

Integrated study of HR-LC/MS and network pharmacology to identify breast cancer-related molecular targets of *Fritillaria cirrhosa* D. Don active constituents in combination with molecular dynamic simulation and experimental evaluation

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Abstract

Fritillaria cirrhosa D. Don is a well-known medicinal plant in Kashmir Himalaya. Traditionally, it has been used to treat several diseases, most notably in the treatment of various cancers particularly lung cancer. However, there is a significant gap between scientific research and its application in conventional medicine. The aim of the current work is to provide first-hand evidences both *in-vitro* and *in silico* showing that *F. cirrhosa* extracts exerts anti-cancer effects against breast cancer. Bulbs of *F. cirrhosa* was extracted with various solvents of increasing polarity. Compounds were identified by High resolution-liquid chromatography-mass spectrometry (HR-LC/MS) technique. Phytocompounds were studied for protein targets involved in pathogenesis of breast cancer using Binding 1DB (similarity index > 0.7). Later, the protein-protein interactions (PPI) network was studied using STRING programme and compound-protein interactions using Cytoscape. In addition, molecular docking was used to investigate intermolecular interactions between the compounds and the proteins software using Autodock tool. Molecular dynamics simulations studies were also used to explore the stability of the representative CDK2 + Peiminine complex. In addition, standard *in-vitro* biochemical assays were used to evaluate the *in-vitro* antiproliferative activity of active extracts of *F. cirrhosa* against several breast cancer cell lines. Bioactive components and potential targets in the treatment of breast cancer were validated through network pharmacology approach. HR-LC/MS detected the presence of several secondary metabolites. Afterward, molecular docking was used to verify the effective activity of the active ingredients against the prospective targets. Additionally, Peiminine showed the highest binding energy score against CDK2 (-12.99 kcal/mol). CDK2 + Peiminine was further explored for molecular dynamics simulations. During the MD simulation study at 100 nanoseconds (ns), a stable complex formation of CDK2 + Peiminine was observed. According to molecular docking results predicted, several key targets of breast cancer bind stably with the corresponding phytocompounds of *F. cirrhosa*. Lastly, *F. cirrhosa* extracts exhibited momentous anticancer activity through *in vitro* studies. Overall, the most important constituents were Imperialine-3- β -glucoside and Peiminine from the *F. cirrhosa* bulbs has effective anti-cancer efficacy by deactivating Akt1 on the PI3K-Akt signaling pathway. Therefore, these findings emphasized the momentous anti-breast cancer activity of *F. cirrhosa* extracts. This may open a new window and provide a theoretical foundation for further development and utilization of *F. cirrhosa* medicinal plant in the treatment of breast cancer.

Introduction

Breast cancer is the most aggressive type of cancer in women's and continues to impact millions of women globally ¹. It is the most diagnosed malignancy in the world, according to information published by the International Agency for Cancer Research (IARC) in December ^{2,3}. More than 2.3 million women were detected with breast cancer, with 685,000 deaths worldwide (WHO; 2022). Hormone receptor positive (HR)-positive breast cancer represents approximately 80% of all the breast cancers ⁴. Numerous current therapeutic techniques (such as targeted therapies) have their own downsides, such as cancer cells developing resistance to anti-breast cancer drugs and the recurrence of breast cancer after treatment ⁵⁻⁷. In recent years, targeted drugs have transformed the treatment of breast cancer, especially the HR-positive subtype ⁸⁻¹⁰. The ineffectiveness of conventional chemotherapeutic strategies necessitates the development of novel drugs for the prevention and treatment of this disease with minimal side effects, and plants can be a significant source of these promising molecules ^{11,12}. They can stimulate several pathways like MAPK-Signaling pathway, JNK-signaling pathway and ERK pathway. Natural Products (NPs) continue to generate a significant number of innovative clinical candidates and medications, especially in anticancer therapeutic areas ^{11,13}.

Several research studies have revealed that about 25–28% of all current medications are believed to be obtained directly or indirectly from higher plants, indicating the immense potential of plants that have been exploited for ages ¹⁴⁻¹⁶. Furthermore, approximately 60% of anticancer drugs are derived from the plant kingdom ^{14,17}. Numerous essential modern drugs, including digitoxin, reserpine, tubocurarine, ephedrine, ergometrine, atropine, and aspirin, were found by tracing their roots to their traditional uses ¹⁵. For example, vinblastine and vincristine, which are used to treat pediatric leukemia and Hodgkin's disease, are produced from *Catharanthus roseus*, an African periwinkle. We are grateful to the plant kingdom for providing these medications ^{18,19}.

Fritillaria (fritillaries) is a genus of perennial herbaceous bulbous lily family (Liliaceae) plants that bloom in the spring. Fritillaria species have been used in traditional Chinese medicine for over two thousand years, and they are today one of the most widely used treatments ²⁰⁻²². It has been estimated that the production of natural medicines from *F. cirrhosa* is worth US\$400 million per annum. Fritillaria, like other Liliaceae family members, contain medicinally important flavonol glycosides, tri- and diferulic-acid sucrose esters, steroidal alkaloids, saponins, and terpenoids ²³.

Fritillaria cirrhosa D. Don is a perennial medicinal plant belonging to the genus Fritillaria and is a critically endangered medicinal herb ²⁴. It is a part of the Astavarga group, a collection of eight herbs utilized in traditional Indian and Himalayan medicine ²⁴. The alkaloids present

in the bulbs of the *F. cirrhosa* displayed unique biological activity especially anti-inflammatory and antitumor^{22,25}.

The emerging field of network pharmacology (NP), founded by Hopkins in 2007, seeks to understand how drugs interact with several targets²⁶. It integrates systematic medicine and informational technology for exploring new drug candidates and their targets. It highlights the concept of "network target, multicomponent therapeutics," altering the paradigm away from the assumption of one gene, one target, and one disease^{27,28}. Network pharmacology is a useful approach used to examine traditional medicine's synergistic actions and underlying mechanisms^{29–31}.

In the present research study, network pharmacology, molecular docking, and *in vitro* validation were used to identify the anti-breast cancer mechanisms of phytochemicals identified from *F. cirrhosa*. Notably, this is the first research study to evaluate the anti-breast cancer mechanism of *F. cirrhosa* using a combination strategy. Therefore, the current study was designed to evaluate the, (1) the phytochemicals present in *Fritillaria cirrhosa* by HR-LC/MS; (2) Mechanism to identify the probable potential protein targets of breast cancer by network pharmacology approach combined with molecular-docking; (3) Gene Ontology (GO) and KEGG pathway enrichment analysis to identify the molecular pathways modulated by phytochemicals of *F. cirrhosa* against breast cancer; and (4) To evaluate the *in-vitro* anticancer activities of the different polarity extracts of *F. cirrhosa* bulb parts, including petroleum ether, ethyl acetate and methanol (Me) against various breast cancer cell lines (MDA-MB-231, MCF-7, MDA-MB-468 and 4T1) as shown in the Fig. 1.

Material And Methods

Plant material

The bulbs of *F. cirrhosa* were collected from several sites of Kashmir Himalaya. The permissions and licenses were obtained from the concerned authorities for collection of the plant material. The plant material collected was identified and authenticated from Centre for Biodiversity and Taxonomy (CBT), Department of Botany, University of Kashmir, vide no. F(voche/specimen) CBT/KASH/21; specimen voucher number 2952-(KASH); Dated:20/7/20.

Permission and Guidelines: It is stated that all the permissions and licenses were obtained from the concerned authorities for collection of the plant material. Further it is stated that all methods were performed in accordance with the relevant guidelines and regulations during collection, identification, preparation of herbarium and extraction.

Data availability

The datasets supposing the current study are available in public database from STITCH (<http://stitch.embl.de/>), Swiss Target Prediction (<http://www.swisstargetprediction.ch/>), GeneCards (<https://www.genecards.org/>), MalaCards (<https://www.malacards.org/>), STRING (<http://www.string-db.org/>), DrugBank (<https://go.drugbank.com/>), and PDB (<https://www.pdb.org>).

Extraction

The air-dried bulbs of the experimental plant was grounded into a fine powder using mixer grinder. Different polarity gradient organic solvents including petroleum ether, ethyl acetate, and methanol were used, to extract the plant material by Soxhlet method. The dried extracts were stored in airtight glass vials at 4°C for future use.

High resolution-liquid chromatography-mass spectrometry (HR-LC/MS)

The phytochemical profile of crude extracts obtained from *F. cirrhosa* bulb parts were analyzed using a HR-LC/MS technique. Compounds were identified based on their mass spectra and unique mass fragmentation patterns. Several public databases such as Compound Discoverer 2.1, ChemSpider, and PubChem were used as the primary resources used to identify the phytochemical constituents in *F.cirrhosa*³².

Screening for active constituents

The phytochemicals from *F. cirrhosa* in the scientific literature and their structures in "canonical smile" and "sdf" file format were retrieved using publicly available small molecule databases such as Dr. Dukes DB (<https://phytochem.nal.usda.gov/phytochem/search>) and Phyto-chemical interactions database (PCIDB; <https://www.genome.jp/db/pcidb>). Using Swiss Target Prediction (<http://www.swisstargetprediction.ch/>), STITCH: chemical association networks (<http://stitch.embl.de/>), PubChem (<https://pubchem.ncbi.nih.gov/>), and Therapeutic Target Database (TTD) (<http://bidd.nus.edu.sg/group/cjttd/>), the predicted targets of the

aforementioned phytochemicals were determined. To acquire complete data, we combined the targets from the aforementioned databases and designated them as phytochemical targets.

Prediction of the potential target gene for the quantitative component and disease

The target genes retrieved from the two databases were merged. Standardization of the gene name and definition of the species as "human" was performed using the UniProtKB function in the UniProt (<https://www.uniprot.org/>) database. GeneCards (<https://www.genecards.org/>) and MalaCards (<https://www.malacards.org/>), the human gene database, were used to retrieve breast cancer-related genes. The keywords used in the search were limited to "breast cancer" and "mammary carcinoma". The targets obtained were compared to those retrieved earlier and target genes linked to breast cancer were selected.

Protein-protein interaction (PPI) network construction

To study the association between putative phytochemical targets and breast cancer-related hub genes, a protein-protein interaction (PPI) network was constructed using the Search Tool STRING (<http://www.string-db.org/>) with a threshold of >0.4. (minimum confidence)³³. On a histogram, the statistical distribution of the 30 most frequent target genes was shown. Cytoscape (3.7.1) program was used to display molecular-interaction networks^{33,34}.

Kyoto Encyclopaedia of Genes and Genomes (KEGG) and Gene Ontology (GO) Enrichment and Network Analyses of the Target Proteins

GO is regarded as an essential bioinformatics technique for identifying genes and analyzing their biological processes^{35,36}. The Kyoto Encyclopaedia of Genes and Genomes (KEGG) database evaluates the complex functions and biological systems of high-throughput sequencing-generated molecular data. KEGG includes genetic, chemical, and system function data³⁷. Shiny GO, a web-based platform, was used for KEGG and GO analysis, which was then applied to examine the activities of the target proteins³⁸.

Compound prescription-active component-disease target gene interaction network analysis

A network of links between prescription-active component-disease-target gene-pathway was constructed using Cytoscape (version 3.8.1). The word "node" refers to the drug, the active component, the disease, the target gene, and the pathways within the framework of the network. The link between the previously stated nodes is referred to as "edge." Following an examination of the quality indicators by degree, screening candidates with degrees above the average were selected from all active network components. When performing network research, the degree of a node's significance is the simplest metric to use.

***In-silico* drug-likeness and toxicity predictions**

In silico drug likeness and toxicity of top hit compounds in the database based on was carried out using SwissADME web browser (<http://www.swissadme.com>)³⁹. Drug-likeness and toxicity filtering was based on Lipinski's rule of five⁴⁰. For example, constituents with predicted oral bioavailability (OB) ≥ 30 were considered active. Constituents that satisfied less than three criteria were considered inactive.

Molecular docking studies

The 3D structure of nine phytochemicals were retrieved from the PubChem data base and performed the docking with breast cancer hub genes such as (AKT-1, TNF, SRC and EGFR) identified by PPI network map. using AutoDock vina programme⁴¹. The crystal structure of breast cancer targets obtained from the RCSB Protein Data Bank (<https://www.pdb.org>). To modify the target proteins by removing water molecules adding and kollman charges and polar hydrogen atoms using PyMol (version 1.7.2.1) programme. After that both target and receptor molecules were saved in pdbqt format. Molecular docking was performed within a grid box of particular dimensions and spacing. Docking studies of the protein–ligand complex were carried out following the Lamarckian Genetic Algorithm (LGA).

Molecular dynamics simulation (MD) studies

Molecular dynamic (MD) simulations investigations were carried out on dock complex with minimum energy using Desmond 2020.1 tool from Schrödinger, LLC programme⁴². This programme employed the SPC water molecules and the OPLS-2005 force field⁴³⁻⁴⁵. In MD simulations, Sodium ions were supplied to the system in order to neutralize the charge, and 0.15M of NaCl solutions were added to replicate the physiological environment. To retrain the system over the peiminine with CDK2 complex, the system was initially equilibrated using NVT ensemble for 100 picoseconds (ps). This was followed by a 12-ps NPT ensemble short run equilibration and minimization. The Nose Hoover chain approach⁴⁶ was used to build up the NPT ensemble, which was maintained throughout simulations at a constant temperature of 27 °C, pressure of 1 bar and relaxation time of 1.0 ps. In the simulation experiment, a 2-fs time step was used. A 12-ns NPT

ensemble run was employed to perform a rapid equilibration and reduction following the previous phase. The NPT ensemble was assembled using the Nose–Hoover chain coupling method ⁴⁷ and operated at 27 °C for 1.0 ps under a pressure of 1 bar for the duration of the investigation. For pressure regulation, the Martyna–Tuckerman–Klein barostat method with a 2 ps relaxation time was adopted. Long-range electrostatic interactions were calculated using Ewald's particle mesh method, with the radius for coulomb interactions fixed at 9 nm. Each trajectory's bonded forces were computed using the RESPA integrator with a 2-fs time step. Using metrics such as the root mean square deviation (RMSD), gyroradius, root mean square fluctuation (RMSF), number of hydrogen atoms (H-bonds), and solvent accessible surface area (SASA), calculations were performed to track the stability of MD simulations ⁴⁸.

***In vitro* cytotoxicity assay**

Cell lines and culture conditions

Multiple breast cancer cell lines were used to test the *in vitro* anticancer properties of *Fritillaria cirrhosa* extracts (MCF-7, MDA-MB-231, MDA-MB-468, and 4T1). The National Centre for Cell Science (NCCS) Pune, India supplied the cells. For purposes of identification, the morphology of these cells was systematically assessed. To culture cells, Dulbecco's Modified Eagle's Medium was employed (DMEM; Thermo Fisher Scientific, Waltham, MA, United States). The medium was supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, United States) and 1% penicillin-streptomycin (Thermo Fisher Scientific, Waltham, MA, United States). The cells were cultivated in a 37 °C CO₂ incubator.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Assay

The MTT assay for determining the cytotoxic activity of extracts was done according to Keepers et al technique (1991). Cancer cells were extracted from culture flasks using trypsin, and trypan blue dye was used to determine their viability. The required number of wells in a 96-well plate were seeded with these cancer cells at a density of 3 x 10³ cells per well in 100 µl of growth medium each well. The plate was incubated for 24 hours in a CO₂ incubator at 5% CO₂ and 37 °C. After 24 hours of incubation, the growth media was carefully removed and varied amounts of plant extracts generated in the growth medium were put to the plate (100µl per well; triplicate) and incubated. After a 4-hour incubation, the medium was discarded and fresh dye (4 mg MTT dye/10 ml growth media without FBS) was added to each well (100 µl/well). The plate was then incubated for another 4 hours. The formazan crystals in the wells were dissolved with 100 µl/well DMSO, and the optical density was measured at 540 nm using a Biotek Synergy HT, USA microplate reader.

Statistical analysis

Network interaction was evaluated via edge count. Docking data are presented as energy in kcal/mol. Interaction stability and fluctuations through MD simulation were analyzed by RMSD and RMSF. All experimental data were presented in mean ± SD. The IC₅₀ was calculated using a linear regression curve by GraphPad *ver* 5 programme.

Results

HR-LC/MS study of *F. cirrhosa* extracts

High resolution liquid chromatography-mass spectrometry (HR-LC/MS) technique in its positive and negative ionization modes permitted the identification of 88 compounds as described in our previous study (Fig. 2) ²¹. Using advanced technique of HR-LC/MS, some additional compounds were identified which primarily included Imperialine, Verticinone or Peiminine, Verticine or Peimine, Ebeiedine, Ebeiedinone, Chuanbeinone (Purapuridine), Sinpeinine A or Puqiedinone, Imperialine-3-β-glucoside and Peonidin (Fig. 3) in *F. cirrhosa* extracts by comparing their output mass data with a reference database and previously published data ^{49–51}. The identified compounds from chromatograms are listed in Table (1). The structures of these compounds were subjected to network pharmacology and *in-silico* docking. These compounds were identified as chemical markers and were listed as candidate compounds for further network pharmacology analysis.

Table 1
Phytocompounds detected in *F. cirrhosa* by HR-LCMS

S.No.	Compound Name	Mol. Weight	Molecular Formula	Canonical Smiles
1	Imperialine	429.6g/mol	C ₂₇ H ₄₃ NO ₃	CC1CCC2C(C3CCC4C(C3CN2C1) CC5C4CC(=O) C6C5(CCC(C6) O) C) (C)O
2	Peiminine	429.6g/mol	C ₂₇ H ₄₃ NO ₃	CC1CCC2C(C3CCC4C(C3CN2C1) CC5C4CC(=O) C6C5(CCC(C6) O) C) (C)O
3	Peimine	431.7g/mol	C ₂₇ H ₄₅ NO ₃	CC1CCC2C(C3CCC4C(C3CN2C1) CC5C4CC(C6C5(CCC(C6) O) C) O) (C)O
4	Ebeiedine	415.7g/mol	C ₂₇ H ₄₅ NO ₂	CC1CCC2C(C3CCC4C(C3CN2C1) CC5C4CC(C6C5(CCC(C6) O) C) O) C
5	Ebeiedinone	413.6g/mol	C ₂₇ H ₄₃ NO ₂	CC1CCC2C(C3CCC4C(C3CN2C1) CC5C4CC(=O) C6C5(CCC(C6) O) C) C
6	Chuanbeinone	413.636g/mol	C ₂₇ H ₄₃ NO ₂	CC1CCC2(C(C3C(O2) CC4C3(CCC5C4CC = C6C5(CCC(C6) O) C) C) C) NC1
7	Sinpeinine A	413.6g/mol	C ₂₇ H ₄₃ NO ₂	CC1CCC2C(C3CCC4C(C3CN2C1) CC5C4CC(=O) C6C5(CCC(C6) O) C) C
8	Imperialine-3-β-glucoside	591.8g/mol	C ₃₃ H ₅₃ NO ₈	CC1CCC2C(C3CCC4C(C3CN2C1) CC5C4CC(=O) C6C5(CCC(C6) OC7C(C(C(C(O7) CO) O) O) C) (C)O
9	Peonidin	301.27g/mol	C ₁₆ H ₁₃ O ₆ ⁺	COC1 = C(C = CC(= C1) C2=[O+][C3 = CC(= CC(= C3C = C2O) O) O) O

Target Gene Screening And Interaction Network Construction

Total of 359 potential target genes were obtained for the 9 quantitative phytocompounds from HR-LC/MS (shown in Fig. 4). Meanwhile, 12063 disease target genes associated with breast cancer were retrieved using Gene Cards platforms. 326 shared common target genes were identified between the quantitative components of HR-LC/MS and breast cancer. All 9 phytocompounds, namely, Imperialine, Verticinone or Peiminine, Verticine or Peimine, Ebeiedine, Ebeiedinone, Chuanbeinone (Purapuridine), Sinpeinine A or Puqiedinone, Imperialine-3-β-glucoside and Peonidin were targeted for further analysis. The common target genes PPI diagram indicated that there were 326 nodes and 3522 edges in PPI (Fig. 5(a)). The frequency of occurrence of the top 30 common target genes is shown in Fig. 5(b). *AKT1*, *TNF*, *SRC*, *EGFR*, *ESR1* and other target genes exhibited high frequency of protein interaction, which may be the node protein of the whole network. The results showed that the selected compounds of *F. cirrhosa* had a high binding activity with *AKT1*, *TNF*, *SRC*, *EGFR*, *ESR1* and could be used as the potential target genes for treating breast cancer.

3.3 Screening Of Key Pathways For Treating Breast Cancer

Gene ontology (GO) analysis of the common target genes showed that the biological process was mainly involved in cellular response to organonitrogen compound and cellular response to nitrogen compound. The molecular functions Integral component of presynaptic membrane and Intrinsic component of presynaptic membrane were leading. In cellular component, G protein-coupled amine receptor activity and Transmembrane receptor protein tyrosine kinase activity were observed (Fig. 6). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of the aforementioned common target genes Fig. 7. After exclusion of broad pathways, the top 20 signalling pathways are listed in Table 2. This suggested that the effect of phytocompounds identified through HR-LC/MS for treating breast cancer may act on multiple pathways, as well as complex interactions among these pathways.

Table 2
Pathway Enrichment Analysis (Top 20).

Pathway	nGenes	Enrichment FDR
Pathways in cancer	66	1.92721E-40
Metabolic pathways	64	2.91743E-14
Neuroactive ligand-receptor interaction	59	2.72087E-43
PI3K-Akt signaling pathway	47	1.41101E-29
Focal adhesion	34	8.28856E-25
Proteoglycans in cancer	34	9.35709E-25
Calcium signaling pathway	34	1.84449E-22
Human papillomavirus infection	33	2.22721E-17
Chemical carcinogenesis	32	6.7506E-23
CAMP signaling pathway	32	1.16924E-21
MicroRNAs in cancer	29	3.5344E-22
Lipid and atherosclerosis	29	8.66778E-19
Human cytomegalovirus infection	29	2.96276E-18
Epstein-Barr virus infection	27	1.91139E-17
Viral carcinogenesis	27	2.07765E-17
Human immunodeficiency virus 1 infection	27	4.45928E-17
Chemokine signaling pathway	26	4.45928E-17
MAPK signaling pathway	26	6.36057E-13
Endocrine resistance	25	3.11535E-23
Ras signaling pathway	25	2.57797E-14

3.4 Compound Prescription-active Component-disease Target Gene Interaction Network

Compound prescription-active component-disease-target gene-pathway interaction network finding is shown in Fig. 8. The network contained a total of 336 nodes (326 target genes, 09 active components. Besides, the interaction network results of 09 active compounds are shown in Table 4. The degree of Imperialine, Verticinone or Peiminine, Verticine or Peimine, Ebeiedine, Ebeiedinone, Chuanbeinone (Purapuridine), Sinpeinine A or Puqiedinone, Imperialine-3- β -glucoside and Peonidin were 104, 107, 104, 103, 106, 105, 106, 110, 104 and 104 respectively. The above-mentioned results show that quality markers in HR-LC/MS may act on the whole biological network system, rather than acting on a single target gene.

PubMed literature review validated the link of the hit compounds to the identified cancer-related 257 proteins (Table 3) where one study demonstrates that imperialine inhibits the non-small cell lung cancer both *in vitro* and *in vivo*⁵². The alkaloid peiminine and peimine inhibits the growth and motility of prostate cancer cells and induces apoptosis by disruption of intracellular calcium homeostasis through Ca_2^+ /CaMKII/JNK pathway⁵³.

Table 3
Summary of literature survey on top scoring *Fritillaria cirrhosa* constituents related to cancer

Compound	Reference	Mechanism	Reference
Imperialine	PMID: 26349052	Imperialine exerts anti-cancer effects against non-small cell lung cancer (NSCLC) both <i>in vitro</i> and <i>in vivo</i> , and this previously unknown function is related to NF-κB centered inflammation-cancer feedback loop.	52
Peiminine	PMID: 35178100; PMID: 31081133	Peimine inhibits the growth and motility of prostate cancer cells and induces apoptosis by disruption of intracellular calcium homeostasis through Ca_2^+ /CaMKII/JNK pathway. Peiminine inhibits glioblastoma <i>in vitro</i> and <i>in vivo</i> via arresting the cell cycle and blocking autophagic flux, suggesting new avenues for GBM therapy.	53,54
Peimine	PMID: 36048972	Peimine plays an anticancer role through endogenous apoptosis pathways and by inhibiting cell migration, and it has the potential to be a useful treatment for gastric cancers.	55

Table 4
Interaction network details of 09 active components

Component	Degree	Target gene
Imperialine	104	AR AKT2 AKT1 AKT3 ALK ADRA1A ADRB2 ADRB3 APP BCL2L1 ADRA2A BAD ACE CCNE2 CCND1 CHRNA4 CHRNA3 ITGA2B HSD17B3 BCHE HSD11B1 CHRM1 REN LSS SLC18A3 UGCG GBA SRD5A2 SIGMAR1 KISS1R MME PRKCA PRKG1 DRD1 INSR CNR1 TACR2 SLC1A2 PLA2G1B ROCK2 MDM2 PTGER4 PTGER2 PTGER3 DRD4 PRKCQ HTR7 HTR6 GLB1 GAA SI NR1H4 DPP4 OPRL1 GPR88 DNPEP JAK3 JAK1 OPRD1 CCR1 CCR3 NR3C1 MAN2B1 PDE10A GANAB CNR2 HSP90AA1 CXCR3 DRD2 FDFT1 RORC SREBF2 HMGCR TNF OPRM1 IDH1 CDK4 RPS6KB1 KIT HTR2A CCR2 MAP3K14 SGPL1 SMO CSF1R FUCA2 GANC GLA CDK2 CHRN4 ITGB3 CCNE1 CHRM2 GBA2 EBP PLA2G2C PLA2G10 ADRA1D MCHR1 MGAM BCL2 FKBP1A MC1R CHRN2
Verticinone or Peiminine	107	AR AKT2 AKT1 AKT3 ALK ADRA1A ADRB2 ADRB3 APP BCL2L1 ADRA2A BAD ACE CCNE2 CCND1 CHRNA4 CHRNA3 ITGA2B HSD17B3 BCHE HSD11B1 CHRM1 REN LSS SLC18A3 UGCG GBA SRD5A2 SIGMAR1 KISS1R MME PRKCA PRKG1 DRD1 INSR CNR1 TACR2 SLC1A2 PLA2G1B ROCK2 MDM2 PTGER4 PTGER2 PTGER3 DRD4 PRKCQ HTR7 HTR6 GLB1 GAA SI NR1H4 DPP4 OPRL1 GPR88 DNPEP JAK3 JAK1 OPRD1 CCR1 CCR3 NR3C1 MAN2B1 PDE10A GANAB CNR2 HSP90AA1 CXCR3 DRD2 FDFT1 RORC SREBF2 HMGCR TNF OPRM1 IDH1 CDK4 RPS6KB1 KIT HTR2A CCR2 MAP3K14 SGPL1 SMO CSF1R FUCA2 GANC GLA CDK2 CHRN4 ITGB3 CCNE1 CHRM2 GBA2 EBP PLA2G2C PLA2G10 ADRA1D MCHR1 MGAM BCL2 FKBP1A MC1R CHRN2
Verticine or Peimine	104	AKT2 AKT1 AKT3 ADRA1A AGL ADRB2 ADRB3 AURKA ADRB1 ADRA2A ALK AR CCNE2 CCND1 FNTA LSS SLC18A3 GAA GBA PLA2G1B SIGMAR1 GANAB HSD17B3 FUCA1 FUCA2 GANC GLA DNM1 IRAK1 IRAK4 MAP3K7 UGCG SI BCHE MANBA DRD2 GLB1 HSP90AA1 MC4R SSTR2 PRKG1 MAN2B1 MAOA JAK3 JAK1 CHRM1 CHRM3 SCN9A OPRL1 HTR2A PRKCD PRKCQ MC3R MDM2 DRD1 PRKCB MELK SLC6A2 DRD4 CCR1 CCR3 NPY1R MAP3K14 RPS6KA3 RPS6KB1 SRC PIK3CD KDR TTK MAPK1 ROCK2 KCNH2 HTR1A PARP1 CCR2 VDR SLC5A7 SYK MTOR PIK3CG PIK3CA CDK9 HTR7 CHEK1 MAP3K8 SOAT1 MMP2 CYP2J2 REN MAOB CDK2 CDK4 FNTB CCNE1 EBP GBA2 MGAM PLA2G2A ADRA1D MC5R MCHR1 CHRM2 HTR2B H1FO
Ebeiedine	103	ACHE AKT2 AKT1 AKT3 ALK AR ADRA1A BCL2L1 BAD ADRB2 ADRB3 APP CHRNA4 FNTA ITGA2B SIGMAR1 SLC18A3 PABPC1 CHRM1 CHRM3 KCNH2 PLA2G1B HRH3 OPRK1 CYP2D6 HTR5A SLC6A2 DRD2 CHRM4 LSS EGFR BCHE HSD17B3 ADRA1B SLC6A3 ADRA2C ADRA2B FYN PRKG1 HRH2 HTR7 DRD3 GBA SRD5A2 DRD1 PRKCD PRKCQ DNM1 INSR TACR2 HRH1 VDR FDFT1 CCR5 OPRL1 ROCK2 PDE10A HDAC1 CCR1 CCR3 MDM2 UGCG GLB1 GAA SI MAP3K14 OPRM1 HSD11B1 DRD4 TNF RPS6KB1 SHH HTR2A MAN2B1 JAK3 JAK1 SRC HSP90AA1 ATP12A BCL2L2 GANAB MANBA HTR1A GPR88 FLT3 JAK2 GC MMP2 CHEK1 CSF1R KIT FNTB ITGB3 EBP PLA2G2A CHRM2 CHRM5 GBA2 MCHR1 ADRA1D MGAM BCL2 CHRN2
Ebeiedinone	106	AR AKT2 AKT1 AKT3 ADRA1A ACHE ALK ADRA2A BAD APP CCNE2 CCND1 CHRNA4 CHRNA3 ITGA2B BCHE HSD11B1 SRD5A2 SIGMAR1 NR3C1 REN HSD17B3 MME KISS1R SLC6A3 SHBG DPP4 CHRM1 SRD5A1 HSD17B7 SLC18A3 PTGER4 PTGER2 PTGER3 PABPC1 CPB2 CCR1 TACR2 PAOX PRKCD PRKCQ CCR5 MDM2 CCR3 OPRL1 HTR7 PRKG1 DRD1 EPHX2 CNR1 NMT1 PRCP TACR1 INSR PDE10A HTR2A CYP2J2 BDKRB1 HTR1B MC4R MC3R SOAT1 PLA2G1B CTSS RORC SREBF2 GPR88 HMGCR ATP12A CXCR3 CNR2 KCNH2 SRC MAP3K14 JAK3 JAK1 JAK2 HPGDS SMO IDH1 LIMK2 PRKCA FDFT1 DRD4 CHRM3 FAAH CDK2 CDK4 CHRN4 ITGB3 CCNE1 EBP FKBP1A PLA2G2C PLA2G10 CHRM2 CPN1 MCHR1 NAAA ADRA1D HTR2B HTR1D MC5R PLA2G2A OGFR1 CHRN2
Chuanbeinone (Purapuridine)	105	AKT2 AKT1 AKT3 ALK ABCB1 ADRA2A ADRA1A ALOX5 AURKA INCENP CCNE2 CCND1 FNTA SIGMAR1 BCHE HTR2A SHH ATP12A UGCG GBA RORC CTSS ESR1 REN ESRRG L3MBTL3 DPP4 PRKG1 DRD3 INSR SREBF2 HMGCR GHSR MAP3K14 SRC PRKCD PRKCQ CHKA MC4R IRAK1 IRAK4 MAP3K7 EZH2 IGF1R GSK3B HRH1 MTOR PIK3CA ADRA2B NPY1R OPRK1 PRKCB HTR7 HTR6 HTR5A MAOA MC3R VDR ADRA1B PIK3CB CTSC MAPK8 MAPK10 MAPK9 PDE10A JAK3 HTR1A JAK1 JAK2 CTSD PIK3CG BACE1 CA2 CDK4 MDM2 TTK TNK2 DYRK1A KDR GSK3A LYN GRK2 CLK1 DYRK2 MAPK7 OPRL1 PFKFB3 ROCK2 CCR3 FLT3 IDH1 RET DRD1 AURKB CDK2 CDK4 FNTB CCNE1 EBP GBA2 MCHR1 MC5R ADRA1D MC1R
Sinpeinine A or Puqiedinone	106	AR AKT2 AKT1 AKT3 ADRA1A ACHE ALK ADRA2A BAD APP CCNE2 CCND1 CHRNA4 CHRNA3 ITGA2B BCHE HSD11B1 SRD5A2 SIGMAR1 NR3C1 REN HSD17B3 MME KISS1R SLC6A3 SHBG DPP4 CHRM1 SRD5A1 HSD17B7 SLC18A3 PTGER4 PTGER2 PTGER3 PABPC1 CPB2 CCR1 TACR2 PAOX PRKCD PRKCQ CCR5 MDM2 CCR3 OPRL1 HTR7 PRKG1 DRD1 EPHX2 CNR1 NMT1 PRCP TACR1 INSR PDE10A HTR2A CYP2J2 BDKRB1 HTR1B MC4R MC3R SOAT1 PLA2G1B CTSS RORC SREBF2 GPR88 HMGCR ATP12A CXCR3 CNR2 KCNH2 SRC MAP3K14 JAK3 JAK1 JAK2 HPGDS SMO IDH1 LIMK2 PRKCA FDFT1 DRD4 CHRM3 FAAH CDK2 CDK4 CHRN4 ITGB3 CCNE1 EBP FKBP1A PLA2G2C PLA2G10 CHRM2 CPN1 MCHR1 NAAA ADRA1D HTR2B HTR1D MC5R PLA2G2A OGFR1 CHRN2
Imperialine-3- β-glucoside	110	ADORA1 ADORA3 ADK ADRB3 ADRB2 ADRB1 AGTR1 ACE ADORA2A AKT2 AKT1 AKT3 AURKA ANPEP FNTA NCOR2 ITGB1 ITGB7 ITGB1 ITGB5 ITGAV ITGA2B SLC5A2 SLC5A1 KCNH2 SI PIK3CA MLNR GLHSR OPRD1 CHEK1 METAP2 BACE1 ESR2 TPSAB1 TYMS XIAP MAOA MAOB F10 ECE1 GRIK1 DPP4 PLAUF PTGFR PTPN1 OPRK1 IDE RORC PDE5A HTR1A CNR1 SLC6A4 CNR2 NTRK1 FYN HRH3 SIRT2 TOP2A ERBB2 DHFR DRD4 MMP2 GABRA5 CTSD CDK7 PRSS1 SYK LNPEP BMP1 HDAC6 HDAC2 HDAC5 ITGA4 HDAC11 FLT3 CAMK2D JAK2 PRSS3 DRD2 DRD1 CDC7 IMPDH1 IMPDH2 MMP3 MMP1 S1PR1 HRH2 MMP13 MNK2 MMP8 EDNRA FNTA FNTB HDAC3 ITGA4 ITGA5 ITGAV ITGB1 ITGB3 PGGT1B MGAM HTR4 PTGDR2 HDAC10 HLA-DRB1 YARS

Component	Degree	Target gene
Peonidin	104	ABCG2 AKR1B1 ADORA3 ABCB1 ALOX5 ACHE ADORA1 ALOX15 ABCC1 ALK AKT1 APEX1 AKR1C2 AKR1C1 AKR1C3 AKR1C4 ADORA2A AKR1A1 ARG1 APP AHR CCNB3 CDK5R1 F2 NR1H3 XDH CA2 CA12 CA4 CYP1B1 CD38 NOX4 MAPT GPR35 AVPR2 TOP2A MAOA IGF1R FLT3 CYP19A1 INSR EGFR PIM1 AURKB DRD4 MYLK MPO PIK3R1 DAPK1 PYGL SYK CA1 GSK3B SRC PTK2 HSD17B2 KDR MMP13 MMP3 CA3 PLK1 CDK1 MMP9 PIK3CG MMP2 PKN1 CA14 CA9 CSNK2A1 MET NEK2 CXCR1 CAMK2B NEK6 PLA2G1B BACE1 AXL NUA1 CA13 ESR1 SIRT1 CDK6 CDK2 PLG TERT OPRD1 ESR2 ST6GAL1 TYR HSD17B1 ESRRA PTGS1 PDE4D CDK1 CDK5 CCNB1 CCNB2 GLO1 CA7 KDM4E CA6 CA5A PLA2G2A PDE4B

3.5 Preparation Of Drug-like Dataset

After applying the Lipinski's Rule of 5, the compounds identified from the HR-LC/MS were evaluated for drug likeliness properties using Swiss ADME software (Table 5). Further, these compounds were selected for molecular docking to assess the binding affinity with many breast cancer targets at the active site of the target.

Table 5
ADMET screening of phytoconstituents identified in *Fritillaria cirrhosa*

Compound name	Pubchem ID	Molecular weight	H/A	H/D	R/B	TPSA (Å)	ADMET	GI	L.R	BBB	CYP2D6	Solubility
Imperialine	442977	429.6	4	1	0	60.77	Yes	High	Yes	Yes	No	Moderately soluble
Verticinone or Peiminine	167691	429.6	4	2	0	60.77	Yes	High	Yes	Yes	No	Moderately soluble
Imperialine-3- β -glucoside	132538	591.8	9	5	3	139.92	Yes	High	No	No	No	Moderately soluble
Sinpeinine A or Puqiedinone	126149	413.6	3	1	0	40.54	Yes	High	Yes	Yes	No	Moderately soluble
Ebeiedine	101324888	415.65	3	2	0	43.7	Yes	High	No	Yes	No	Poorly Soluble
Ebeiedinone	764778	413.6	3	0	4	23.55	Yes	High	Yes	Yes	Yes	Moderately soluble
Chuanbeinone	5315861	399.61	3	1	0	40.54	Yes	High	No	Yes	No	Moderately soluble
Verticine or Peimine	131900	431.65	4	3	0	63.93	Yes	High	Yes	Yes	No	Moderately soluble
Peonidin	441773	301.27	6	4	2	103.29	Yes	High	Yes	No	No	Moderately soluble
Imperialine	442977	429.6	4	1	0	60.77	Yes	High	Yes	Yes	No	Moderately soluble
Verticinone or Peiminine	167691	429.6	4	2	0	60.77	Yes	High	Yes	Yes	No	Moderately soluble
Imperialine-3- β -glucoside	132538	591.8	9	5	3	139.92	Yes	High	No	No	No	Moderately soluble

3.6 Molecular docking studies of hit compounds in the active sites of AKT1, TNF, SRC, EGFR, ESR1 and CDK2 Proteins Virtual screening studies were performed to decipher the binding aspects of compounds identified by HR-LC/MS with top 30 breast cancer drug targets. Out of nine compounds, Peiminine showed best docking score with CDK2 protein. The images of docked complexes, molecular surfaces, 2D and 3D interactive plots for peiminine with CDK2 protein are shown in Fig. 9. Molecular docking studies revealed that the peiminine bound significantly with protein CDK2 with lowest binding energy – 12.99 kcal/mol (Table 6). The molecular surface view displayed deeply bound ligand in the saddle shaped core of binding cavity (Fig. 9 (I)). The ribbon structure displayed the ligand buried inside the cavity (Fig. 9 (II)). From 2D and 3D plots it is depicted that ligand peiminine formed alkyl interaction with Val18, Ala31, Leu134, Ala144 and conventional hydrogen bonds with Lys33 residue (Fig. 9 (II)). Other non-bonded interactions such as van der Waal's interactions are also depicted Fig. 9 (II). All the binding energy scores are calculated from the best cluster (95%) that fall within the lowest RMSD 0.25 Å. Therefore, from the docking studies it can be suggested that peiminine has high affinity for the protein CDK2 and further considered for MD simulation studies.

Table (6): Binding energy obtained from the docking calculations of tested compounds with AKT1, TNF SRC, EGFR, ESR1 Protein, CDK2 Proteins

Compound Name	AKT1 Protein Energy score (kcal/mol)	TNF Protein Energy score (kcal/mol)	SRC Protein Energy score (kcal/mol)	EGFR Protein Energy score (kcal/mol)	ESR1 Protein Energy score (kcal/mol)	CDK2 Protein Energy score (kcal/mol)
Imperialine	9.87	-3.46	-7.73	-10.11	-8.38	-6.19
Peiminine	-11.1	-7.5	-10.2	-11.1	-7.5	-12.99
Peimine	-10.1	-6.6	-9.5	-10.1	-9.4	-6.7
Ebeiedine	-8.9	-6.6	-8.6	-10.1	-9.3	-8.9
Ebeiedinone	10.2	-7.6	-9.7	-10.1	-6.8	-9.7
Chuanbeinone	-10	-6.9	-10	-7.9	-9.3	-7.9
Sinpeinine A	-9.8	-7.2	-9.4	9.5	-7.4	-8.9
Peonidin	9.7	-8	-9.5	9.3	-7.2	-10.1
Imperialine-3- β -glucoside	-11.02	-7.59	-8.52	-9.58	-12.05	-11.7

3.7 Molecular Dynamics Simulation

Molecular dynamics and simulation (MD) studies were carried out in order to determine the stability and convergence of CDK2 + Peiminine complex. Each simulation of 100 nanoseconds (ns) displayed stable conformation while comparing the root mean square deviation (RMSD) values. The C α -backbone of CDK2 + peiminine complex exhibited a deviation of 2.8 Å (Fig. 10A), while complex 3 exhibited a deviation of 2.0 Å (Fig. 10A, black), while the ligand RMSD is little high 3.1 Å. RMSD plot is within the acceptable range signifying the stability of ligand bound state before and after simulation and it can also be suggested that the complex is quite stable due to higher affinity of the ligand. The plots for root mean square fluctuations (RMSF) displayed significant spike of fluctuation (5.9 Å) at amino acid residues 40–60 in protein while the rest of the residues less fluctuating during the entire 100 ns simulation (Fig. 10B). The higher fluctuating residues are due to loop and turn conformation. Therefore, for RMSF plot it can be suggested that the protein structure is stable during simulation and having flexible regions for acquiring best conformations. Radius of gyration is the measure of compactness of the protein. Here in this study, protein forming complex with ligand peiminine displayed less fluctuating radius of gyration (Rg) from 20.01 to 19.89 and became stable (Fig. 10C). The overall quality analysis from RMSD and Rg it can be suggested that peiminine bound to the protein targets posthumously in the binding cavities and played a significant role in stability of the proteins. Number of hydrogen bonds formed between protein and ligand is an important factor to analyze for a stable complex throughout the simulation time. Here in this case, the number of H-bonds formed in complex displayed constant interactions with an average of 3 numbers at the end of 100 ns simulation (Fig. 10D). The stabilization of the ligand must be maintained via weak non-bonded interactions along with Hydrogen bonds.

Followed by Rg analysis, similar patterns were also observed in solvent accessible surface area (SASA) in both ligand bound and unbound state. It is clearly visible from the Fig. 10E that in the unbound state of Peiminine to protein CDK2 displayed high surface area accessible to solvent (Fig. 10E, red). The SASA value lowered as compared to unbound state while bound with CDK2 (Fig. 10E, black). The overall study of Rg signifies the ligands binding compel the respective proteins to become more compact and less flexible.

3.8 In vitro cytotoxicity assay: MTT assay on breast cancer cell lines

Graph 1 shows the cell viability (%) of various breast cancer cell lines; MDA-MB-231, MCF-7, MDA-MB-468, and 4T1 when treated with different concentrations of petroleum ether, ethyl acetate, and methanolic extracts of *F. cirrhosa*. The IC₅₀ values of petroleum ether, ethyl acetate, and methanolic extracts against various breast cancer cell lines are shown in Table 7. The ethyl acetate extract of the plant had shown a maximum cytotoxic effect on MDA-MB-231 with an IC₅₀ value of 83.7 µg/mL, followed by methanolic and petroleum ether extract with IC₅₀ values of 141.3 and 164.9 µg/mL, respectively. The ethyl acetate extract of the plant had shown a maximum reduction in the growth of the MCF-7 breast cancer cell line with the lowest IC₅₀ value of 95.75 µg/mL, followed by petroleum ether and methanolic extract with IC₅₀ values of 109.8 and 150.2 µg/mL respectively. The ethyl acetate extract showed maximum antiproliferative potential against the

MDA-MB-268 cell line with an IC₅₀ value of 110 µg/mL. Methanolic and petroleum ether extracts have shown less antiproliferative potential against MDA-MB-268 with IC₅₀ values of 224.7 and 216.2 µg/mL, respectively. The ethyl acetate extract is highly specific to 4T1 cell lines with an IC₅₀ value of 95.23 µg/mL, followed by methanolic and petroleum ether extract with IC₅₀ values of 172.1 and 308.7 µg/mL, respectively. Hence ethyl acetate extract of *F. cirrhosa* had showed the highest anticancer activity against the MDA-MB-231, MCF-7, and MDA-MB-468 and 4T1 cell lines as compared to other extracts.

Table 7
IC₅₀ values in µg/mL of various extracts of *F. cirrhosa* against different breast cancer cell lines.

Extracts	MDA-MB-231	MCF-7	MDA-MB-468	4T1
Petroleum ether	164.9	109.8	216.2	308.7
Ethyl acetate	83.7	95.75	110	95.23
Methanol	141.3	150.2	224.7	172.1

Discussion

Breast cancer is the leading cause of death in women ⁵⁶. There is still no effective treatment for the vast majority of patients with advanced breast cancer, despite the fact that current therapies have shown some promise against the disease ⁵⁷. It is necessary to develop efficient treatments to prevent, delay, or reverse the occurrence of breast cancer in high-risk women. Natural products are better at chemoprevention of breast cancer than synthetic ones because they have fewer adverse effects and lower toxicity ¹⁵. Natural product research has attracted a lot of attention recently for the treatment of breast cancer. Based on the network pharmacology we explored the molecular mechanism of the therapeutic effects of *F. cirrhosa* on breast cancer. Network pharmacology analysis aids in understanding the complex interactions that occur between drugs and their targets, as well as the likely mechanisms of action.

In the present study many secondary metabolites were identified using HR-LC/MS of *F. cirrhosa* of which 09 phytocompounds propose a probable mechanism against oxidative stress and breast cancer. 359 potential target genes were obtained for the 9 quantitative phytocompounds from HR-LC/MS. Out of which, 326 represented breast cancer targets which were found to be involved in top 20 signalling pathways. Among 326 breast cancer targets predicted, 30 were identified as a major druggable targets. The compound-genes network revealed that the selected compounds of *F. cirrhosa* had a high binding affinity with target genes of breast cancer and could be used as the potential target genes for treating breast cancer. On the basis of degree value of compounds in the network, we obtained the Imperialine-3-β-glucoside compound as most active ingredient of *F. cirrhosa*. However, the degree of other compounds also found high which might indicate that quality markers may act on the whole biological network system, rather than acting on single gene.

The Gene ontology (GO) enrichment and KEGG pathway enrichment further revealed the associations with disease occurrence and development. Analysis of top 20 pathways; Pathways in cancer, Metabolic pathways, Neuroactive ligand-receptor interaction, PI3K-Akt Signaling Pathway, Focal adhesion, Proteoglycans in Cancer, Calcium Signaling Pathway, Human Papillomavirus Infection, Chemical Carcinogenesis, CAMP Signaling Pathway, Micromas in Cancer, Lipid and Atherosclerosis, Human Cytomegalovirus Infection, Epstein Barr virus Infection, Viral Carcinogenesis, Human Immunodeficiency Virus 1 Infection, Chemokine Signaling Pathway, MAPK Signaling Pathway, Endocrine Resistance and Ras signaling pathway suggested the direct contribution in cancer disease. This suggested that the effect of phytocompounds for treating breast cancer may act on multiple pathways, as well as complex interactions among these pathways. Based on the frequency of each gene in compound-gene network AKT1 exhibited high frequency of protein interaction, followed by TNF, SRC, EGFR, CDKs, ESR1 and other target genes. The gene *AKT1* is recognized to administer the breast cancer and the PI3K-Akt signaling pathway is known to be altered in breast cancer. Its activation leads to the amplified tumor growth and reduced survival. The identification of highly implicated gene and pathway from this study, corroborate the previous findings along with the potential active compounds against these genes. Imperialine as a promising novel anti-cancer compound against NSCLC both *in vitro* and *in vivo* approaches ⁵². The natural product peiminine represses various types of cancers including colorectal, lung cancer, gastric cancer etc as reported in previous studies ^{58–60}. Peiminine has been previously demonstrated to reduce the viability of HepG2 cells in a time- and dose-dependent manner and had an IC₅₀ of 4.58 µg/mL at 24h. The result suggests that peiminine can induce apoptosis in human hepatocellular carcinoma HepG2 cells through both extrinsic and intrinsic apoptotic pathways ⁶¹. Peiminine exhibits its anticancer activity by inhibits glioblastoma *in vitro* and *in vivo* through cell cycle arrest and autophagic flux blocking ⁵⁴. However, the role of peiminine in supressing breast cancer is poorly understood.

Additionally, using AutoDock version 4.2.6, an in-silico docking analysis of the nine most prevalent compounds was carried out against 30 top breast cancer targets (Fig. 5B). While open-source drug design tools like AutoDock, which also meet the primary criteria of the Open-Source Initiative, have sped up the drug research and development process⁶². All tested compounds showed promising results against CDK2 proteins based on docking score. This is consistent with previous studies, which revealed the importance of 3-hydrazonoindolin-2-one scaffold. The molecular docking studies confirms that 3-hydrazonoindolin-2-one scaffold inhibits the CDK2. Out of nine compounds, peiminine and imperialine 3-glucoside bioactives showed the best docking results. All bioactives fit comfortably into the binding sites on CDK2 protein and interact favourably with critical amino acid residues.

Cyclin-dependent kinases (CDKs), a group of regulatory complexes that phosphorylate cell cycle substrates, are necessary for cell cycle progression. CDKs enzymes play a key role in regulating the cell cycle development through G1-phase^{63–66}. Previous research showed that, in comparison to normal cells, these enzymes are also overexpressed in a variety of human malignancies, including breast cancer⁵⁷. Therefore, inhibiting CDKs is a remarkable goal in searching for breast cancer targeted therapies. Also, the stability of the representative CDK2 + Peiminine complex was further explored using molecular dynamics simulations. The RMSD plots show that the MD results showed stable patterns throughout the entire simulation run (Figs. 10A and 10B). The results revealed that the compounds did not introduce any abnormal behaviour into the complex system. The small spikes seen during RMSF may be due to the protein loop region's increased flexibility. The majority of RMSF values were seen to fall within the acceptable range over the course of 100 nanoseconds. The RMSF plots suggest that CDK2 has a lot of flexibility to accommodate the ligand at the binding pocket. The protein remained compact throughout the simulation process, as shown by the Rg plots. According to the average results observed, the protein backbone was compact. To understand how the residues behaved throughout the simulation run, the fluctuations of the residues were analyzed. Additionally, after ligand binding, the target's surface area that was accessible to solvent decreased. Additionally, the compound SMILES id was obtained from the ChemSpider to determine the compound's originality⁶⁷. The results showed that peiminine compound had not been tested for CDK2 previously. Furthermore, our study further aimed to investigate the *in vitro* cytotoxic potential of active extracts of *F. cirrhosa*. The main goal of cancer chemotherapy is to target cancer cells without exhibiting toxicity to normal cells and this is a limitation of the use of current chemotherapy agents⁶⁸. In the present study, Ethyl acetate extract of *F. cirrhosa* had showed the highest anticancer activity against the MDA-MB-231, MCF-7, and MDA-MB-468 and 4T1 cell lines as compared to other three extracts. This is consistent with the previous study, which revealed that alkaloids possess significant antitumor activity^{69,70}. Previous reports also revealed that alkaloids of *F. cirrhosa* displays significant anti-inflammatory activity and attenuates acute lung injury⁷¹. The results of network pharmacology and docking studies revealed that these 09 phytochemicals could be employed to treat breast cancer due to their capacity to bind stably to core targets. In this regard, modern high throughput techniques, including chromatography can be utilized; liquid chromatography and mass spectrometry can aid in the resolution of this problem. Even though we have provided some promising information, additional study and clinical trials are necessary to properly evaluate potential of bioactives of *F. cirrhosa* and validate its medical applications.

Conclusion

In the current study, we explore the potential mechanisms of phytochemicals in inhibiting breast cancer through a combination of bioinformatics analysis, network pharmacological prediction, and experimental verification *in vitro* experiments. We described molecular interactions between a protein involved in the pathogenesis of breast cancer and bioactive components from *F. cirrhosa*. It was found that Peimine, Imperialine 3-glucoside, and other important phytochemicals of *F. cirrhosa* efficiently binds with CDK2, indicating their role in inhibiting CDK2 and other protein targets in breast cancer.

Abbreviations

HR-LC/MS: High Resolution Liquid Chromatography Mass Spectrometry

STRING: Search Tool for the Retrieval of Interacting Genes/Proteins

CDKs: Cyclin Dependent Kinases

RCSB: Research Collaboratory for Structural Bioinformatics

PDB: Protein Data Bank

LGA: Lamarckian Genetic Algorithm

MD: Molecular Dynamic Simulation

RMSD: Root Mean Square Deviation

RMSF: Root Mean Square Fluctuation

OPLS: Optimized Potentials for Liquid Simulations

RG: Radius of Gyration

SASA: Solvent Accessible Surface Area

DPPH: 2,2-diphenyl-1-picrylhydrazyl

MTT: (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)

GO: Gene ontology

KEGG: (Kyoto Encyclopaedia of Genes and Genomes)

Declarations

CRedit authorship contribution statement

Corresponding author (Manzoor Ahmad Mir): Conceptualization, Investigation, Supervision, Visualization, and Analysis of Results and Review.

Basharat Ahmad Bhat: Writing-original Draft, Perform Docking Study, and Review.

Wajahat Rashid Mir: Antioxidant assay, docking and review.

Mustfa Alkhanani: Network Pharmacology and Review

Abdullah Almilaibary: Network Pharmacology and Review

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Declaration of competing interest

The authors declare that they have no conflict of interest financial or otherwise

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Graphs

Graph 1 is available in the Supplementary Files section

Figures

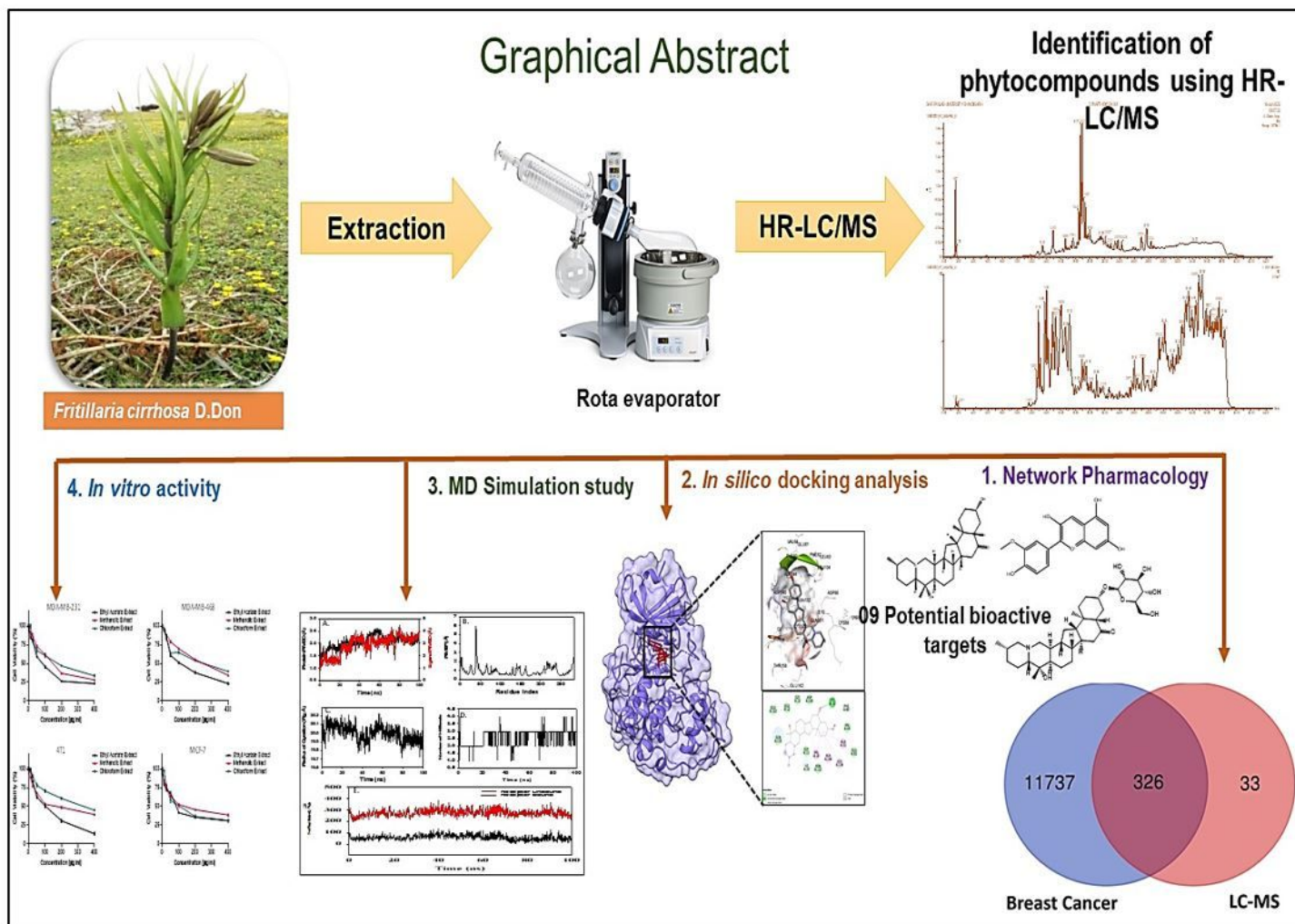


Figure 1

Overall flow chart of the study

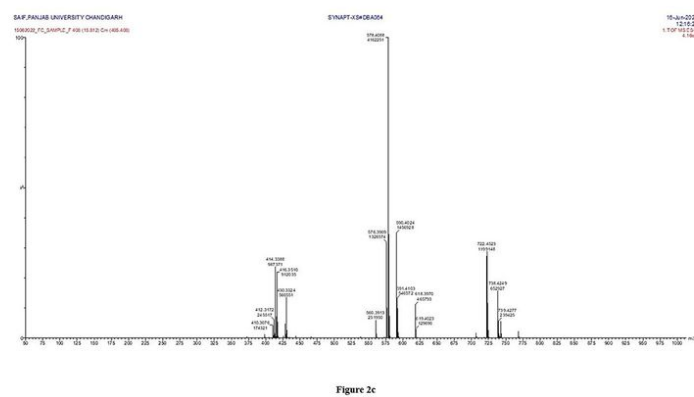
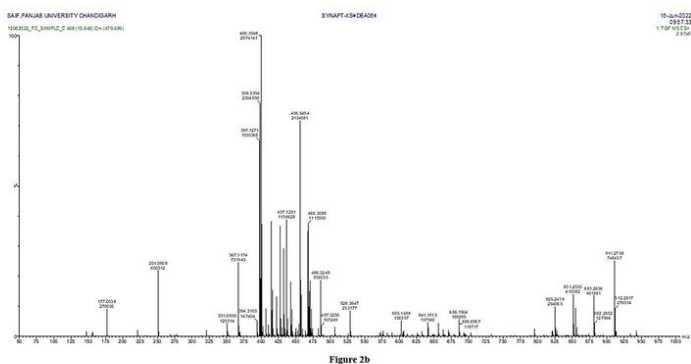
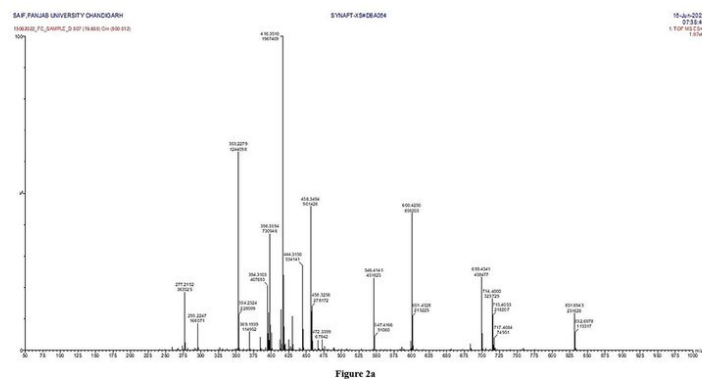


Figure 2

2a. HR-LC/MS chromatogram of methanolic extract in positive mode of *F. cirrhosa*

2b. HR-LC/MS chromatogram of Ethyl acetate extract in positive mode of *F. cirrhosa*

2c. HR-LC/MS chromatogram of Petroleum ether extract in positive mode of *F. cirrhosa*

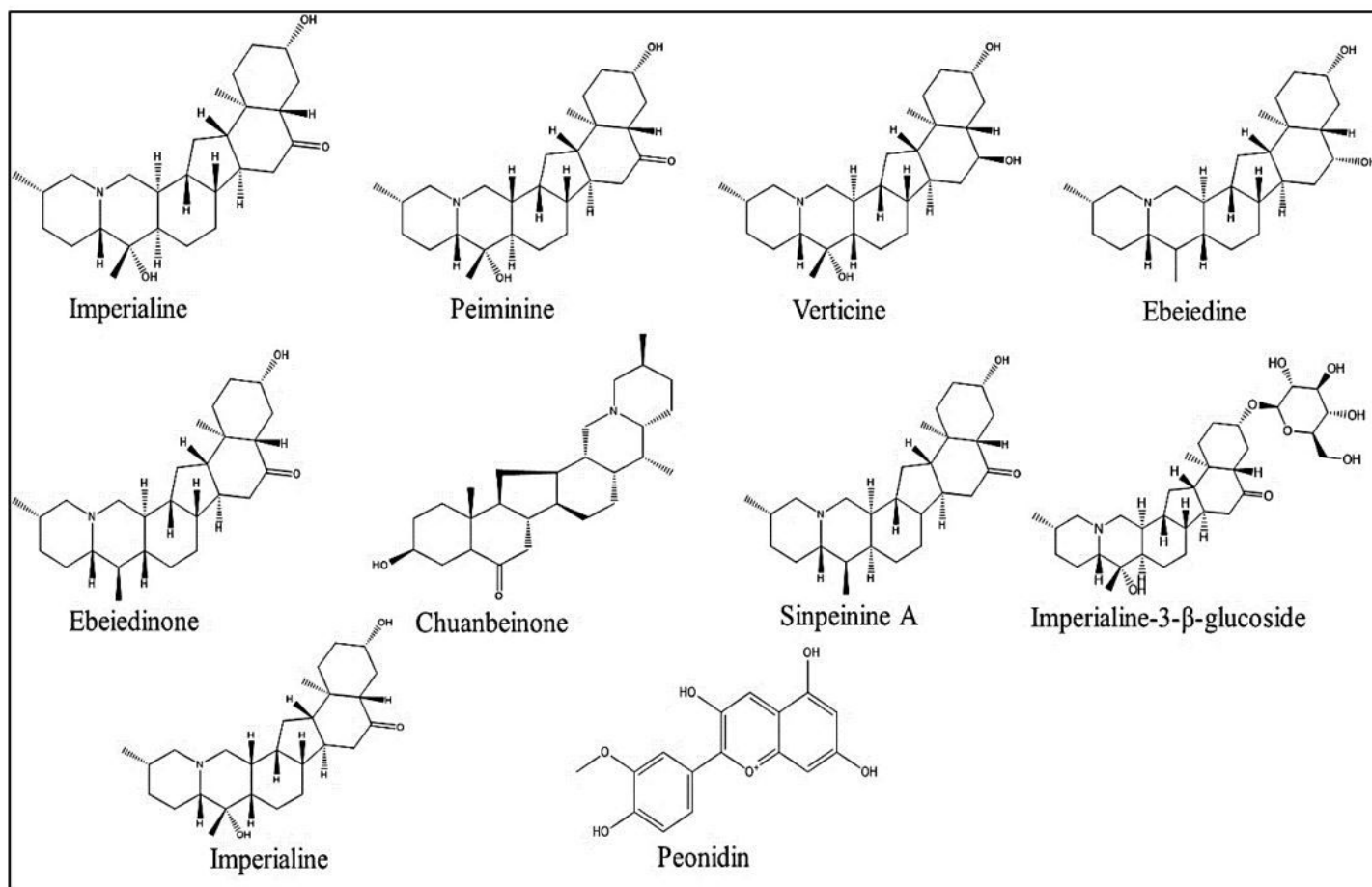


Figure 3

Structures of the compounds identified based on HR-LC/MS from *F. cirrhosa* drawn by ChemDraw Pro 16.0 Suite (Perkin Elmer, USA).

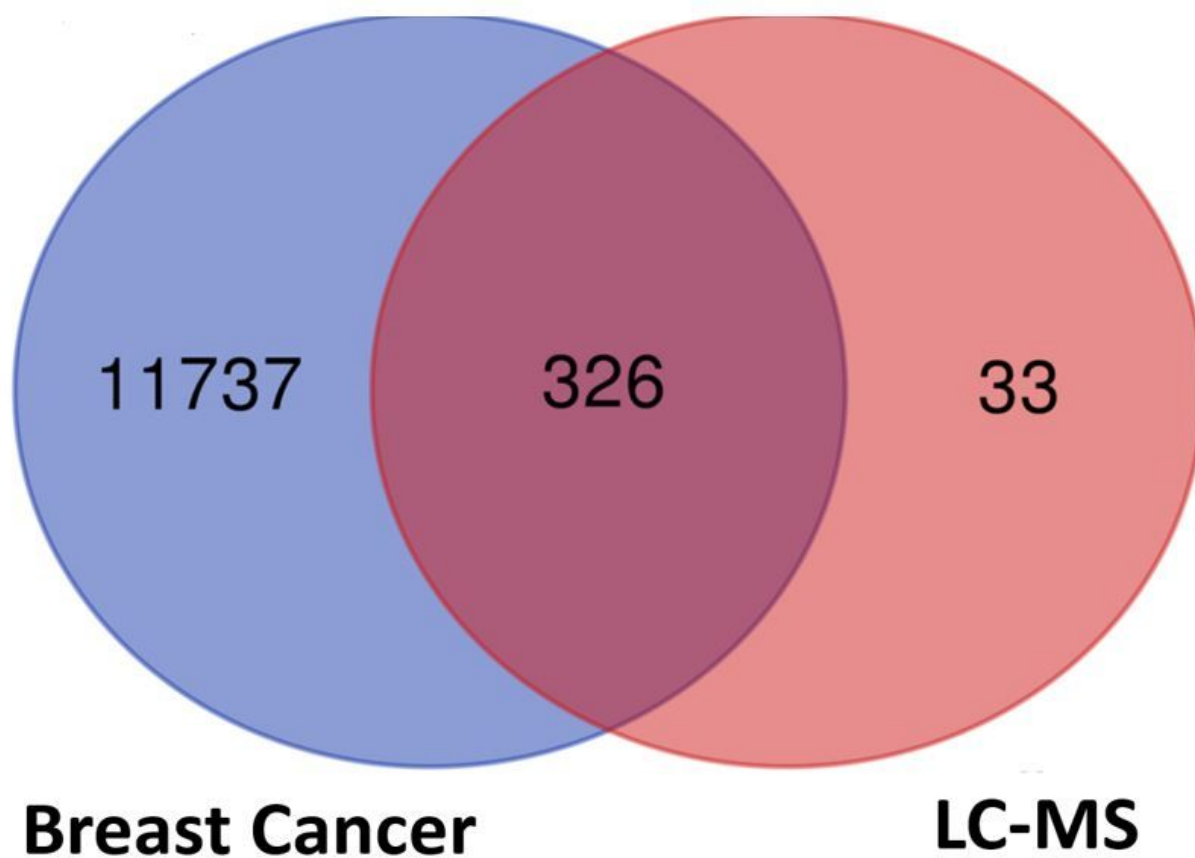


Figure 4

Venn diagram represents common target genes for compound prescription and disease. The size denotes the number of the target genes, the blue circle symbolizes the target genes of breast cancer, the red circle symbolizes the target genes of 09 quantitative phytocompounds detected by HR-LC/MS, and the coincident part depicts the common target genes.

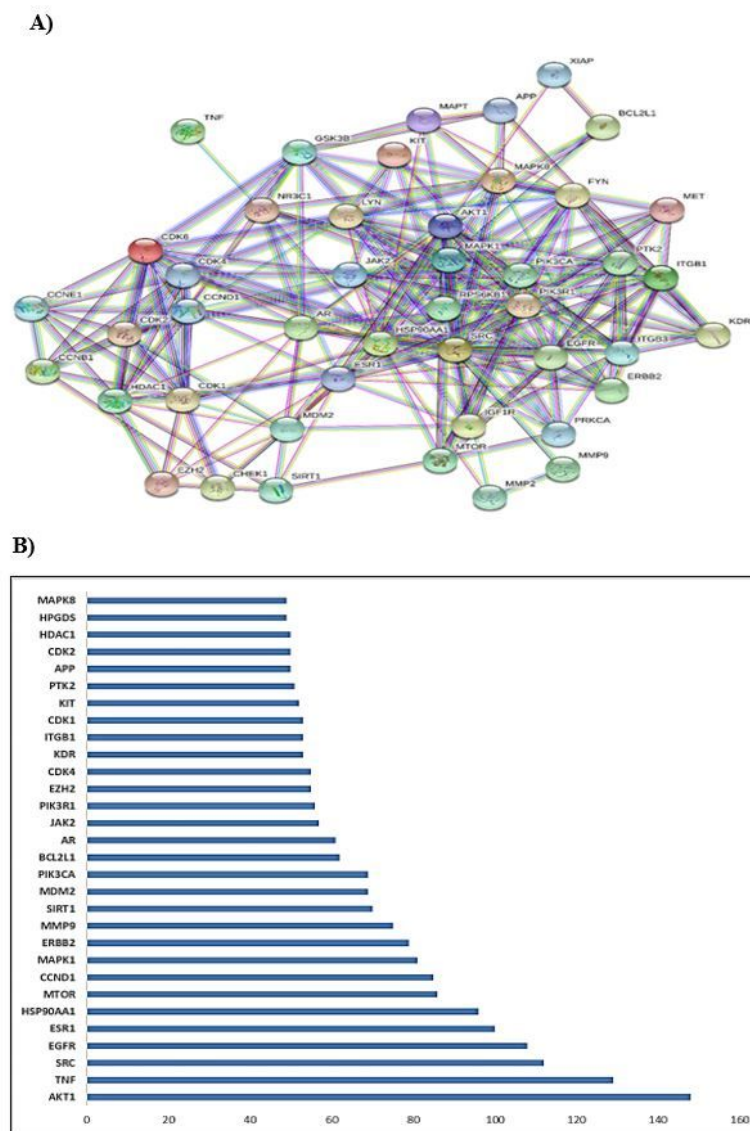


Figure 5

The result of the common target genes network interaction. **(A)** PPI network of the common target genes. The nodes represent target genes; the stuffing of the nodes represent 3D structure of target genes; the edges represent target genes-target genes associations; the colours of the edges represent different interactions; cyan and purple represent known interactions; green, red, and blue purple represent predicted interactions; chartreuse, black, and light blue represent others.

(B): Frequency of the top 30 common target genes.

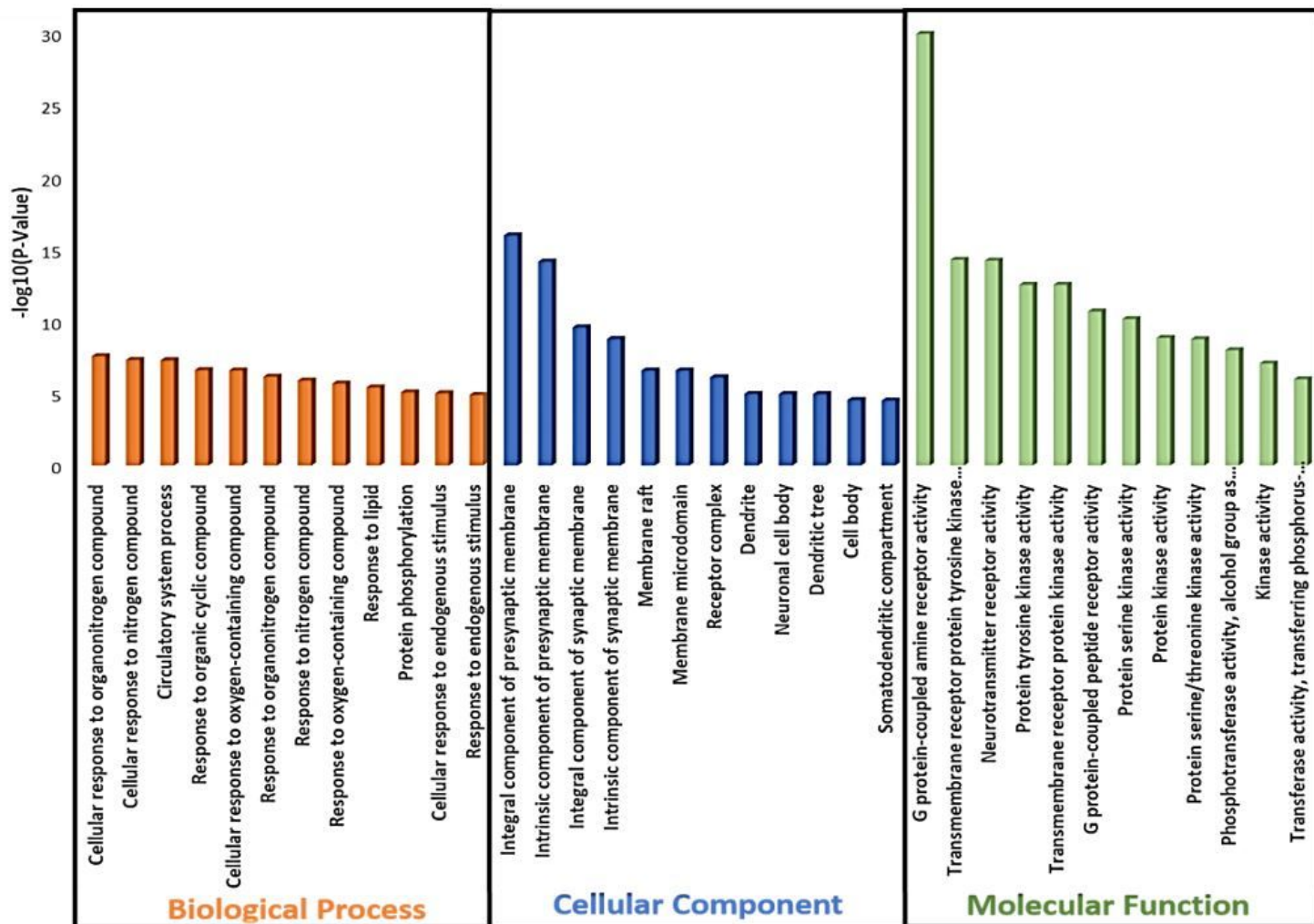


Figure 6

GO (Biological process, molecular function and cellular component) analysis (top 20). The node length represents the number of target genes enriched, and the node colour from blue to red represents the P value from large to small.

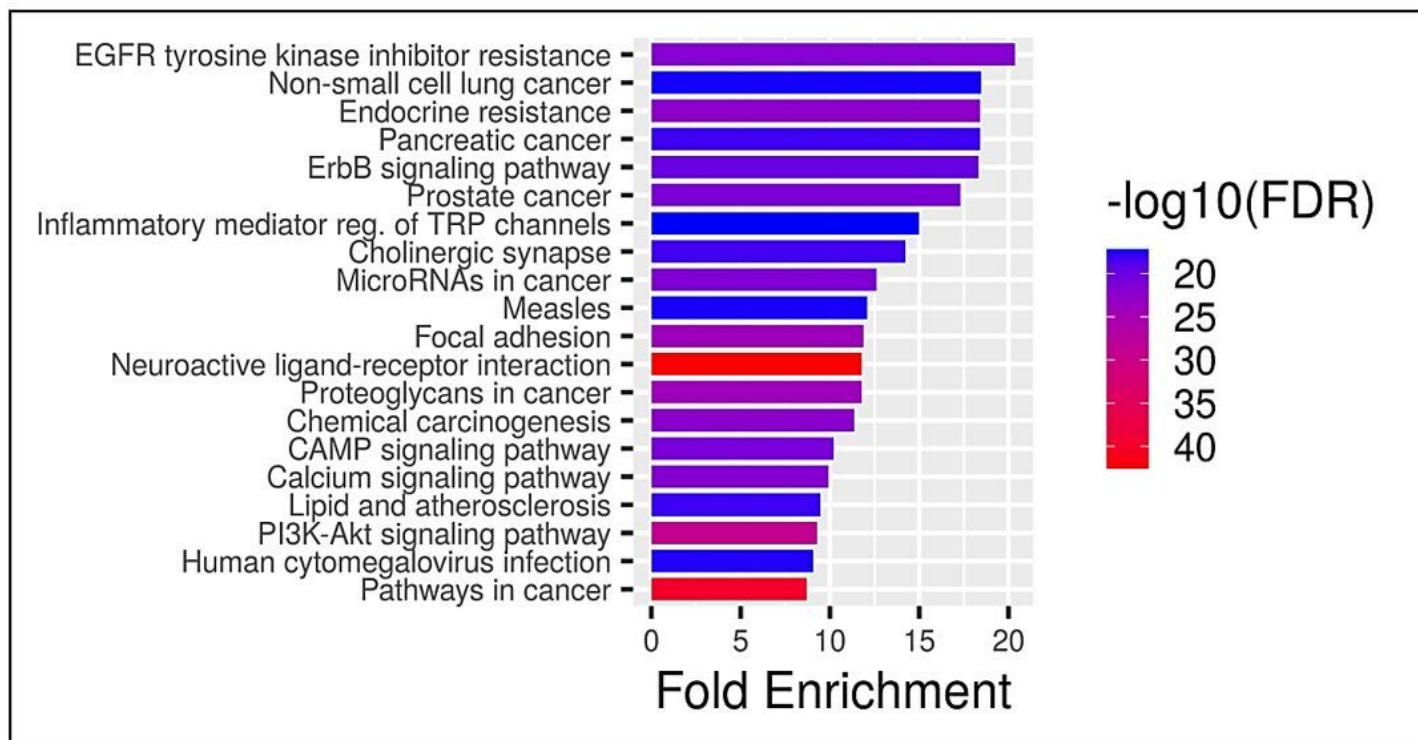


Figure 7

KEGG pathway enrichment analysis (top 20). The node size represents the number of target genes enriched, and the node colour from blue to red represents the P value from large to small.

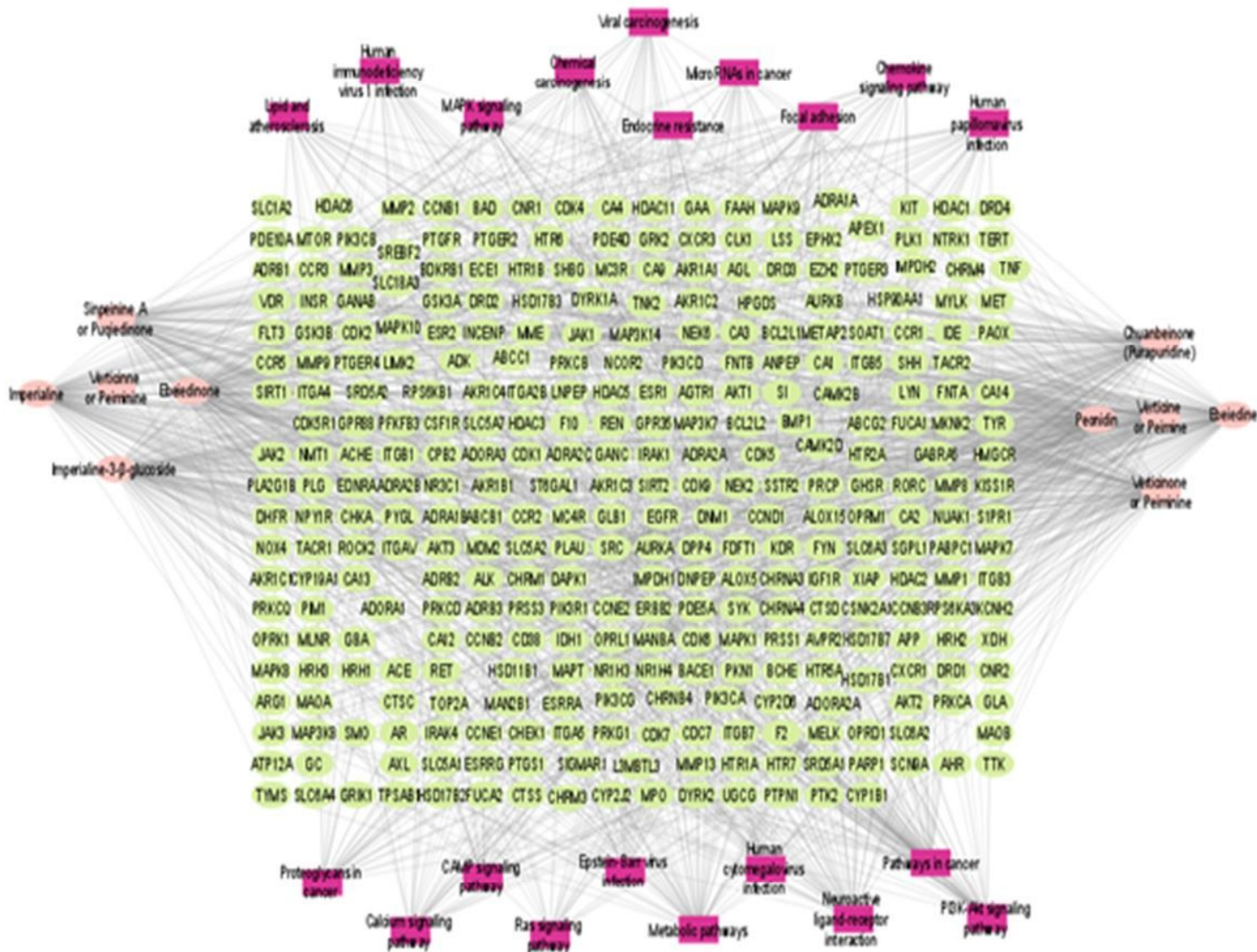


Figure 8

Compound prescription-active component-disease-target gene. The node colors represent different groups: purple represents the components and blue represents target genes. Component node size represent the degree; the degree from low to high represents the node size from small to large

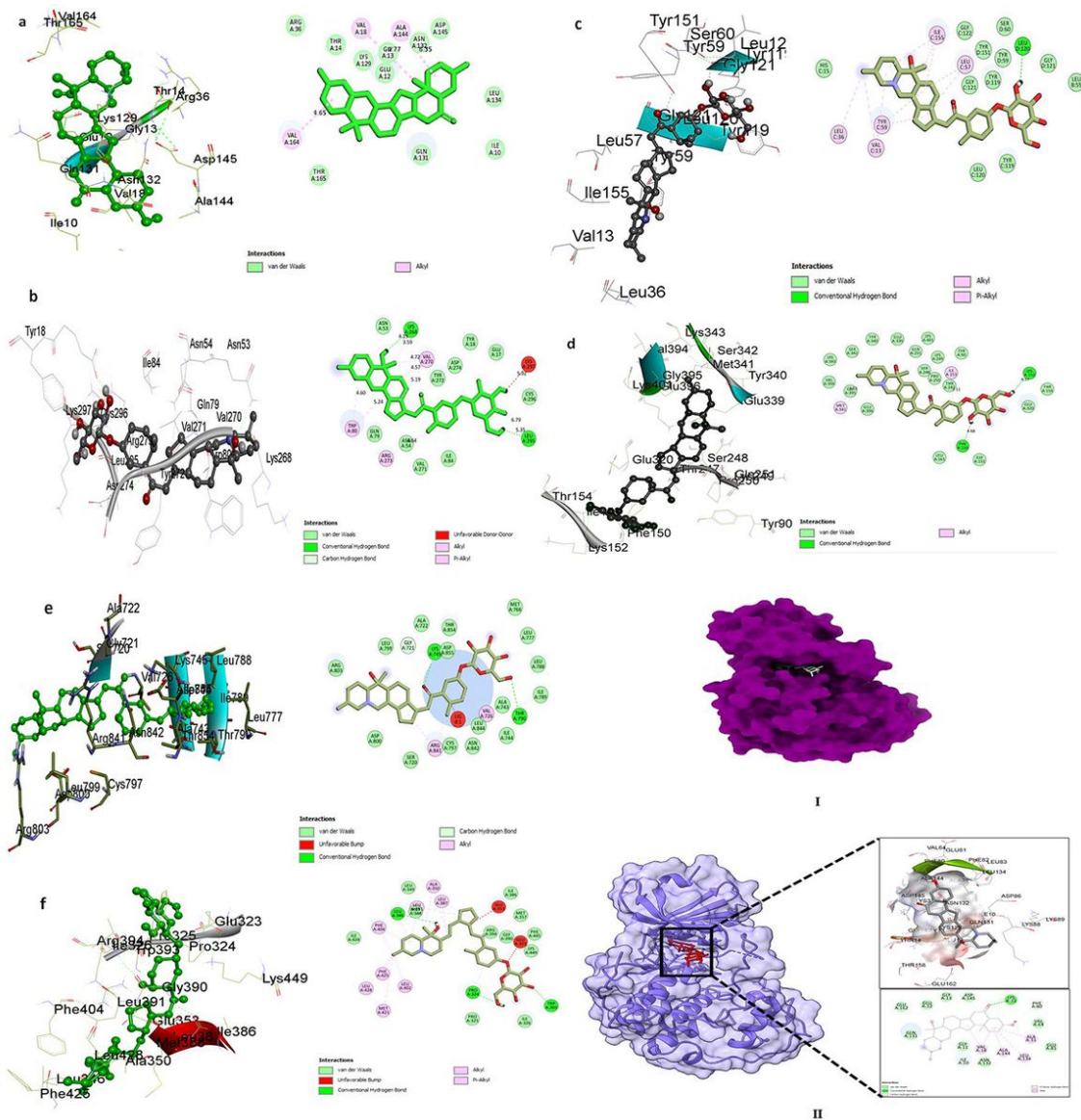


Figure 9

2D and 3D representation of predicted binding mode for compounds (a) Peiminine with CDK2 (b) Imperialine-3-β-glucoside with AKT1 (c) Imperialine-3-β-glucoside with TNF proteins active sites.

2D and 3D representation of predicted binding mode for compounds (d) Imperialine-3-β-glucosidewith SRC (e) Imperialine-3-β-glucosidewith EGFR (f) Imperialine-3-β-glucosidewith ESR1 proteins active sites.

Molecular surface view of CDK2 exhibiting the deep core accommodating the ligand peiminine (I). (II) Complex view rendered in ribbon displayed the ligand binding at the core (left), 3D ligand interaction with the binding cavity residues (up) and 2D interaction with non-bonded interactions (down)

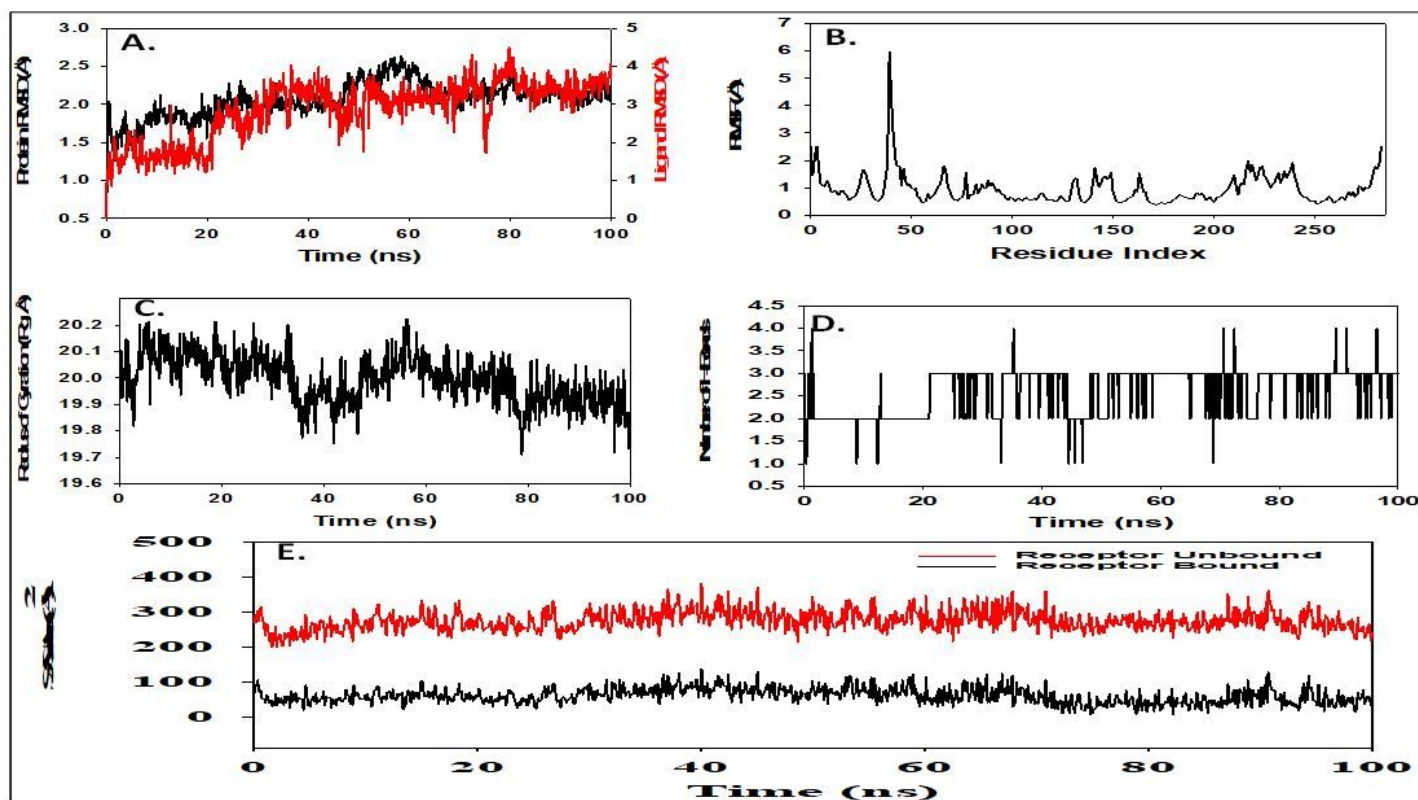


Figure 10

Analysis of MD simulation trajectories of 100 ns time scale. (A) RMSD plot displaying the molecular vibration of Ca backbone of CDK2 (black) and Peiminine (red). (B) RMSF plot showing the fluctuations of respective amino acids throughout the simulation time 100 ns for CDK2 bound to Peiminine. (C) Radius of gyration plots for the deduction of compactness of protein CDK2+Peiminine. (D) Number of hydrogen bonds formed between CDK2+Peiminine during 100 ns simulation time scale. (E) Solvent accessible surface area (SAS Area) displaying the unbound area at the binding pocket (Red) and bound CDK2+Peiminine (Black).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Graph1.jpg](#)