used to assess the induction of apoptosis in MCF-7 cells after GA treatment at concentrations of 20, 40, and 80 μM . Fluorescence microscopy revealed a clear concentration-dependent increase in the apoptotic cell population. In the untreated control group, cells predominantly exhibited uniform green fluorescence, demonstrating healthy cell populations and intact membrane integrity (Fig. 15). At the IC_{50} concentration (40 μM), a marked increase in apoptotic cells was observed, with enhanced red fluorescence indicating compromised membrane integrity and progression toward late apoptosis. Collectively, Our studies suggest that GA causes apoptosis in MCF-7 cells, thereby confirming its potent cytotoxic and pro-apoptotic activity.

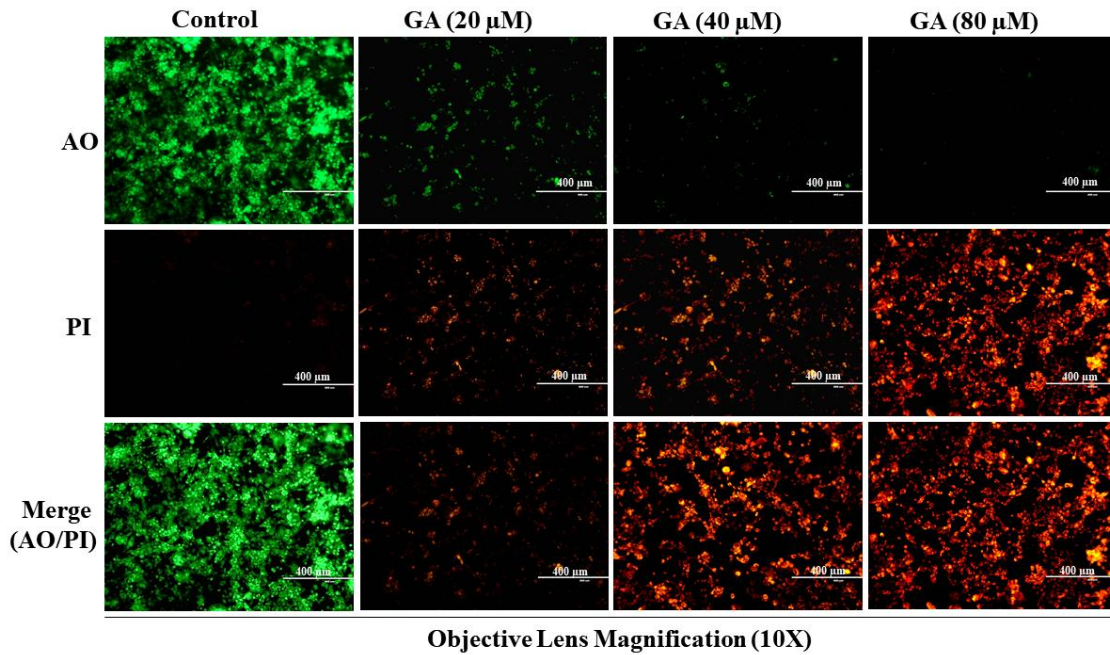


Fig. 15. The effect of GA on MCF-7 cell viability was assessed by AO/PI dual staining.

We assessed Intracellular reactive oxygen species (ROS) content in MCF-7 cells following treatment with GA at 20, 40, and 80 μM (Fig. 16). Control cells showed weak basal fluorescence, indicating minimal endogenous ROS levels. At the IC_{50} concentration (40 μM), ROS generation was markedly elevated, as evidenced by a substantial increase in fluorescence signal and a higher proportion of ROS-positive cells. This elevated ROS level is indicative of disrupted redox homeostasis and is likely associated with mitochondrial dysfunction and activation of apoptotic pathways. These results confirm that GA induces oxidative stress in a concentration-dependent manner, contributing to its cytotoxic and pro-apoptotic effects in MCF-7 cells.

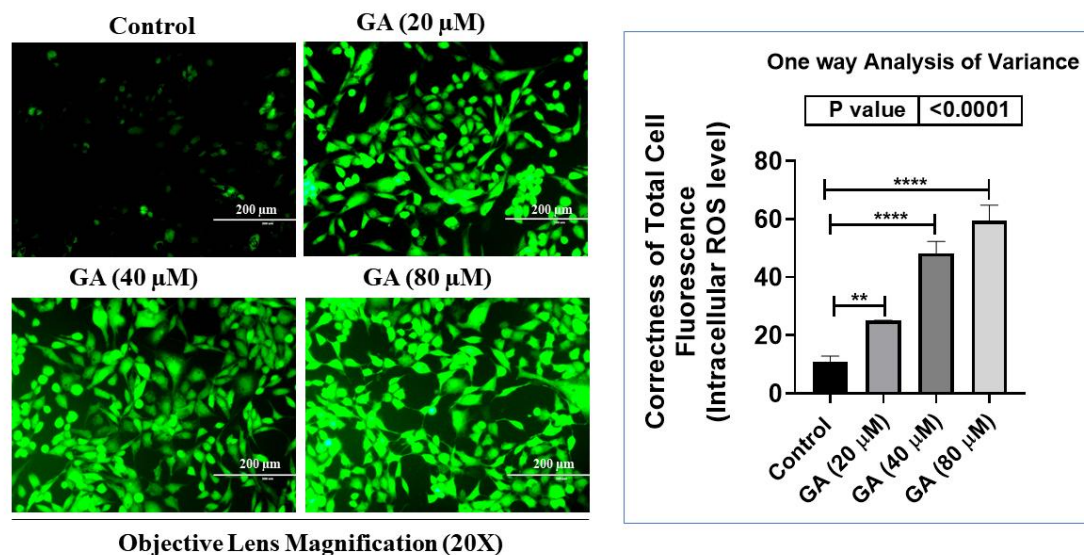


Fig. 16. ROS generation induced by GA treatment showed a concentration-dependent increase in intracellular reactive species levels. The bar graph represents the Corrected Total Cell Fluorescence (CTCF) analysis comparing fluorescence intensity between control (untreated) and treated groups.

The effect of GA on the migratory potential of MCF-7 cells was evaluated using a cell migration/ Scratch assay at concentrations of 20, 40, and 80 μM , as highlighted in Fig. 17. At the IC_{50} concentration (40 μM), migration was substantially reduced, as indicated by limited movement of cells into the gap. These findings demonstrate that GA effectively suppresses the migratory capacity of MCF-7 cells at 40 μM , indicating its possible function in preventing the spread of cancer cells.

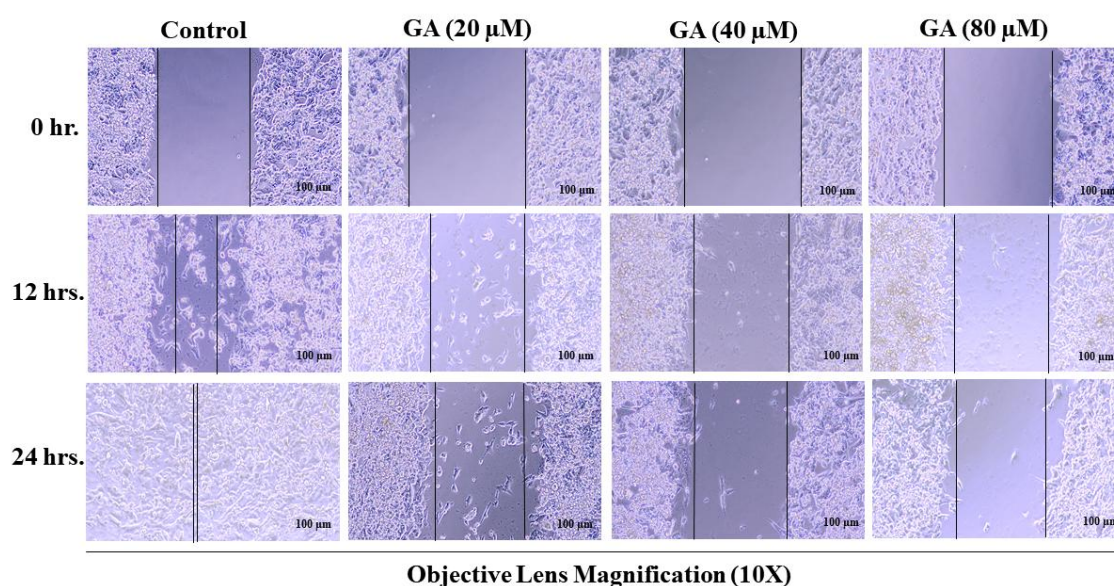


Fig. 17. The effect of GA treatment on MCF-7 cell migration was evaluated in comparison to untreated controls

The anti-proliferative effect of GA was evaluated using a colony formation assay to assess its long-term impact on clonogenic survival. In the control group, cells exhibited robust colony-forming ability, characterised by dense, uniformly distributed colonies, indicating a high proliferative potential. At the IC_{50} concentration (40 μ M), a substantial reduction in colony formation was evident (Fig. 18), with fewer, smaller colonies, indicating significant impairment of proliferative capacity. This pronounced inhibitory effect demonstrates GA's strong cytotoxicity at higher doses. Overall, the results indicate that GA effectively inhibits long-term cell survival at 40 μ M.

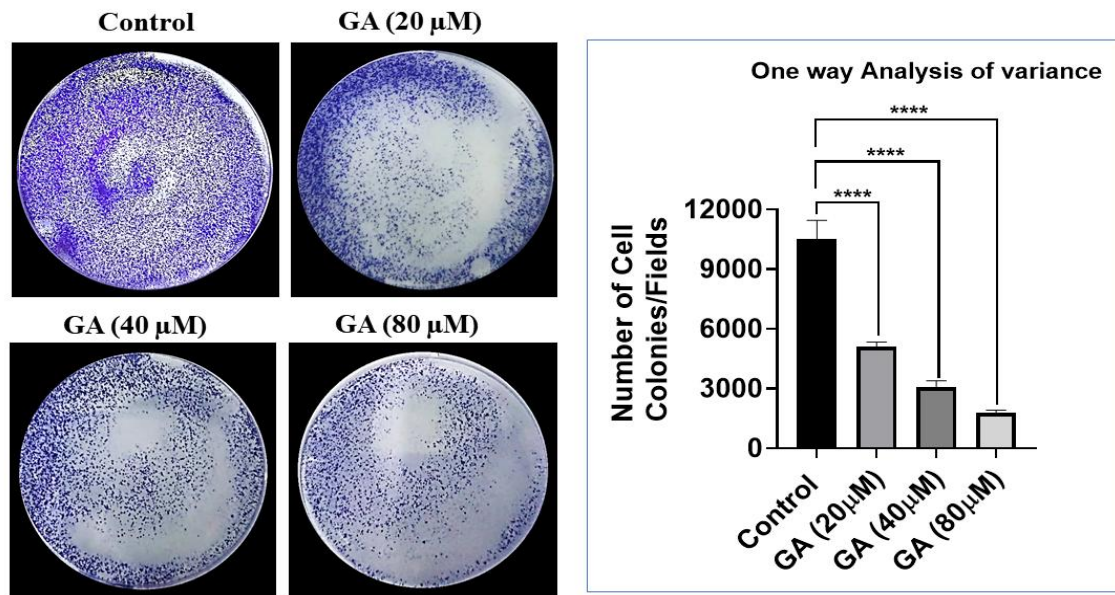


Fig. 18. GA concentrations were increased when exposed to cells, followed by a colony formation assay. GA treatment resulted in a significant, dose-dependent reduction in colony number and size relative to control cells, demonstrating its anti-proliferative effect.

The impact of GA on mitochondrial membrane potential (MMP) was evaluated using a fluorescence-based assay at 20 μ M, 40 μ M (IC_{50}), and 80 μ M. At the IC_{50} concentration (40 μ M), a marked transition in fluorescence pattern was observed. Red fluorescence was decreased while green fluorescence intensity significantly increased, as shown in Fig. 18. This shift indicates a substantial loss of mitochondrial membrane potential, suggesting that GA effectively induces mitochondrial dysfunction at this concentration. Overall, these findings suggest that GA may trigger apoptosis via mitochondrial signalling.

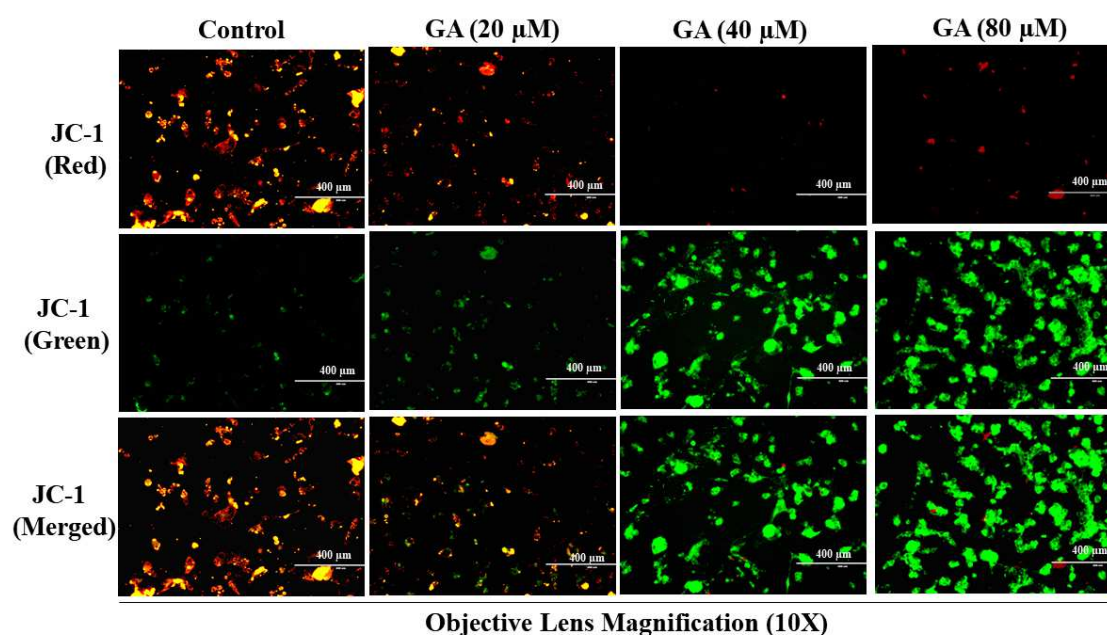


Fig. 18 JC-1 Staining shows representative photographs illustrating changes in mitochondrial membrane potential in MCF-7 cells upon GA treatment

The effect of GA on gene expression was quantitatively assessed using qRT-PCR at three concentrations: 20 μ M, 40 μ M (IC_{50}), and 80 μ M (Fig. 19). At the IC_{50} dose (40 μ M), a considerable elevation of pro-apoptotic genes (Bax) was detected, accompanying a reduction in the expression of anti-apoptotic genes (Bcl-2) associated with cell survival. In addition, increased caspase-3 expression is observed, indicating cell death via apoptosis at the IC_{50} concentration. Overall, the qRT-PCR data demonstrate that GA induces alterations in gene expression at 40 μ M.

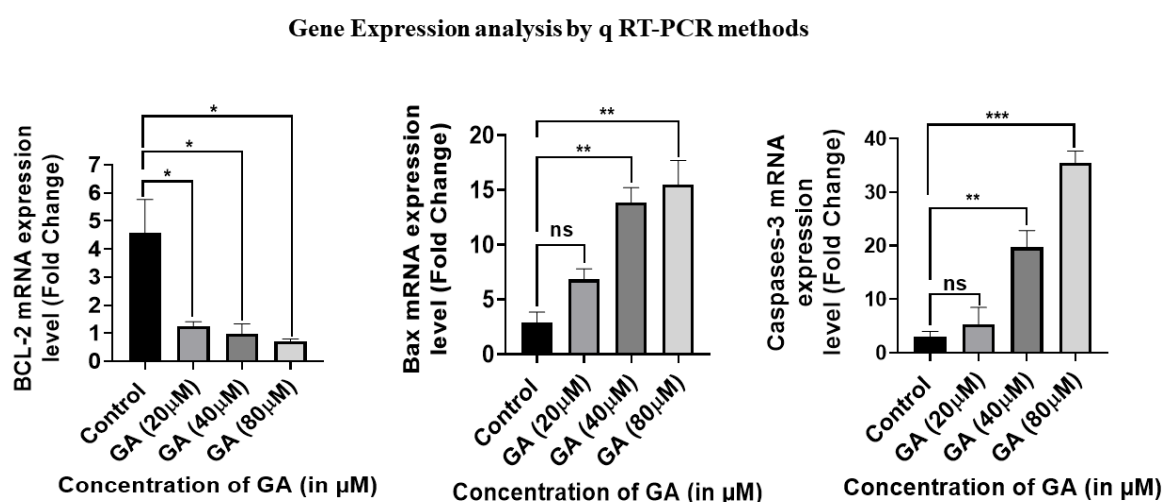


Fig. 19. The mRNA expression of apoptotic and anti-apoptotic genes was evaluated. treatment with various concentrations. of GA demonstrating a marked downregulation of the anti-apoptotic gene Bcl-2 and an upregulation of the pro-apoptotic genes Bax and Caspase-3.

4. Conclusion

This work demonstrates that GA is a mechanistically significant anti-cancer drug that acts by triggering apoptosis in cancerous cells and coordinating the control of mitochondrial integrity. Biological assays showed that GA inhibits MCF-7 cell motility, proliferation, and clonogenic potential at a 40 μ M concentration. These results call for additional research using in vivo models and clinical trials to assess GA's translational potential as a therapeutic candidate in the treatment of BC. Overall, current studies' results make GA a viable multi-target treatment option for BC.

Acknowledgements

The author is thankful to the Indian Institute of Technology (BHU) and MHRD, India, for the fellowship. The authors would like to acknowledge Prof. Sairam Krishnamurthy, Head of the Department of Pharmaceutical Engineering and Technology, IIT (BHU), Varanasi-221005, Uttar Pradesh, India.

Funding

This study was not funded by any govt and private agency.

Author information

Authors and Affiliations

Department of Pharmaceutical Engineering and Technology, Indian Institute of Technology (Banaras Hindu University), Varanasi, 221005, Uttar Pradesh, India

Rohit Singh Negi, Nitish Kumar, Sunil Kumar Mishra

Centre for Genetic Disorders, Institute of Science, Banaras Hindu University, Varanasi-221005, India

Rakesh Kumar Gupta, Pawan Kumar Dubey

Contributions

Conceptualization: RSN have developed the concept of the study. Methodology: RSN have prepared methodology. Validation: RSN and RKG, have validated study. Formal Analysis: RSN and RKG contributed for analysis. Investigation: RSN, RKG were main investigators of the study. Data Curation: NK Writing: RSN has written manuscript. Review and Editing: PKD and SKM done reviewing and editing of the manuscript. Supervision: PKD and SKM. Project Administration: PKD and SKM handled administrative tasks for this study. The authors declare that all data were generated in-house and that no paper mill was used.

Corresponding author

Correspondence to Sunil Kumar Mishra.

Ethics declarations

Consent for publication

Not applicable.

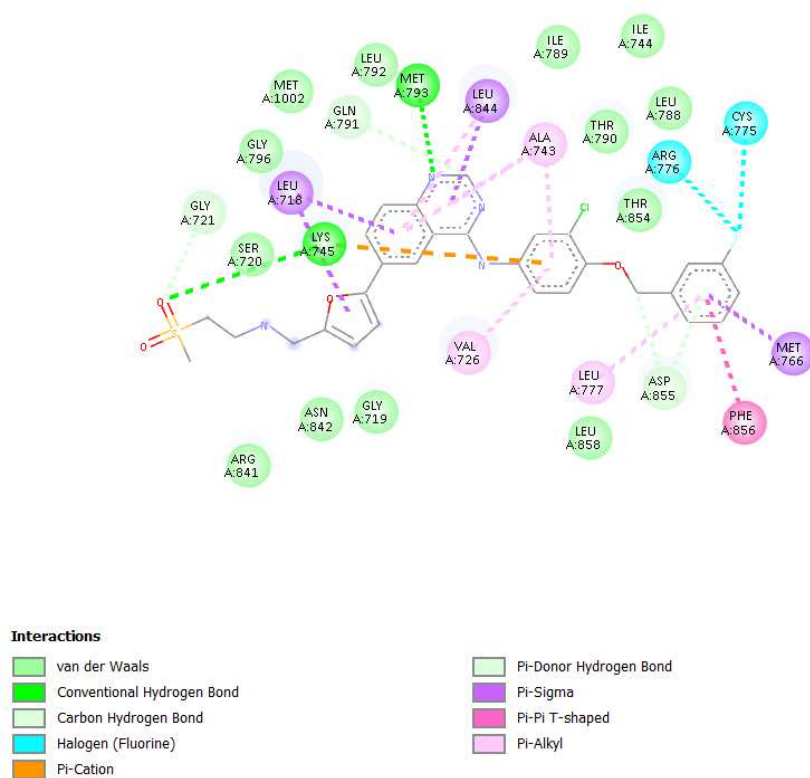
Conflict of interest

The authors declare no competing interests

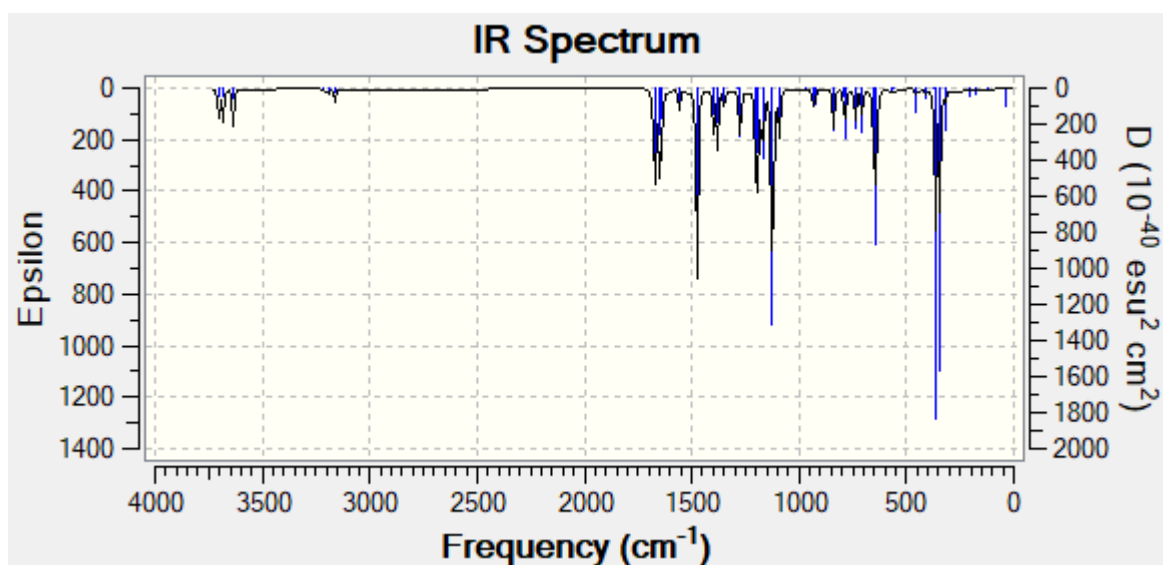
Data availability

The datasets generated during and/or analyzed during the current study are included in the manuscript and are available from the corresponding author upon reasonable request.

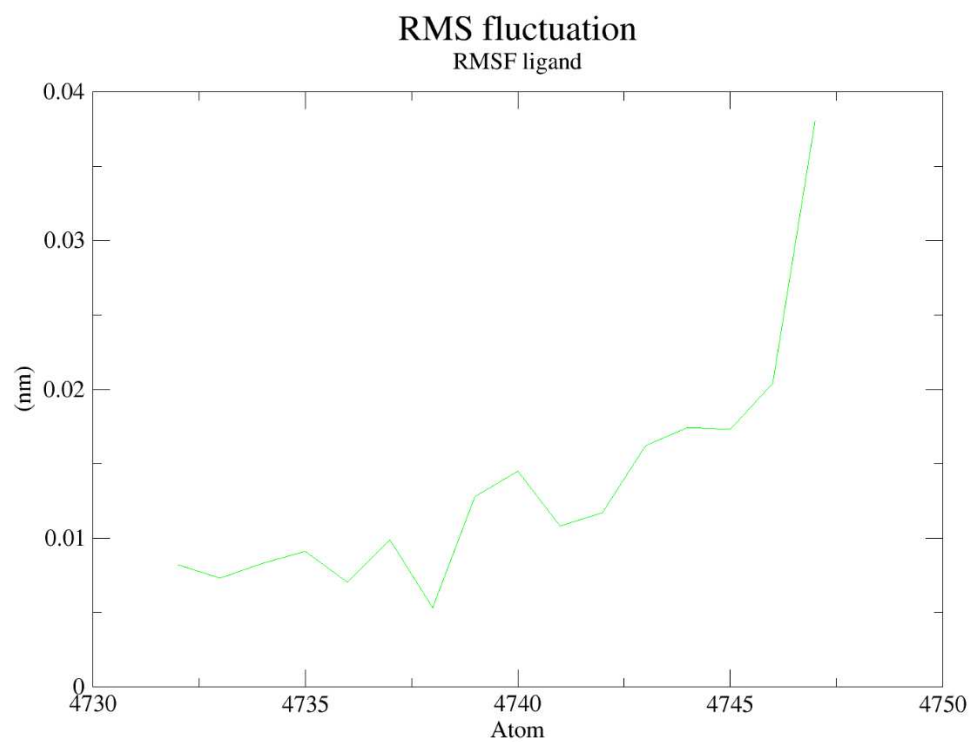
Supplementary files



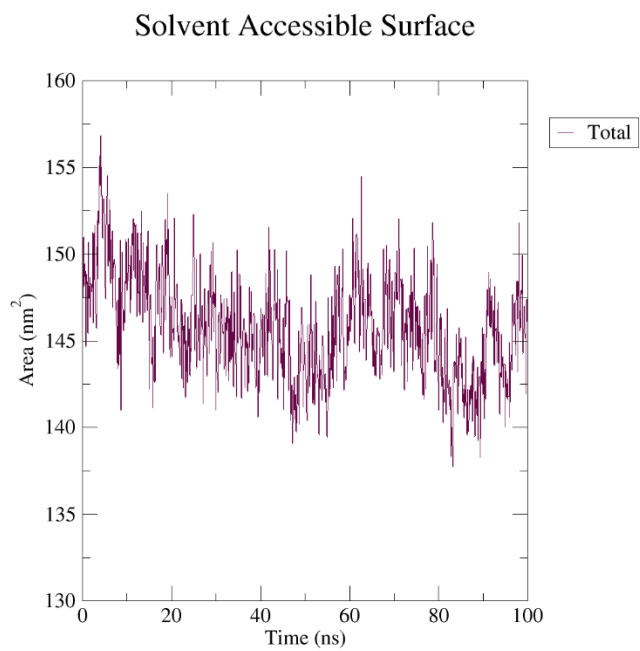
S1. 2D interaction of Lapatinib (Co-crystallised ligand) with Protein 1XKK.



S2. IR spectrum of GA calculated by Gaussian09W

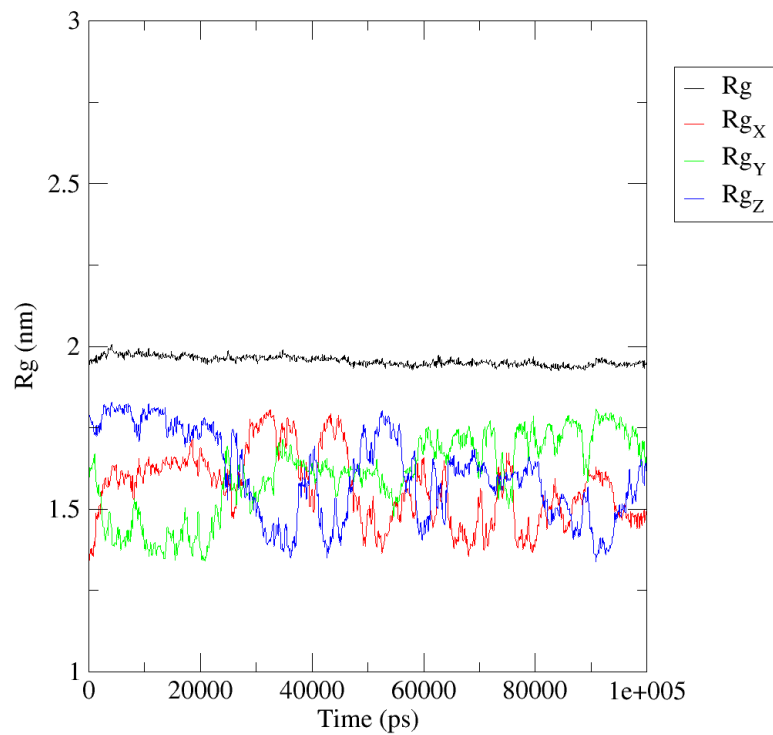


S3. RMSF of GA



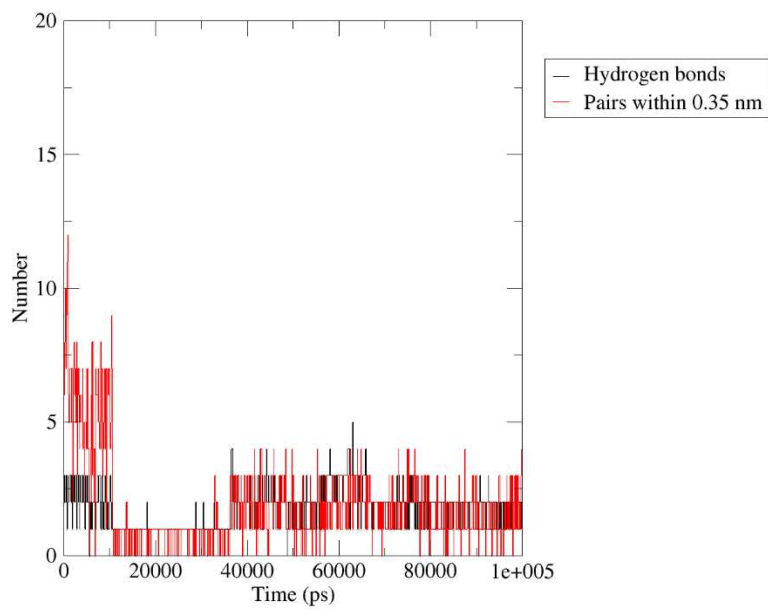
S4. SASA of protein-ligand complex

Radius of gyration (total and around axes)



S5. R_g of protein 1XKK

Hydrogen Bonds



S6. H-bond numbers of complex

References

- [1] E. Wiechec, L.L. Hansen, The effect of genetic variability on drug response in conventional breast cancer treatment, *Eur J Pharmacol* 625(1-3) (2009) 122-30.
- [2] Y.S. Sun, Z. Zhao, Z.N. Yang, F. Xu, H.J. Lu, Z.Y. Zhu, W. Shi, J. Jiang, P.P. Yao, H.P. Zhu, Risk Factors and Preventions of Breast Cancer, *Int J Biol Sci* 13(11) (2017) 1387-1397.
- [3] C. DeSantis, R. Siegel, P. Bandi, A. Jemal, Breast cancer statistics, 2011, *CA Cancer J Clin* 61(6) (2011) 409-18.
- [4] M.I. Nounou, F. ElAmrawy, N. Ahmed, K. Abdelraouf, S. Goda, H. Syed-Sha-Qhattal, Breast Cancer: Conventional Diagnosis and Treatment Modalities and Recent Patents and Technologies, *Breast Cancer* 9(Suppl 2) (2015) 17-34.
- [5] S. Saxena, C.I. Szabo, S. Chopin, L. Barjhoux, O. Sinilnikova, G. Lenoir, D.E. Goldgar, D. Bhatanager, BRCA1 and BRCA2 in Indian breast cancer patients, *Hum Mutat* 20(6) (2002) 473-4.
- [6] S. Vijayakumar, T. Ramamoorthy, A.D. George, A. Nath, Understanding female breast cancer risk in the Indian population: Evidence from a systematic review and meta-analysis, *Cancer Epidemiol* 100 (2026) 102979.
- [7] K. Sathishkumar, M. Chaturvedi, P. Das, S. Stephen, P. Mathur, Cancer incidence estimates for 2022 & projection for 2025: Result from National Cancer Registry Programme, India, *Indian J Med Res* 156(4&5) (2022) 598-607.
- [8] A. Suwalska, M. Rozenblit, M. Harigopal, J. Dai, M. Liu, J.P. Townsend, M. Marczyk, L. Pusztai, Mutational landscape of normal breast tissues adjacent to invasive breast cancer, *Cell Rep Med* 7(1) (2026) 102543.
- [9] Z.S. Lima, M. Ghadamzadeh, F.T. Arashloo, G. Amjad, M.R. Ebadi, L. Younesi, Recent advances of therapeutic targets based on the molecular signature in breast cancer: genetic mutations and implications for current treatment paradigms, *J Hematol Oncol* 12(1) (2019) 38.
- [10] R. Ali, M.K. Wendt, The paradoxical functions of EGFR during breast cancer progression, *Signal Transduct Target Ther* 2 (2017) 16042-.
- [11] H. Mechchate, I. Es-Safi, O. Mohamed Al Kamaly, D. Bousta, Insight into Gentisic Acid Antidiabetic Potential Using In Vitro and In Silico Approaches, *Molecules* 26(7) (2021).
- [12] R. Joshi, R. Gangabhairathi, S. Venu, S. Adhikari, T. Mukherjee, Antioxidant activity and free radical scavenging reactions of gentisic acid: in-vitro and pulse radiolysis studies, *Free Radic Res* 46(1) (2012) 11-20.
- [13] F. Abedi, B.M. Razavi, H. Hosseinzadeh, A review on gentisic acid as a plant derived phenolic acid and metabolite of aspirin: Comprehensive pharmacology, toxicology, and some pharmaceutical aspects, *Phytother Res* 34(4) (2020) 729-741.
- [14] H. Ando, Y. Hirai, M. Fujii, Y. Hori, M. Fukumura, Y. Niiho, Y. Nakajima, T. Shibata, K. Toriizuka, Y. Ida, The chemical constituents of fresh Gentian root, *J. Nat. Med.* 61(3) (2007) 269-279.
- [15] A. Mardani-Ghahfarokhi, R. Farhoosh, Antioxidant activity and mechanism of inhibitory action of gentisic and α -resorcylic acids, *Sci Rep* 10(1) (2020) 19487.
- [16] L.B. Cunha, E.D. Lepore, C.C.B. Medeiros, R. Sorrechia, R. Pietro, M.A. Corrêa, Can Gentisic Acid Serve as a High-Performance Antioxidant with Lower Toxicity for a Promising New Topical Application?, *Life* 14(8) (2024).
- [17] X. Dong, Q. Zhang, F. Zeng, M. Cai, D. Ding, The protective effect of gentisic acid on rheumatoid arthritis via the RAF/ERK signaling pathway, *J Orthop Surg Res* 17(1) (2022) 109.
- [18] M. Kabra, S. Bhandari, A. Sharma, R. Gupta, Evaluation of anti-parkinson's activity of gentisic acid in different animal models, *J. Acute Dis.* 3(2) (2014) 141-144.
- [19] R.N. Razliqi, A. Ahangarpour, S.A. Mard, L. Khorsandi, Gentisic acid ameliorates type 2 diabetes induced by Nicotinamide-Streptozotocin in male mice by attenuating pancreatic oxidative stress and inflammation through modulation of Nrf2 and NF- κ B pathways, *Life Sci* 325 (2023) 121770.
- [20] S. Sun, H.J. Kee, Y. Ryu, S.Y. Choi, G.R. Kim, H.S. Kim, S.J. Kee, M.H. Jeong, Gentisic acid prevents the transition from pressure overload-induced cardiac hypertrophy to heart failure, *Sci Rep* 9(1) (2019) 3018.

- [21] M.A. Altinoz, I. Elmaci, S. Cengiz, E. Emekli-Alturfan, A. Ozpinar, From epidemiology to treatment: Aspirin's prevention of brain and breast-cancer and cardioprotection may associate with its metabolite gentisic acid, *Chem Biol Interact* 291 (2018) 29-39.
- [22] R.S. Negi, A.K. Singh, A.K. Pathak, R.K. Gupta, N. Kumar, J. Behera, M.K.S. Gond, A.K. Singh, P.K. Dubey, S.K. Mishra, Incorporation of network pharmacology, molecular docking, survival, density functional theory, and experimental studies to explore the potential key targets of formononetin by TERT-mediated anti-cancer effects in MCF-7 breast cancer, *Naunyn Schmiedeberg's Arch Pharmacol* (2026).
- [23] A.K. Singh, A. Kumar Pathak, P. Kumar, A. Kumar Singh, M. Kumar Sah Gond, R. Singh Negi, R. Das, S. Agrawal, S. Kumar Mishra, K.N. Tiwari, Effects of Asiatic acid on brain cancer by altering astrocytes and the AKT1-PRKCB signaling pathway: A genomic and network pharmacology perspective, *Brain Res* 1859 (2025) 149652.
- [24] J. Zhou, H. Li, B. Wu, L. Zhu, Q. Huang, Z. Guo, Q. He, L. Wang, X. Peng, T. Guo, Network pharmacology combined with experimental verification to explore the potential mechanism of naringenin in the treatment of cervical cancer, *Sci Rep* 14(1) (2024) 1860.
- [25] P. Zou, J. Li, Y. Zhang, Z. Qian, H. Nie, N. Yang, L. Zhang, L. Lin, D. Peng, C. Zhang, Network pharmacology and molecular docking analysis on Shenfu Qiangxin indicate mTOR is a potential target to treat heart failure, *Eur J Med Res* 29(1) (2024) 173.
- [26] Y. Wu, X. Liu, G. Li, Integrated bioinformatics and network pharmacology to identify the therapeutic target and molecular mechanisms of Huangqin decoction on ulcerative Colitis, *Sci Rep* 12(1) (2022) 159.
- [27] T. Saha, S. Chatterjee, S. Das, An in silico DNA binding investigation using DFT, molecular docking and molecular dynamics simulation on mono and tetra acetylated derivatives of quinalizarin to enable a comparison with experimental DNA binding data, *Archives of biochemistry and biophysics* 776 (2026) 110680.
- [28] A. Rampadarath, J.O. Aribisala, N.P. Makunga, S. Mazibuko-Mbeje, S. Sabiu, Molecular bioprospection of *Helianthus annuus* L.(sunflower) cypselae for antidiabetic therapeutics through network pharmacology, density functional theory and molecular dynamics simulation, *S. Afr. J. Bot.* 162 (2023) 72-95.
- [29] X. Song, Y. Zhang, E. Dai, L. Wang, H. Du, Prediction of triptolide targets in rheumatoid arthritis using network pharmacology and molecular docking, *Int. Immunopharmacol.* 80 (2020) 106179.
- [30] X. Zha, R. Ji, Y. Li, R. Cao, S. Zhou, Network pharmacology, molecular docking, and molecular dynamics simulation analysis reveal the molecular mechanism of halocline against gastric cancer, *Mol. Diversity* 29(5) (2025) 4693-4703.
- [31] R. Kaundal, S. Kumar, D. Singh, D. Kumar, Underutilized edible Himalayan herb, *Viola canescens* Wall.; chemical composition, antioxidant and antimicrobial activity against respiratory tract pathogens, *Food Biosci.* 62 (2024) 105201.
- [32] D. Chauhan, R. Kumar, R.K. Gupta, R. Pratap, P.K. Dubey, D. Kushwaha, Synthesis, X-ray crystal structure, anticancer activity, DFT, and molecular docking studies of 1, 2, 4-triazole linked indolyl thioglycosides, *Journal of Molecular Structure* (2025) 144243.
- [33] R.K. Gupta, A. Priyadarshan, S. Tiwari, Y. Patel, A.K. Bind, G. Tripathi, A. Tripathi, A. Kumar, P.K. Dubey, THPM (1, 2, 3, 4-Tetrahydro pyrimidine) and Berberine Exhibit Synergistic Impact on Inhibition of Cell Migration and Colonization Through ROS-Mediated Apoptotic Pathways in the Breast Cancer Cells, *ChemistrySelect* 10(16) (2025) e202404148.
- [34] P. Kumari, P. Kumar, R.K. Gupta, A. Tripathi, P.K. Dubey, A. Ambasta, J.K. Chauhan, J. Goura, A. Kumar, G. Tripathi, Synthesis, crystallographic study, in silico and in vitro investigation of novel flavonol-amino acid conjugate as an anti-proliferative agent, *Journal of Molecular Structure* 1343 (2025) 142768.
- [35] N.A. Franken, H.M. Rodermond, J. Stap, J. Haveman, C. Van Bree, Clonogenic assay of cells in vitro, *Nature protocols* 1(5) (2006) 2315-2319.

- [36] V. Pandey, A. Tripathi, A. Rani, P.K. Dubey, Deoxyelephantopin, a novel naturally occurring phytochemical impairs growth, induces G2/M arrest, ROS-mediated apoptosis and modulates lncRNA expression against uterine leiomyoma, *Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie* 131 (2020) 110751.
- [37] X.D. Zhou, Y.M. Agazie, Inhibition of SHP2 leads to mesenchymal to epithelial transition in breast cancer cells, *Cell Death Differ* 15(6) (2008) 988-96.