Pharmacogn. Res., 2023; 15(4):716-726.
https://www.phcogres.com

Original Article

Phytochemical Crosstalk in Indian Gooseberry Preparation to Explain Therapeutic Potentials of Dietary Supplements

Md Rafi Uz Zama Khan, Vishal Trivedi*

Department of Biosciences and Bioengineering, Malaria Research Group, Indian Institute of Technology-Guwahati, Guwahati, Assam, INDIA.

ABSTRACT

Background: The therapeutic properties of phytochemicals found in medicinal plants, as well as in dietary plants and herbs, have had a significant impact on the development of several herbal remedies for a range of ailments. *Amalaki churna*, a well-known ayurvedic composition, has been traditionally used to treat a number of disorders and has been shown to have anti-inflammatory, anti-diabetic, and anti-cancer properties, among others. The presence of large number of phytochemicals in this ayurvedic formulation seems to be responsible for its diverse actions. However, little is known about the precise molecular interaction between phytochemicals and their protein targets. This study puts forward a methodical and structured approach to determine the targets for the phytochemicals recovered from the aqueous extract of *Amalaki churna* and to establish the phytochemical-protein cross talk occurring at the molecular level. **Materials and Methods:** Various phytochemicals were identified from AMCAE using spectroscopic techniques such as HPLC, NMR (^{1}H, ^{13}C, and 2D NMR), and UPLC-MS analysis. The phytochemical-protein cross talk was discovered utilizing network-based pharmacology, with phytochemicals serving as core nodes. Seven hub proteins were considered most important from the string network of the protein targets using variable parameters such as Degree Centrality (DCY), Closeness Centrality (CCY) and Betweenness Centrality (BCY). Molecular docking and molecular dynamic simulations were carried out to ascertain the stability of the best docked phytochemical-protein complexes. The *in silico* findings were further validated using c-Src kinase protein as a model. **Results:** In this study, we have fractionated and identified 8 phytochemicals from *Amalaki churna*. We were able to find a total of 387 protein targets from the Drug bank and Binding DB by using structural similarity search. A network with 273 nodes and 417 edges that was acquired from Drug Bank demonstrated the substantial cross talk between phytochemicals and their protein targets. In a similar manner, phytochemical similarity search from Binding data bank resulted in a protein-phytochemical network with 143 nodes and 275 edges. Further, seven hub proteins with most interconnectedness were selected as the top most protein targets of the phytochemicals. Molecular modelling and docking experiments show that phytochemicals fit well into the target proteins' active sites. Molecular dynamic studies were used to further demonstrate the durability and stability of the protein phytochemical complexes. Phytochemicals were also shown to alter cell cycle regulation, disrupt cell survival pathways, limit cell migratory capacity, and induce apoptosis. The disruption of many cellular pathways was demonstrated using c-Src kinase as a model, in which down-regulation of the tyrosine kinase by phytochemicals led in the disruption of its downstream proteins such as Akt1, cyclin D1 and vimentin. **Conclusion:** Network analysis, followed by molecular docking, molecular dynamics modelling, and *in vitro* studies, clearly underline the importance of protein-phytochemical cross-talk.

Keywords: Phytochemical, *Amalaki churna*, Network pharmacology, Protein enrichment, Molecular dynamics.

Correspondence:
Dr. Vishal Trivedi
Professor, Department of Biosciences and Bioengineering, Malaria Research Group, Indian Institute of Technology-Guwahati, Guwahati-781039, Assam, INDIA.
Email: vtrivedi@iitg.ernet.in, Vishalash_1999@yahoo.com
ORCID ID: 0000-0001-8477-4326 (Md Rafi Uz Zama Khan)

Received: 01-05-2023;
Revised: 04-06-2023;
Accepted: 23-06-2023.

INTRODUCTION

The potential therapeutic qualities of plant and herbs have greatly influenced in the development of several herbal treatments for a variety of disorders across the world.[1-3] The presence of phytochemicals in these medicinal plants is primarily responsible for their wide range of effects.[4,5] In addition to high-value medicinal plants, even regular diets include phytochemicals like carotenoids, phytosterols, polyphenols, terpenoids, etc., that are directly beneficial to the human body.[3,5,6] One such dietary supplement is *amalaki churna*, a well-known ayurvedic remedy that is prepared from the fruits of *Phyllanthus emblica* Linn. for treating indigestion, gastritis, ulcer, constipation, vomiting, and gastrointestinal issues among other malaises.[7-9] *Amalaki churna* powder is consumed orally or it is soaked in water and then

DOI: 10.5530/pres.15.4.076

Copyright Information :
Copyright Author (s) 2023 Distributed under Creative Commons CC-BY 4.0

Publishing Partner : EManuscript Tech. [www.emanuscript.in]

taken after meal to supplement the cellular digestion process and treat disorders. Different parts of this plant are known to show anti-inflammatory, anti-diabetic, anti-microbial, analgesic, anti-pyretic, hepatoprotective, antiulcer, and anti-cancer activities.[8,10] However, little is known about the explicit molecular interaction between phytochemicals and their protein targets. In this study, the identity of 8 bioactive polyphenols in the formulation was structurally determined by biochemical fractionation of aqueous extract of *Amalaki churna* by high performance liquid chromatography, followed by IR, UPLC-MS and NMR analysis. A structure-based similarity cheminformatic search was conducted to identify drug-like molecules that had structural identity with the phytochemicals discovered in AMCAE. The cheminformatics search helps us in discovering the unique and shared protein target interactions between AMCAE phytochemicals and their predicted protein targets. Each protein target in this complex network of proteins is connected to a number of other proteins, and the disruption or inhibition of a single protein can have an effect on proteins in several pathways. The results obtained after molecular docking and MD simulations studies signifies the role of protein-phytochemical cross talk and crucial protein as drug target. Moreover, c-Src kinase was used as a model to confirm the *in silico* results. It was discovered that AMCAE treatment down-regulated c-Src kinase along with its downstream targets such as including Akt1, cyclin D1, and vimentin.

MATERIALS AND METHODS

Chemicals

Ayurvedic medicine *Amalaki churna* was purchased from Baidyanath, India. DMSO-d_6 (Deuterated DMSO), Acetone-d_6 (Deuterated acetone), D_2O (Deuterated water), and CD_3OD (Deuterated methanol), were purchased from Kanto Chemical Co. Inc., Tokyo, Japan. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was purchased from Sigma Aldrich (St. Louis, MO, USA). Dulbecco's modified eagle's medium, DMSO (Dimethyl sulphoxide), Fetal Bovine Serum (FBS), antibiotic solution (100X), Phosphate Buffer Saline, and trypsin were obtained from HiMedia (Mumbai, India). Akt1 (cat. # BB-AB0300) and β-actin (cat. # BB-AB0024) were purchased from BioBharathi Life Science Pvt, Ltd., (India). Cyclin D1 (cat. # ab134175) and vimentin (cat. # ab92547) were purchased from Abcam (UK). Src-kinase (cat. # 2123T) was purchased from Cell Signaling Technology Inc., (USA). The Clarity™ Western ECL substrate kit (cat. # 170-5061) was purchased from Bio-Rad. All chemicals and reagents were of analytical grade purity.

Preparation of aqueous extract of *Amalaki churna* (AMCAE)

1 g of *Amalaki churna* was dissolved in 5 mL of water and heated to 37°C for 2 hr while being spun at 150 rpm. The resulting mixture was centrifuged at room temperature for 10 min at 6000 rpm. The procedure was repeated using 5 mL of water, and the resulting supernatant was put to use in further experiments. Until usage, the supernatant was kept at 4°C.

Identification of the main chemicals contained in the *Amalaki churna* Aqueous Extract by fractionation, isolation, and identification (AMCAE): The phytochemicals found in AMCAE were separated using gradient HPLC. A C_{18} column (YMC-TRIART C_{18}- (4.6ID X 250mm)) was used with a gradient of acetonitrile and water, and a flow rate of 1 mL/min was used to record the HPLC chromatogram at 254 nm. Small compounds found in AMCAE were extracted further, and they were then isolated using HPLC and open column chromatography. With the use of several spectrometric methods, including HPLC, NMR (^1H, ^{13}C, and 2D NMR), and UPLC-MS analysis, the structures of the isolated compounds were determined.

Collection of similar compounds from databases

Drugs and small molecules with structural similarity to the phytochemicals extracted from AMCAE that was higher than or equal to 50% were selected using a cheminformatics technique from the Binding DB (https://www.bindingdb.org/rwd/bind/index.jsp)[11] and Drug bank (https://go.drugbank.com/).[12,13] Networks were built using the chemical candidates and their corresponding protein targets, which makes it easier to comprehend how the phytochemicals and their protein targets interact. Later, the discovered proteins were changed to their matching ensemble gene IDs (https://david.ncifcrf.gov/conversion.jsp) for further analysis.[14]

Network construction between phytochemicals and proteins (targets) using Cytoscape: Using Cytoscape, a pharmacology network was created to correlate the phytochemical-protein interactions discovered through similarity searches (Version-3.9.1).[15] Before creating the network, the phytochemicals and proteins were labelled as source and target nodes, respectively.

Protein-Protein Interaction (PPI) network development using STRING and Cytoscape

The targets of similarity search, proteins (UniProt IDs), were subsequently examined to create the protein-protein interaction network. The PPI network was developed using "Search Tool for the Retrieval of Interacting Genes-STRING" software available online (https://string-db.org/).[16] Setting a confidence level of 0.990 enhanced the PPI network. The UniProt IDs are automatically recognized by the STRING programme, which then converts them to the matching canonical Gene names. Cytoscape was used to study the STRING network that resulted after the nodes with no neighbors were removed (Version-3.9.1). The parameters Degree Centrality (DCY), Betweenness Centrality (BCY) and Closeness Centrality (CCY) were employed to further

enrich the PPI network. The network's key targets were chosen as the leading protein with the most connections.

GO functional enrichment and KEGG pathway analysis

The Gene Ontology (GO) studies comprising of Molecular Functions (MF), Cellular Components (CC), and Biological Processes (BP) of the targets obtained from similarity search against AMCAE isolated compounds were analyzed using Database for Annotation, Visualization and Integrated Discovery (DAVID-2022) (https://david.ncifcrf.gov/) software.[14] Based on gene count, *p*-value (< 0.05), and fold enrichment value, which would statistically be significant for indicating the essential functions that are being targeted, the top 10 GO terms for each process were chosen. Using Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, the functional pathways connected to the discovered targets were examined.

Molecular docking of AMCAE isolated compounds against protein targets: The chemical compounds retrieved from AMCAE were collected from PubChem in SDF format and afterwards transformed into Mol2 format after their energy was minimized using the Chem3D pro programme. RCSB PDB (https://www.rcsb.org/) was used to retrieve the appropriate PDB files for the protein targets found. The Swiss-PDB reader performed energy reduction on all protein PDB files. Using Autodock tools-1.5.6, molecular docking of ligands and proteins was carried out.[17] A maximum of 2500000 energy evaluations were performed for calculations of energy binding while the GA runs were set to 100. The acquired binding energy was utilized to assess the binding affinity of ligands to the various targets. Discovery studio was used to visualize the ligand-protein complex's 2D structure.

Molecular dynamic simulation studies

The molecular dynamics simulation of the target proteins in complex with the phytochemicals obtained were studied using GROMACS 2018.1.[18] The topology files were generated using CHARMM-GUI server (https://charmm-gui.org/) and the force fields were generated using CHARMM36. The system was neutralized with Sodium (Na^+) and Chloride (Cl^-) wherever necessary and the energy minimization was done with Lincs algorithm. The temperature and the pressure of the system were set to 300K and 1 atmospheric pressure to pretend physiological conditions. The vanderwaals short range distance cut off was set to 1.2nm. The 10ns MD simulation run was performed with integration step of 2fs under NVT (Amount of substance (N), Volume (V), and Temperature (T)) thermal equilibration conditions. The MD trajectories obtained were analyzed with standard parameters such as Radius of gyration (Rg), Root Mean Square Deviation (RMSD), Root Mean Square Fluctuation (RMSF), and number of hydrogen bonds throughout the simulation time of 10ns. The MM-PBSA (Molecular Mechanics Poison-Boltzmann Surface Area) was used to predict the binding energy of the protein-ligand complexes obtained using GROMACS function. The results obtained were analyzed and the images were developed in QtGrace version 0.2.6.

ADMET studies of phytochemicals

In order to study the physicochemical characteristics of the phytochemicals derived through AMCAE, ADMET (Absorption, distribution, metabolism, excretion and toxicity) analysis was performed using SwissADME (http://www.swissadme.ch/). Lipinski's rule of five was applied to important characteristics such as Molecular Weight (MW), octanol/water Partition coefficient (logP), Total Polar Surface Area (TPSA), Number of Hydrogen Bond Donors/Acceptors (HBD and HBA), and Total Number of Rotatable Bonds (TRB). These metrics are widely used as benchmarks to forecast their molecular flexibility, oral bioavailability, gastro-intestinal absorption, and other properties that decide whether a certain phytochemical/chemical compound may be evaluated as a prospective drug like molecule.

Cell culture

HCT-116 cells (Colorectal cancer cell line) were grown in DMEM: F12 High glucose, 10% Fetal Bovine Serum (FBS), and 1% penicillin-streptomycin anti-microbial solution. Cells were grown at 37°C in an incubator with 5% CO_2 humidity.

Cell Viability assay

Prior to the experiment, $1*10^4$ cells were planted in 200 µL complete medium overnight. After washing the cells twice with cell culture grade Phosphate Buffered Saline (PBS), various doses of treatments (0-500 µg/mL) in serum-free medium were given. The viability of cells was measured using MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide) cell viability method as described previously.[19,20] The findings are presented as a percentage of survival in comparison to the control group.

Western blotting

Prior to the experiment, HCT-116 ($1*10^6$) cells were seeded in a 6 well plate. Cells were treated with AMCAE at doses of 7.5, 20, 30, 50, and 100 µg/mL for 24 and 48 hr after being washed twice with PBS. Cells were collected and lysed in RIPA lysis solution, and protein levels were determined using conventional protein assays. Proteins were separated on a 10% SDS-polyacrylamide gel, and then transferred to nitrocellulose membrane (Bio-Rad cat. # 162-0112) using a Trans-Blot Turbo (Bio-Rad). The membrane was blocked with 5% BSA at room temperature for 1-2 hr. The blots were then incubated with primary antibodies (Akt1 (1:2500), Src-kinase (1:2000), vimentin (1:2500), cyclin D1 (1:5000), and β-actin (1:5000)) overnight at 4°C followed by incubation with appropriate HRP conjugated secondary antibodies for 1-2 hr at room temperature. Proteins bands were

analysed by using Bio-Rad Clarity™ Western ECL substrate kit and images were developed in Bio-Rad chemiDoc system.

RESULTS

Amalaki churna Aqueous Extract (AMCAE) is repleted with polyphenols

8 prominent peaks were detected when AMCAE was spiked at 254 nm in gradient HPLC chromatography (Figure 1A). The 8 polyphenols present in AMCAE were identified to be mucic acid-2-O-gallate, β-glucogallin, gallic acid, 5-hydroxymethylfurfural, macabarterin, corilagin, ellagic acid, and transcinnamic acid whose structures were solved using various spectroscopic techniques such as ^{1}H, ^{13}C NMR (600 MHz), UPLC-MS, IR and HPLC (Figure 1B). The isolated 8 compounds accounted roughly 85% of aqueous extract makeup. Macabarterin, a higher order polyphenol was also found which was reported only once which came from another plant source.[21] The discovery of macabarterin in our study illustrates ayurvedic medicine's ability to seek for innovative and bioactive drug like molecules.

Polyphenol cross talk is manifested by sharing of drug targets

Polyphenols, the secondary metabolites derived from higher plants are present abundantly in plant based supplements.[22] Polyphenol rich food is shown to have oxidative, metabolic and anti-inflammatory effects.[23] Given its wide range of potential therapeutic applications, polyphenols tend to have a wide range of interacting proteome network.[24] A phytochemical-protein network was constructed using the 8 polyphenols isolated from AMCAE by mapping with their protein targets (Supplementary Data 1a and 1b). A total of 273 nodes and 417 edges with 3.02 average number of neighbors highlighted the cross-talk between phytochemicals ant their protein targets obtained from Drug Bank (Figure 1C). Because protein and drug interactions are critical in finding relevant targets, we constructed a network of protein targets with a total number of 168 nodes and 262 edges with 3.1 average number of neighbors using Binding DB to aid in understanding the kinetics of the protein targets (Figure 1D). The protein network analysis revealed that each phytochemical has distinct protein targets, whereas a single protein is a target for numerous phytochemicals. The generated networks differentiate between protein targets based on the number of neighbors they have. For example, the network from Drug Bank showed that β-glucogallin, corilagin and macabarterin exclusively target 3, 10 and 31 proteins respectively. Also, both corilagin and macabarterin were found to share 26 protein targets amongst them. All the three polyphenols share a sum of 14 targets between them (Figure 1C). The protein targets with most neighbors were selected from a sum of 387 proteins (159 from Binding DB and 271 from Drug Bank (after removing 43 duplicates)) which were having 6 neighbors. The top most connected targets revealed from both phytochemical-protein networks were cytochrome P450-3A4 (P08684), tyrosine protein phosphatase (P18031), carbonic anhydrase 1 (P00918), and carbonic anhydrase 2 (P00915). The results illustrate the importance of synergistic and individualistic actions of phytochemicals from AMCAE.

Protein-Protein Interaction (PPI) of proteins targeted by AMCAE

The complex nature of different diseases, and their interaction patterns are widely explained by the help of a biological protein network.[25] The list of protein targets collected from Drug Bank and Binding DB was utilized to examine the PPI interaction network and its most significant proteins that are responsible for network stability. STRING analysis performed at a confidence level of 0.990 yielded a network with PPI enrichment values of 1.0e-16. The PPI network comprised of 372 nodes and 135 edges with average number of neighbors as 3.6 (Figure 2A). A total of 96 proteins had at least one neighbor in the network even though the confidence level was set to 0.99 (Supplementary data-2). In order to find the most important proteins responsible for the integrity of the network, certain parameters (DCY ≥ 7, BCY ≥ 0.08 and CCY > 0.3) were employed. This screening of proteins resulted a total of 8 proteins namely HSP90AA1 (Heat Shock Protein HSP90 Alpha), PI3KR1 (Phosphatidylinositol 3-Kinase Regulatory subunit alpha), ESR-α (Estrogen Receptor-Alpha), c-Src (Proto-oncogene tyrosine-protein kinase Src (c-Src)), EGFR (Epidermal Growth Factor Receptor), AR (Androgen Receptor), NCOA1 (Nuclear Receptor Coactivator 1), and Akt1 (RAC-alpha serine/threonine-protein kinase) (Figure 2B).

Perturbance of multiple pathways by phytochemicals from AMCAE

The insights provided by STRING analysis of protein targets and phytochemical-protein network are indicators of proteins being involved in multiple cellular and metabolic pathways. The Gene Ontology (GO) studies on the molecular targets provided us insights about their functional make up. The GO enrichment performed using DAVID software yielded 724, 129 and 251 GO terms for BP, CC and MF respectively (Supplementary data 3). The top 10 hits of all three functional annotations sheds light on the localization, function and which cellular process they affect the most. The protein targeted by AMCAE are related mostly to interacting kinases that are localized in plasma membrane and cytoplasm which affect multiple signal transduction pathways (Figure 2C). The KEGG pathway analysis of molecular targets revealed that there are 178 significant pathways (p-value < 0.02) that can be restricted or suppressed by phytochemical from AMCAE (Supplementary data-4). The effectuated pathways from KEGG analysis are mostly metabolic and cellular pathways that have a role in cancer related signaling (Figure 2D).

Phytochemicals from AMCAE possess strong affinity towards their protein targets

Ligand-protein interaction studies are a crucial step in determining the signal transduction events that is being modulated by a particular ligand. The comparison between the binding affinities of known inhibitors versus the ligand of interest might help us decide which is better. A ligand can interact with its target at several places, but in this study, the protein targets with their known inhibitors were considered. The findings of the molecular docking showed that the phytochemicals from AMCAE have a higher affinity for binding to their targets than their respective controls or known inhibitors (Table 1). Resveratrol is a well-known inhibitor of Akt1 that binds to its kinase domain. Corilagin a polyphenol isolated from AMCAE was found to have identical binding energy as suggested by molecular docking studies. The identical affinities or binding energies of both resveratrol and corilagin towards with kinase domain of Akt1 is largely due to sharing of common amino acids (Trp80, Val270, Val271, and Asp292). In addition, corilagin was shown to have the highest affinity for HSP90AA1 among the phytochemicals identified from AMCAE, despite the fact that its recognized inhibitor rifabutin had a higher affinity. Further, ellagic acid was found to be the most potent of all phytochemicals from AMCAE. Ellagic acid showed lowest binding energies (strongest affinity) towards EGFR, AR, ESR-α and c-Src kinase with binding energies of -7.6, -7.8, -7.7 and -7.7 kcal/mole respectively. Higher order polyphenol macabarterin was discovered to have negligible interactions with HSP90AA1, PI3KR1, and EGFR, but no affinity for AR, Akt1, ESR-, and c-Src. The interactive amino acids of the best docked protein-ligand complexes are demonstrated in Figure 2E.

Phytochemicals from AMCAE exhibit drug likeliness based on ADMET studies

The key factors in drug discovery are the pharmacodynamics and pharmacokinetic properties of a compound or molecule that manifests its drug likeliness. As per Lipinski's rule of 5, there should not be more than one violation in the parameters such as molecular weight, number of rotatable bonds, number of hydrogen bond acceptor and donors, lipophilicity, and total polar surface area. Four of the eight phytochemicals that were separated from AMCAE exhibited zero violations, one showed one violation, and three showed more than two violations (Supplementary data-5). The phytochemicals that showed more than 2 violations are macabarterin, mucic acid 2-O-gallate and corilagin. Although, these compounds are known to have high molecular weights, which are contributing to violations in Lipinski's rule of 5, further validation is required to exert their drug likeliness.

Molecular dynamic modelling studies show that AMCAE phytochemicals are stable with their protein targets: The ligand and protein interactions revealed by molecular docking states imply the ability of phytochemicals from AMCAE to specifically inhibit target proteins. Our findings were corroborated further by employing molecular dynamics simulations, which provided a better and recognized understanding of the changes in the dynamics of the protein ligand system. The stability of the ligand-protein complex with its backbone (only protein) were compared using various parameters such as RMSD, RMSF, radius of gyration, Number of H bonds formed in the system, and paired distance for 10,000 picoseconds. The MD simulations of seven distinct proteins (c-Src, PI3KR1, ESR-α, EGFR, Akt1, AR, and HSP90) demonstrated that the average values of protein properties and their complexes with phytochemicals from AMCAE are insignificant (Supplementary data 5 and Table 2). For instance, c-Src kinase in complex with its inhibitor ellagic acid remained stable across the MD simulation period, as evidenced by ellagic acid being present in the binding pocket during the run time gathered every 3000 picoseconds (Figure 3A). The RMSD difference between c-Src and its complex with ellagic acid was determined to be 0.002nm, which is extremely small. Hence, the slight indifference between the RMSD values of c-Src protein backbone and its complex with ellagic acid suggests that the protein-ligand system is stable at 300K (Figure 3B). The complex was also found to be stable at higher temperatures when it was further noted that the difference between average RMSD values of c-Src and its complex with ellagic acid was determined to be 0.039 and 0.009 nm at 400K and 500K respectively (Figure 3C and 3D). The average RMSF values of the c-Src protein backbone and its complexes varied by 0.0018, 0.032, and 0.02 nm, respectively, suggesting that the change in RMSF values, which indicates changes in the protein structure following ligand binding, is stable not only at 300K but also at 400K and 500K (Figure 3E). Additionally, it was discovered that there was little difference in the average radius of gyration between the c-Src protein backbone and its complex with ellagic acid (0.008 nm at 300K, 0.012 nm at 400K, and 0.014 nm at 500K), suggesting that there is no change in the folding pattern of c-Src upon binding with ellagic acid (Figure 3F). Similarly, the average number of H-bonds for c-Src complexes with ellagic acid at 300K, 400K, and 500K was found to be 1.14, 2 and 2.1, indicating that the complex forms stable hydrogen bonds even at higher temperatures (Figure 3G). Furthermore, the protein-ligand (complexes other than that of c-Src and ellagic acid) complexes' RMSD variation from the protein backbone was found to be extremely small, falling between 0.002 and 0.13 nm for. Similar to this, the variation in gyration's radius fluctuated between 0.005 and 0.062 nm (Table 2).

Using the MMPBSA (g_mmpbsa) tool [26] on gromacs, the binding energies of all seven protein-ligand complexes were retrieved from the MD trajectory data. Androgen receptor coupled with ellagic acid showed least binding energy value of -82.2kJ/mol (Table 3). Ellagic acid was the most potent of all the

Table 1: Binding energy values of AMCAE isolated compounds against candidate protein targets.

Protein name	PDB ID	Binding Site	Binding residues	Binding Energy (kcal/mol)													
				Controls							AMCAE isolated compounds						
				Ho	Res	PG	Rif	Wor	EA	EA	C1	C2	C3	C4	C5	C6	C7	C8
AR	2AX6	LBD	665-919	-8.6							-5.4	-6.3	-4.9	-4.6	1856	11.9	-7.8	-6.1
Akt1	3O96	PH and KD	6-108 and 150-447		-7.5						-5.6	-6.5	-5.1	-5	102.7	-7.4	-7.1	-6.3
ESR-α	1X7R	LBD	355-549			-6.4					-4.9	-7.5	-4.9	-5	759	-4.2	-7.7	-5.1
HSP90AA1	1YET	NTD	624-718				-11.1				-3.1	-6.1	-4.7	-4	-3.3	-7.9	-5.6	-4.4
PI3KR1	5AUL	C-SH2	9-232					-6.4			-5	-4.2	-5.6	-4.7	-2.7	-7.9	-5.7	-6.6
EGFR	2ITY	KD	685-953						-7.6		-3.2	-4.8	-5.2	-5.3	-6.1	-7	-7.6	-4.7
c-Src	2H8H	KD	270-523							-7.7	-5.1	-6.7	-4.7	-4.6	11	-6.8	-7.7	-5.1

Note: Rif- Rifabutin, Ho- Homosalate, Res- Resveratrol, PG- Propyl gallate, Wor- Wortmannin, EA- Ellagic acid C1- Mucic acid-2-O-gallate, C2- β-glucogallin, C3- Gallic acid, C4- 5-Hydroxymethylfurfural, C5- Macabarterin, C6- Corilagin, C7- Ellagic acid, C8- Trans-cinnamic acid.

Table 2: Mean molecular dynamic simulation parameter values for proteins and their complexes. Note: (P = Protein, P+L = Protein + ligand). MD simulations of all the proteins and their complexes are run at ambient temperature (300K).

	c-Src (300K)		c-Src (400K)		c-Src (500K)		Androgen receptor		Estrogen receptor		EGFR		PI3KR1		Akt1		HSP90	
	P	P+L	P	P+L	P	P+L	P	P+L	P	P+L	P	P+L	P	P+L	P	P+L	P	P+L
Radius of gyration (nm)	1.841	1.849	2.654	2.642	1.88	1.866	1.771	1.787	1.431	1.369	2.095	2.071	2.471	2.466	2.47	2.454	2.473	2.449
RMSD (nm)	0.106	0.108	0.391	0.43	0.183	0.174	0.25	0.248	0.469	0.328	0.197	0.179	0.185	0.174	0.271	0.225	0.437	0.567
RMSF (nm)	0.0949	0.0967	0.261	0.229	0.136	0.116	0.153	0.147	0.17	0.132	0.136	0.142	0.129	0.126	0.19	0.176	0.32	0.379
Number of H bonds	-----	1.148	-----	2.009	-----	2.108	-----	1.762	-----	0.059	-----	3.445	-----	1.267	-----	2.009	-----	2.237
Pair distance (nm)	-----	0.187	-----	0.182	-----	0.166	-----	0.191	-----	1.118	-----	0.177	-----	0.208	-----	0.192	-----	0.188

Table 3: MMPBSA binding energies of protein and ligand complexes.

	Vanderwaal energy (kJ/mol)	Electrostatic energy (kJ/mol)	Polar solvation energy (kJ/mol)	SASA energy (kJ/mol)	Binding energy (kJ/mol)
c-Src_ellagic acid (300K)	-122.01	-16.4	91.2	-13.03	-60.2
c-Src_ellagic acid (400K)	-113.3	-50.3	139.3	-13.3	-37.7
c-Src_ellagic acid (500K)	-107.6	-69.9	156.9	-14.6	-35.3
Androgen receptor_ellagic acid	-167.2	-23.9	123.5	-14.6	-82.2
Estrogen receptor_ellagic acid	-131.6	-67.3	134.5	-15.09	-79.5
EGFR_ellagic acid	-123	-63.5	138	-14.01	-62.6
PI3KR1_transcinnamic acid	-12.3	-18.7	3.6	-2.2	-29.8
Akt1_corilagin	-202.4	-117.2	275.9	-21.6	-65.4
HSP90AA1_corilagin	-153.02	-58.5	249.6	-18.6	19.4

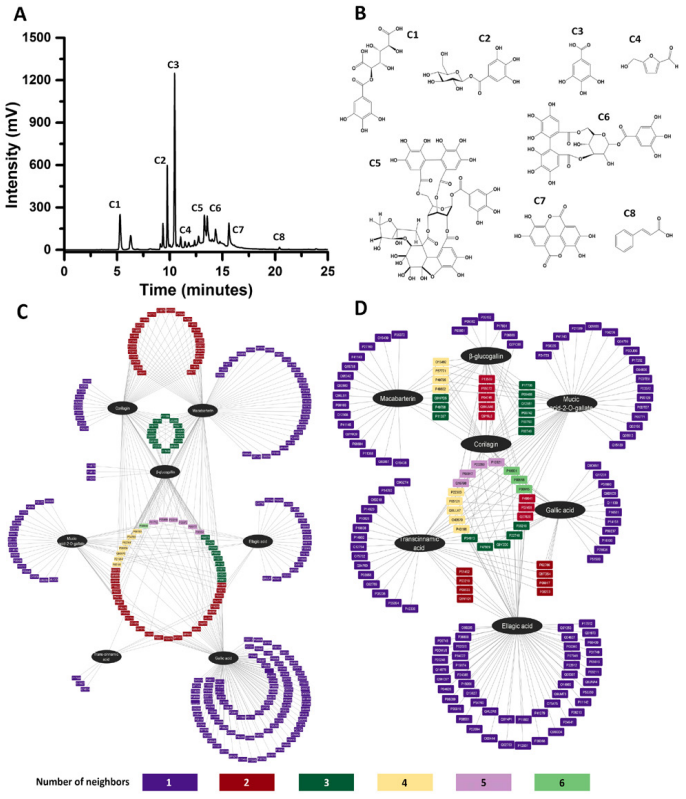

Figure 1: Several bioactive phytochemicals are present in AMCAE (*Amalaki churna* Aqueous Extract). (A) Fractionation of *Amalaki churna*. Gradient HPLC chromatogram developed at 254 nm gives 8 distinctive peaks. (B) Chemical structures of compounds isolated from AMCAE. The compounds identified are C1- Mucic acid-2-*O*-gallate, C2- β-glucogallin, C3- Gallic acid, C4- 5Hydroxymethylfurfural, C5- Macabarterin, C6- Corilagin, C7- Ellagic acid, C8- Transcinnamic acid. (C) Phytochemical-protein network depicts sharing of protein targets amongst them. Cytoscape network for phytochemical from Drug Bank. (D) Target-phytochemical interaction shows importance of key proteins. Cytoscape network for phytochemical-target network from Binding DB.

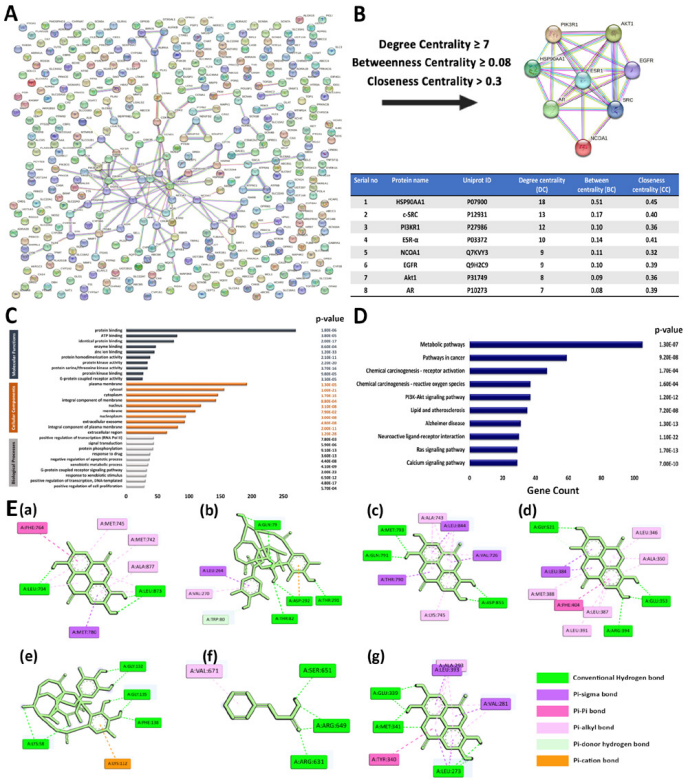

Figure 2: Phytochemicals targets multiple cellular pathways. (A) Protein-protein interaction analysis of protein targets at 0.990 level of confidence. (B) The top 8 targets of AMCAE based on Cytoscape network analysis. (C) GO enrichment analysis of protein targets obtained by DAVID software. (D) KEGG Pathway analysis for candidate targets obtained from similarity search. (E) Phytochemicals bind well with their protein targets. Molecular modelling and docking of phytochemicals into specified protein targets to produce a phytochemical-protein target model. The molecular model analysis shows that phytochemicals have extensive interactions with protein targets. (a) Androgen receptor- Ellagic acid, (b) Akt1-Corilagin, (c) EGFR-Ellagic acid, (d) Estrogen receptor α-Ellagic acid, (e) HSP90AA1-Corilagin, (f) PI3KR1-Transcinnamic acid (g) c-Src-Ellagic acid.

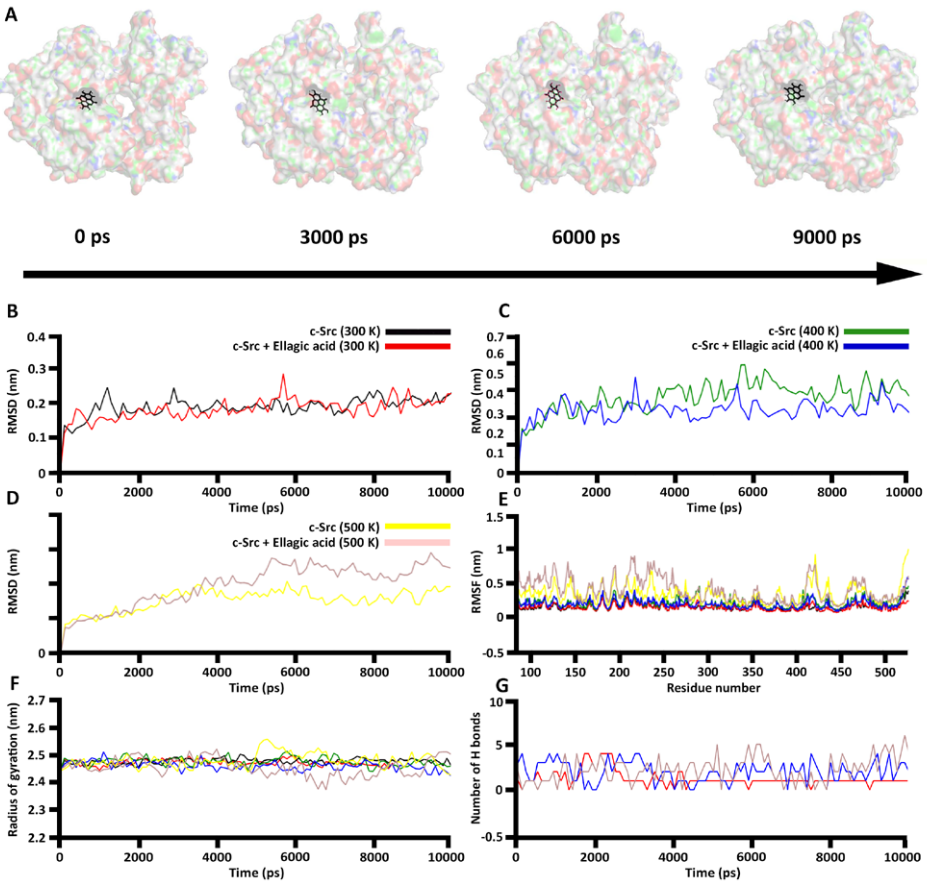

Figure 3: Molecular dynamic simulation results of phytochemicals with their protein targets. (A) Ellagic acid possess stable binding with c-Src kinase throughout its simulation time at 300K. (B) RMSD plot of Src kinase and its complex with Ellagic acid at 300 K. (C) RMSD plot of Src kinase and its complex with Ellagic acid at 400 K. (D) RMSD plot of Src kinase and its complex with Ellagic acid at 500 K. (E) RMSF plot of c-Src and its complex with Ellagic acid at various temperatures. (F) Radius of gyration plot of c-Src and its complex with Ellagic acid at various temperatures. (G) Number of H-bonds of c-Src in complex with Ellagic acid at various temperatures.

phytochemicals from AMCAE since it formed stable complexes with c-Src, AR, ESR-αand EGFR. Although the system is stable at a variety of temperatures, the binding energies of c-Src in combination with ellagic acid show that there are significant differences between them.

Down regulation of c-Src kinase and its related protein in colorectal cancer cells

Amalaki churna is made from the fruits of amla, a plant that is very well known for its variable properties such as antibacterial, antifungal, antioxidant, and anti-inflammatory among other properties.[7] Extracts of amalaki are also known for having anti-cancer and anti-ulcerative properties.[7-9] Since, the churnas are made with fundamental idea of increasing the absorption of ayurvedic formulations in gastrointestinal tracts, we treated colorectal cancer cells (HCT-116) with AMCAE to observe its effect on loss of cellular viability and cellular signaling using c-Src kinase as a model. A decrease in cellular viability was observed upon treatment of colorectal cancer cells in a dose dependent manner with an IC_{50} of 76.2 ± 6.3 µg/mL after 48 hr of treatment (Figure 4A and 4B). c-Src kinase which is a non-tyrosine kinase and explicitly involved in many cellular processes such as proliferation, motility, adhesion and differentiation is related to progression of cancer of colorectal origin.[27] Several receptors such as VEGFR, EGFR, IL-4 and GPCR activate and regulate c-Src kinase.[28-33] In CRC cells, the activation of NF-B by Akt1, a process made possible by the active EGFR/Src complex, suppresses the apoptotic mechanism.[34] Both c-Src kinase and Akt1 were found to be down regulated in HCT-116 cells upon treatment with AMCAE after 24 and 48 hr of treatment (Figure 4C and 4D). It's possible that c-Src, a regulator of Akt1, has been suppressed or that a direct phytochemical intervention by AMCAE has directly inhibited Akt1. Akt1 was also found to be

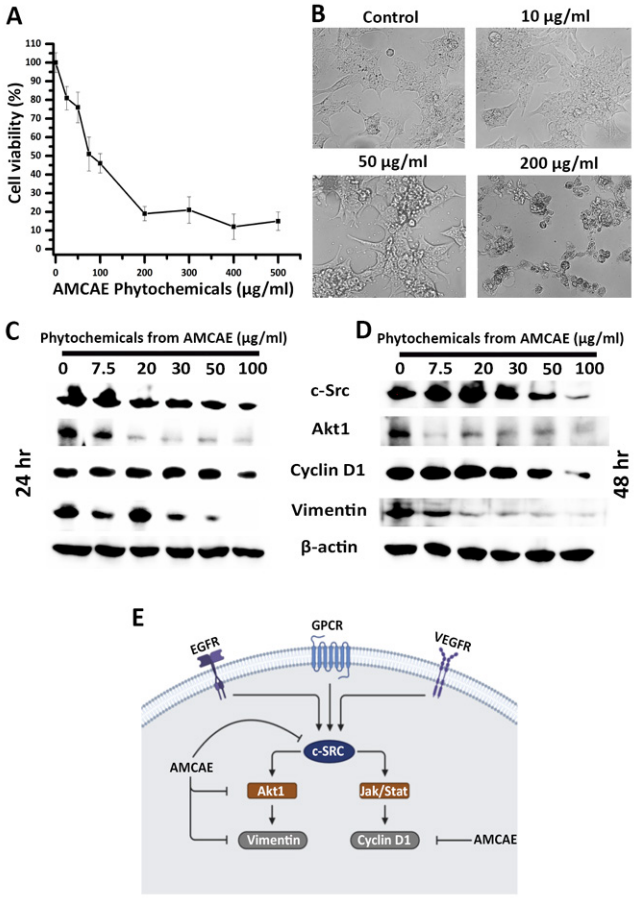

Figure 4: *In vitro* validation of *in silico* based approach with c-Src kinase as a model. (A) AMCAE kills colorectal cancer cells. AMCAE was used to treat colorectal cancer cells (HCT-116) at different concentrations (0-500 µg/mL), and the MTT test was used to determine the loss in cell viability. Images from different fields were taken utilizing the Cytell cell imaging (GE Healthcare) after observing under a microscope. (B) Representative images are shown. (C and D) After 24 and 48 hr of AMCAE treatment, western blot images demonstrate decreased expression of c-Src kinase and its downstream targets such as Akt1, cyclin D1, and vimentin. β-actin was considered as internal reference. (E) Mechanism of AMCAE induced cell signaling disruption in colorectal (HCT-116) cancer cells.

inhibited by corilagin as discussed in earlier sections. To further validate our findings, we examined the expression of cyclin D1 and vimentin, both of which are downstream targets of c-Src kinase. AMCAE down regulated expression of vimentin and cyclin D1. These two proteins are related to cell cycle progression and regulation of EMT process. Vimentin is not directly regulated by c-Src kinase; hence its down regulation may be caused by the inhibition of Akt1 or vimentin inhibitors from AMCAE (Figure 4E).

DISCUSSION

It is rather acknowledged that the complex interactions among phytochemicals in an extract, their protein targets, and their underlying mechanisms continue to impede the creation of a suitable therapeutic candidate. Plants and herbal sources continue to be a significant source of phytochemicals that can be used to create powerful pharmaceuticals, and understanding plants gives a solid foundation for the hunt for novel therapies. An interdisciplinary approach encompassing medicinal chemistry, natural product chemistry, biochemistry, pharmacology, and even molecular biology is required to comprehend the effect from a certain plant or herb. In this study, using various spectroscopic techniques such as ^{1}H, ^{13}C NMR (600 MHz), UPLC-MS, and IR, 8 polyphenols were identified from AMCAE. Given that polyphenols have a wide range of potential therapeutic applications,[35] a phytochemical-protein network that was created using the 8 polyphenols extracted by mapping with their protein targets illustrate the importance of synergistic and individualistic actions of phytochemicals from AMCAE. Certain metrics (DC ≥ 7, BC ≥ 0.08 and CC > 0.3) were used to identify the most significant proteins accountable for the network's integrity. This screening of proteins resulted a total of 8 proteins namely HSP90AA1, PI3KR1, ESR-α, c-Src kinase, EGFR, AR, NCOA1, and Akt1. Further, among all the phytochemicals from AMCAE, ellagic acid was discovered to be the most powerful. Ellagic acid displayed the lowest binding energies (highest affinity) for EGFR, AR, ESR-α, and c-Src kinase. The molecular dynamic simulation investigations revealed information about the stability and power of the selected protein ligand complexes when compared to their protein backbones, which is consistent with our molecular docking experiments. All the complexes displayed little variation in their RMSD, RMSF, number of H-bonds, and radius of gyrations, indicating that the selected phytochemicals might make good drug-like compounds. Apart for HSP90 and its complex with corilagin, the stability of all the complexes was also validated by the MMPBSA binding energies. In addition, five of the eight phytochemicals that make up AMCAE's ADMET profiles complied with Lipinski's criteria, indicating that AMCAE as a whole can be viewed as a candidate for a medication. We further conducted *in vitro* studies using c-Src as a model in order to further validate our conclusions from data obtained from *in silico* analysis. Several important protein targets, including c-Src Akt1, cyclin D1, and vimentin, were down regulated after AMCAE treatment of HCT-116 cells. The inhibition of these proteins demonstrates how the previously described cross-talk between phytochemicals and their protein targets is interrelated. Since protein targets revealed have a range of functions to perform, it is fair to assume that phytochemicals from AMCAE derived from herbal medicines have substantial therapeutic usefulness.

CONCLUSION

In conclusion, the present study provides a systematic approach towards understanding the molecular mechanism of action of AMCAE. The qualitative and quantitative analysis of phytochemicals from AMCAE revealed the presence of numerous bioactive compounds. Molecular docking and molecular dynamic simulations with the AMCAE phytochemicals and their respective

protein targets revealed the potential of phytochemicals to act as drug like molecules and showed their stability, strength and specificity. Biological experiments with HCT-116 cells confirmed the *in silico* results and showed the down regulation of proteins involved in cancer and other metabolic pathways. The present study provides a base for further in-depth investigation of the potential of AMCAE in treatment of various diseases and the potential of phytochemicals to act as drug like molecules.

ACKNOWLEDGMENT

This work was supported by the Department of Science and Technology (DST-SERB) grant to V.T. MR acknowledges the financial support in the form of a fellowship from Indian Institute of Technology-Guwahati.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

BCy: Betweenness Centrality; **BP:** Biological Processes; **CC:** Cellular Components; **CCy:** Closeness Centrality; **DAVID:** Database for Annotated Visualization and Integrated Discovery; **DCy:** Degree Centrality; **HCAE:** Haritaki Churna Aqueous Extract; **HPLC:** High Pressure Liquid Chromatography; **KEGG:** Kyoto Encyclopedia of Genes and Genomes; **MF:** Molecular functions; **NMR:** Nuclear Magnetic Resonance; **PDB:** Protein Data Bank; **PPI:** Protein-Protein Interaction; **STRING:** Search Tool for Retrieval of Interacting Genes/Proteins; **UPLC-MS:** Ultra Performance Liquid Chromatography-Mass Spectrometer.

SUMMARY

Understanding the complex interactions among phytochemicals, protein targets, and mechanisms is crucial for developing effective therapeutic candidates. An interdisciplinary approach involving various scientific fields is necessary to comprehend the effects of plant extracts. In this study, 8 polyphenols were identified from an Indian Gooseberry extract using spectroscopic techniques. A phytochemical-protein network was created, highlighting the synergistic and individualistic actions of the phytochemicals. Ellagic acid emerged as the most potent compound, exhibiting strong binding affinity with multiple protein targets. Molecular simulations confirmed the stability of the phytochemical-protein complexes. *In vitro* studies validated the downregulation of key proteins, supporting the therapeutic potential of Indian Gooseberry extract.

REFERENCES

1. Petrovska BB. Historical review of medicinal plants' usage. Pharmacogn Rev. 2012;6(11):1-5. doi: 10.4103/0973-7847.95849, PMID 22654398.
2. Prasathkumar M, Anisha S, Dhrisya C, Becky R, Sadhasivam S. Therapeutic and pharmacological efficacy of selective Indian medicinal plants–a review. Phytomed Plus. 2021;1(2):100029. doi: 10.1016/j.phyplu.2021.100029.
3. Makhaik MS, Shakya AK, Kale R. Dietary phytochemicals: as a natural source of antioxidants. Antioxid-Benefits Sources Mech Action. 2021.
4. Craig WJ. Phytochemicals: guardians of our health. J Am Diet Assoc. 1997;97(10):S199-204. doi: 10.1016/s0002-8223(97)00765-7, PMID 9336591.
5. Guan R, Van Le Q, Yang H, Zhang D, Gu H, Yang Y, *et al*. A review of dietary phytochemicals and their relation to oxidative stress and human diseases. Chemosphere. 2021;271:129499. doi: 10.1016/j.chemosphere.2020.129499, PMID 33445014.
6. Han X, Shen T, Lou H. Dietary polyphenols and their biological significance. Int J Mol Sci. 2007;8(9):950-88. doi: 10.3390/i8090950.
7. Srivasuki K. Nutritional and health care benefits of Amla. J Pharmacogn. 2012;3(2):147-51.
8. Bandyopadhyay SK, Pakrashi SC, Pakrashi A. The role of antioxidant activity of *Phyllanthus emblica* fruits on prevention from indomethacin induced gastric ulcer. J Ethnopharmacol. 2000;70(2):171-6. doi: 10.1016/s0378-8741(99)00146-4, PMID 10771207.
9. Zhao T, Sun Q, Marques M, Witcher M. Anti-cancer properties of *Phyllanthus emblica* (Indian gooseberry). Oxid Med Cell Longev. 2015;2015:950890. doi: 10.1155/2015/950890, PMID 26180601.
10. Gaire BP, Subedi L. Phytochemistry, pharmacology and medicinal properties of *Phyllanthus emblica* Linn. Chin J Integr Med. 2014:1-8. doi: 10.1007/s11655-014-1984-2, PMID 25491539.
11. Gilson MK, Liu T, Baitaluk M, Nicola G, Hwang L, Chong J. BindingDB in 2015: a public database for medicinal chemistry, computational chemistry and systems pharmacology. Nucleic Acids Res. 2016;44(D1):D1045-53. doi: 10.1093/nar/gkv1072, PMID 26481362.
12. Wishart DS, Feunang YD, Guo AC, Lo EJ, Marcu A, Grant JR, *et al*. DrugBank 5.0: a major update to the DrugBank database for 2018. Nucleic Acids Res. 2018;46(D1):D1074-82. doi: 10.1093/nar/gkx1037, PMID 29126136.
13. Wishart DS, Knox C, Guo AC, Shrivastava S, Hassanali M, Stothard P, *et al*. DrugBank: a comprehensive resource for *in silico* drug discovery and exploration. Nucleic Acids Res. 2006;34(S1):D668-72. doi: 10.1093/nar/gkj067, PMID 16381955.
14. Huang DW, Sherman BT, Tan Q, Kir J, Liu D, Bryant D, *et al*. David Bioinformatics Resources: expanded annotation database and novel algorithms to better extract biology from large gene lists. Nucleic Acids Res. 2007;35(S2):W169-75. doi: 10.1093/nar/gkm415, PMID 17576678.
15. Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, *et al*. Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res. 2003;13(11):2498-504. doi: 10.1101/gr.1239303, PMID 14597658.
16. Szklarczyk D, *et al*. The STRING database in 2017: quality-controlled protein–protein association networks, made broadly accessible. Nucleic Acids Res. 2016:gkw937.
17. Morris GM, Huey R, Lindstrom W, Sanner MF, Belew RK, Goodsell DS, *et al*. AutoDock4 and AutoDockTools4: automated docking with selective receptor flexibility. J Comp Chem. 2009;30(16):2785-91. doi: 10.1002/jcc.21256, PMID 19399780.
18. Van Der Spoel D, Lindahl E, Hess B, Groenhof G, Mark AE, Berendsen HJ. GROMACS: fast, flexible, and free. J Comp Chem. 2005;26(16):1701-18. doi: 10.1002/jcc.20291, PMID 16211538.
19. Deka SJ, Roy A, Ramakrishnan V, Manna D, Trivedi V. Danazol has potential to cause PKC translocation, cell cycle dysregulation, and apoptosis in breast cancer cells. Chem Biol Drug Des. 2017;89(6):953-63. doi: 10.1111/cbdd.12921, PMID 27933735.
20. Deka SJ, Mamdi N, Manna D, Trivedi V. Alkyl cinnamates induce protein kinase C translocation and anti-cancer activity against breast cancer cells through induction of the mitochondrial pathway of apoptosis. J Breast Cancer. 2016;19(4):358-71. doi: 10.4048/jbc.2016.19.4.358, PMID 28053624.
21. Ngoumfo RM, Ngounou GE, Tchamadeu CV, Qadir MI, Mbazoa CD, Begum A, et al. Inhibitory effect of macabarterin, a polyoxygenated ellagitannin from *Macaranga barteri*, on human neutrophil respiratory burst activity. J Nat Prod. 2008;71(11):1906-10. doi: 10.1021/np8004634, PMID 19006373.
22. Pandey KB, Rizvi SI. Plant polyphenols as dietary antioxidants in human health and disease. Oxid Med Cell Longev. 2009;2(5):270-8. doi: 10.4161/oxim.2.5.9498, PMID 20716914.
23. Vauzour D, Rodriguez-Mateos A, Corona G, Oruna-Concha MJ, Spencer JP. Polyphenols and human health: prevention of disease and mechanisms of action. Nutrients. 2010;2(11):1106-31. doi: 10.3390/nu2111106, PMID 22254000.
24. Lacroix S, Klicic Badoux J, Scott-Boyer MP, Parolo S, Matone A, Priami C, *et al*. A computationally driven analysis of the polyphenol-protein interactome. Sci Rep. 2018;8(1):2232. doi: 10.1038/s41598-018-20625-5, PMID 29396566.
25. Safari-Alighiarloo N, Taghizadeh M, Rezaei-Tavirani M, Goliaei B, Peyvandi AA. Protein-Protein Interaction networks (PPI) and complex diseases. Gastroenterol Hepatol Bed Bench. 2014;7(1):17-31. PMID 25436094.
26. Kumari R, Kumar R, Open-Source Drug Discovery Consortium, Lynn A. G_MMPBSA–a GROMACS tool for high-throughput MM-PBSA calculations. J Chem Inf Model. 2014;54(7):1951-62. doi: 10.1021/ci500020m, PMID 24850022.
27. Jin W. Regulation of Src family kinases during colorectal cancer development and its clinical implications. Cancers. 2020;12(5):1339. doi: 10.3390/cancers12051339, PMID 32456226.
28. Mao W, Irby R, Coppola D, Fu L, Wloch M, Turner J, *et al*. Activation of c-Src by receptor tyrosine kinases in human colon cancer cells with high metastatic potential. Oncogene. 1997;15(25):3083-90. doi: 10.1038/sj.onc.1201496, PMID 9444956.

29. Ellis LM, Staley CA, Liu W, Fleming RY, Parikh NU, Bucana CD, *et al*. Down-regulation of vascular endothelial growth factor in a human colon carcinoma cell line transfected with an antisense expression vector specific for c-src. J Biol Chem. 1998;273(2):1052-7. doi: 10.1074/jbc.273.2.1052, PMID 9422768.
30. Barderas R, Bartolomé RA, Fernandez-Aceñero MJ, Torres S, Casal JI. High expression of IL-13 receptor α2 in colorectal cancer is associated with invasion, liver metastasis, and poor PrognosisRoleIL-13Rα2colorectalcancermetastasis. Cancer Res. 2012;72(11):2780-90. doi: 10.1158/0008-5472.CAN-11-4090, PMID 22505647.
31. Bartolomé RA, García-Palmero I, Torres S, López-Lucendo M, Balyasnikova IV, Casal JI. IL13 receptor α2 signaling requires a scaffold protein, FAM120A, to activate the FAK and PI3K pathways in colon cancer MetastasisIL13Rα2signalingcolorectalcancer metastasis. Cancer Res. 2015;75(12):2434-44. doi: 10.1158/0008-5472.CAN-14-3650, PMID 25896327.
32. Pai R, Soreghan B, Szabo IL, Pavelka M, Baatar D, Tarnawski AS. Prostaglandin E2 transactivates EGF receptor: a novel mechanism for promoting colon cancer growth and gastrointestinal hypertrophy. Nat Med. 2002;8(3):289-93. doi: 10.1038/nm0302-289, PMID 11875501.
33. Fukuda R, Kelly B, Semenza GL. Vascular endothelial growth factor gene expression in colon cancer cells exposed to prostaglandin E2 is mediated by hypoxia-inducible factor 1. Cancer Res. 2003;63(9):2330-4. PMID 12727858.
34. Lien GS, Wu MS, Bien MY, Chen CH, Lin CH, Chen BC. Epidermal growth factor stimulates nuclear factor-κB activation and heme oxygenase-1 expression via c-Src, NADPH oxidase, PI3K, and Akt in human colon cancer cells. PLOS ONE. 2014;9(8):e104891. doi: 10.1371/journal.pone.0104891, PMID 25122478.
35. Rasouli H, Farzaei MH, Khodarahmi R. Polyphenols and their benefits: a review. Int J Food Prop. 2017;20(S2):1700-14.

Cite this article: Khan MRUZ, Trivedi V. Phytochemical Crosstalk in Indian Gooseberry Preparation to Explain Therapeutic Potentials of Dietary Supplements. Pharmacog Res. 2023;15(4):716-26.