

Network pharmacology-based anti-colorectal cancer activity of piperlonguminine in the ethanolic root extract of *Piper longum* L.

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Research Article

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Abstract

The most frequent cancer to affect the human colon is colorectal cancer (CRC). CRC has the second highest incidence and fatality rates of any malignancy, at 10.2 and 9.2 percent, respectively. Plants and plants-based products for thousands of years, have been utilized to treat cancer along with other associated health issues. Alkaloids are a valuable class of chemical compounds with great potential as new medicine possibilities. Many of the alkaloids tested from different types of medicinal plants and herbs were found to impede proliferation of cells and kill many types of cancer cells. *Piper longum* Linn contains various types of alkaloids. In this research, the ethanolic root extract of *Piper longum* (EREPL) is the subject of study based on network pharmacology. Two alkaloids were picked from the gas chromatography mass spectrometry (GC-MS) analysis. However, only piperlonguminine received preference because it adhered to Lipinski's rule and depicted no toxicity. Web tools which are available online, like, Swiss ADME, pkCSM and ProTox-II were used to evaluate the pharmacokinetics and physiochemical properties of piperlonguminine. The database that SwissTargetPrediction and TCMSP maintain contains the targets for piperlonguminine. Using DisGeNET, GeneCards and Open Targets Platform databases, we were able to identify targets of CRC. The top four hub genes identified by Cytoscape are SRC, MTOR, EZH2, and MAPK3. The participation of hub genes in colorectal cancer-related pathways was examined using the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database. The colorectal cancer pathway, the ErbB signaling pathway and the mTOR signaling pathway emerged to be important. Our findings show that the hub genes are involved in the aforementioned pathways for tumor growth, which calls for their downregulation. Additionally, piperlonguminine has the potential to become a successful medicine in the future for the treatment of CRC.

Introduction

Colorectal cancer (CRC) develops in the lining of the colon, namely in the mucosal epithelium. The occurrence of CRC has increased in recent years. It has been on the rise throughout the years, and its prevalence among young people has accelerated in recent years. Human CRC has the second highest new incidence and fatality rates of any malignancy, at 10.2 and 9.2 percent, respectively [1]. Clinical symptoms of colorectal cancer, which might include gastrointestinal unease, constipation, intestinal obstruction, mucopurulent stool, and metastatic lymph nodes are all symptoms of lymphoma and others, may not appear until later stages due to the disease's complex etiology. Surgery, chemotherapy, and biological antibody therapy are the mainstays of current CRC treatment [2]. Fluorouracil, capecitabine, and oxaliplatin are the most often used adjuvant therapies, but they have detrimental side effects, like, diarrhea, neuropathy and neutropenia [3]. The above therapeutic constraints require the development of effectual therapeutic procedures for the treatment of CRC, thereby reducing harmful side effects and consequences [4]. Herbal treatments have gained popularity because of their reduced potential for negative side effects. Many different diseases have traditionally been treated using thousands of different herbs. Among these, members of the genus *Piper* are among the most widely utilized medicinal herbs in human history. *Piper longum*, also called as pippali or long pepper, is a Piperaceae pinnacle vine. Long pepper is primarily found in subtropical and tropical regions. The plant contains numerous creeping, integrated branches that are dense at the nodes. In addition, it also possesses a massive woody root [5]. The Long pepper has been used in traditional medicinal and nutritional practices for centuries. It is detailed in length in ancient Indian Ayurvedic textbooks and was a popular spice prior to the Columbian exchange. Long pepper has been investigated for its possible health benefits, and it has been discovered to possess antioxidant, anti-inflammatory, antiamoebic, acaricidal, antiviral and antibacterial properties [6]. In traditional medicine, the fruit of the *Piper longum* plant is used to cure a wide range of conditions, including, respiratory associated infections, bronchitis, malaria, asthma, cholera, gonorrhea, cough, diarrhea, cold and tumours [7]. Piperlonguminine, piperundecalidine, piperine, pipermonaline, piperlongumine, diaeudesmin, sesamin and sylvatinare the major components extracted from different sections of *P. longum* [7]. Piperlonguminine is an amide alkaloid found in the *P. longum* root, which is first time isolated

in 1966 from this plant along with piperlongumine, besides piperine [7]. Piperlonguminine has traditionally been utilized for the treatment of cough, bronchitis, respiratory associated infections, asthma, malaria, constipation, hepatitis, gonorrhea, cholera, diarrhea and tumours [8]. Piperlonguminine and its variants have been investigated as potential TrxR inhibitors, a protein involved in cellular redox balance regulation. Piperlonguminine has also been demonstrated in studies to induce ROS and autophagy, as well as modulate p38 and Akt/mTOR signaling [9]. Piperlonguminine is now being studied for its anticancer properties as well as its immunosuppressive properties. However, few studies have demonstrated anticancer efficacy in silico, in vivo, and in vitro. In order to discover new drugs utilizing computational biological methods, the area of network pharmacology integrates system biology with genomic technology. A method for deciphering intricate connections between biological systems, treatments, and diseases is called network pharmacology [10]. By analyzing large sets of data, it also pinpoints synergistic effects in cancer and reveals potential mechanisms of complicated bioactives [11].

Materials and methods

Plant material

Fresh *Piper longum* Linn. roots were acquired from the CSIR-Central Institute of Medicinal and Aromatic Plants (CSIR-CIMAP), Lucknow, India. The roots were separated from the branch and thoroughly cleaned. To flush out pollutants, we employed double-distilled water. The roots were then dried in the shade before being cut using a knife. Automatic grinders were used for grinding the cut roots, and the roots powder was stored in a sealed container for later use.

Preparation of plant extracts and chemical profiling

The dried roots of the plant were ground into a rough powder. Overnight, 200g of dried powder of root was mixed with 1000 mL of 95% ethanol at 35°C (thrice). After passing through Whatman filter paper No. 1, the extract that contained the solvent was subsequently concentrated using a rotating evaporator at temperatures below 45° C and under a vacuum. The extract was put in container that kept it cool until it was used again. GC-MS was used to find different alkaloids, the size of their peaks, and mass-to-charge ratio (M/Z). Two alkaloids were screened among the 61 phytoconstituents.

ADMET screening of alkaloids

The Swiss ADME (<http://www.swissadme.ch/>), pkCSM (<http://biosig.unimelb.edu.au/pkcsml/>) and ProTox-II (https://tox-new.charite.de/protox_II/), were the 3 online web tools utilized to forecast the physicochemical characteristics of two alkaloids related to ADME that are present in the EREPL. The hydrogen bond donors (HBD) and acceptors (HBA), topological polar surface area (TPSA), lipophilicity (MLOGP), molar refractivity (MR) and druglikeness (DL), were all determined using the Swiss ADME. Blood-brain barrier permeability (BBB), P-glycoprotein substrate, fraction unbound, renal OCT2 substrate, central nervous system permeability (CNS), CYP3A4 and CYP2D6 inhibitor, hERG I inhibitor, oral rat chronic toxicity (LOAEL), and oral rat acute toxicity (LD50) were all determined using the pkCSM. Protox-II was used to test for toxicity, including mutagenicity, hepatotoxicity, and carcinogenicity.

Identification of possible piperlonguminine and colorectal cancer targets

By adding the canonical smile of piperlonguminine in two web tools, Traditional Chinese medicine systems pharmacology database and analysis platform (TCMSP) (<https://tcmsp-e.com>) and Swiss Target Prediction (<http://www.swisstargetprediction.ch/>), the possible targets of piperlonguminine were identified. The 3 databases, GeneCards (<https://www.genecards.org/>), Open Targets Platform (<https://platform.opentargets.org/>), and DisGeNET

(<https://www.disgenet.org/>), were used to assess potential CRC targets. The targets of CRC and the active phytoconstituents of EREPL were linked using the web tool called Venny 2.1.0 (<https://bioinfogp.cnb.csic.es/tools/venny/>).

Compound–target network construction

Piperlonguminine and colorectal cancer target genes were exported to the software called Cytoscape (Version 3.8.2) (<https://cytoscape.org>) to construct a compound-target network. Cytoscape is an open-source application that allows anyone to investigate and join massive networks based on a wide variety of criteria.

Methods for constructing protein–protein interaction (PPI) networks and screening hub genes

The PPI network was generated with STRING database (Version 11.5) (<https://string-db.org/>). STRING stores information about both observed and anticipated interactions. Species-wide dissemination of the STRING-derived PPI interactions is reported. Multiple proteins option was selected to enter the set of piperlonguminine and colorectal cancer target genes by selecting the organism *Homo sapiens*. The STRING data was sent to Cytoscape for further investigation of the network. Hub genes were analyzed using topological measures like betweenness, degree and closeness.

Gene ontology (GO) analysis and pathways retrieval

For GO we utilized ShinyGo 0.76 (<http://bioinformatics.sdstate.edu/go/>). Utilizing the KEGG Pathway database (<https://www.genome.jp/kegg/>) and the DAVID database (<http://david.ncifcrf.gov/tool.jsp>), the pathways in which the hub genes are engaged were discovered.

Molecular docking of the ligand with proteins

Molecular docking of the phytoconstituents piperlonguminine was conducted with the hub genes SRC (PDB ID: 1A07), MAPK3 (PDB ID: 2ZOQ), MTOR (PDB ID: 4JT6), and EZH2 (PDB ID: 5HYN). The SDF file of piperlonguminine was downloaded from PubChem. The SDF file was converted to PDB file using cactus server (<https://cactus.nci.nih.gov/translate/>). The Protein Data Bank (<https://www.rcsb.org>) provided the PDB file for the proteins for *Homo sapiens*. Through Autodock 4, molecules of water from the protein were eliminated before docking. Later, the Kollman charges and polar hydrogen were added, and the ligand and protein PDB data were converted into PDBQT files. The screened protein and ligand were molecularly docked using Auto Dock 4 and the Discovery studio program was employed for its visualization.

Protein expression analysis as a means of validating screened hub genes

The Human Protein Atlas, a web-based software application (<https://www.proteinatlas.org/>) was employed to look at protein expression in both healthy and cancerous cells of colon.

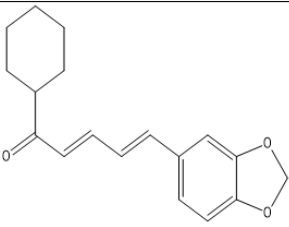
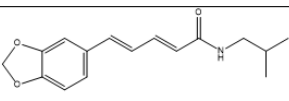
Results

GC-MS analysis of EREPL

The EREPL contained a variety of compounds, which were screened out using GCMS. Two of the 61 different compounds that were discovered belonged to the class of alkaloids. As shown in Fig. 1 and Table 1, M/Z ratio, the

retention duration, negative ion mode, positive ion mode, and chromatogram were used to determine the alkaloids, piperlonguminine and piperine.

Table 1 Alkaloids present in the ethanolic root extract of *Piper longum* Linn. based on GC-MS

S.No.	Name of alkaloids	Structure	MW (g/mol)	Chemical formula	Class of the alkaloids	M/Z	Analysis mode
1	Piperine		285.34	C ₁₇ H ₁₉ NO ₃	Alkaloid	201.10	GC-MS
2	Piperlonguminine		273.33	C ₁₆ H ₁₉ NO ₃	Alkaloid	221.10	GC-MS

Pharmacokinetics and physiochemical analysis of alkaloids in the EREPL

The two alkaloids present in the extract were examined for ADMET. Only piperlonguminine among these alkaloids showed no deviations from Lipinski's criterion and no toxicity. According to Tables 2, 3, 4, and 5, piperine was carcinogenic. As a result, only piperlonguminine was selected for additional research based on two criteria: no toxicity and no violations of Lipinski's criterion. The intestinal absorption of piperlonguminine was high, at 94.721%.

Table 2

Principle computed physiochemical properties of alkaloids present in the ethanolic root extract of *Piper longum* Linn

S. No	Alkaloids	HBA	HBD	MLOGP	MR	TPSA	DL
1	Piperine	3	0	2.39	85.47	38.77	Yes
2	Piperlonguminine	3	1	2.14	78.77	47.56	No
*HBA Hydrogen bond acceptor, HBD Hydrogen bond doner, MLOGP Moriguchi octanol-water partition coefficient, MR Molar refractivity, TPSA Topological polar surface area, DL Druglikeness							

Table 3

Important computed pharmacokinetic properties of alkaloids present in the ethanolic root extract of *Piper longum* Linn

S. No	Alkaloids	CaCo2	HIA	skin permeability	PGS	VDs	fraction unbound	BBB	CNS
1	Piperine	1.596	94.444	-3.131	Yes	0.158	0.134	-0.102	-1.879
2	Piperlonguminine	1.326	94.721	-2.936	Yes	0.008	0.101	-0.334	-1.356
*CaCo2 Human colorectal carcinoma cell line, HIA Human intestinal absorption, PGS P-glycoprotein substrate, VDs Virtual decoy sets, BBB Blood-brain barrier, CNS Central nervous system									

Table 4

Toxicity profiling of the Alkaloids on the basis of ADMET, present in the ethanolic root extract of *Piper longum* Linn

S. No	Alkaloids	CYP2D6 Inhibitor	CYP3A4 Inhibitor	Herg1	Total clearance	Renal OCT2 substrate	AMES toxicity	Oral rat Acute toxicity (LD50)	Oral rat chronic toxicity (LOAEL)
1	Piperine	No	No	No	0.232	Yes	No	2.811	1.51
2	Piperlonguminine	No	No	No	0.229	No	No	2.572	1.888
*CYP2D6 Cytochrome P450 2D6, CYP3A4 Cytochrome P450 3A4, hERG human Ether-a-go-go Related Gene									

Table 5

Toxicity profiling of the alkaloids using Prottox-II, present in the ethanolic root extract of *Piper longum* Linn

S. No	alkaloids	Hepatotoxicity	Carcinogenicity	Mutagenicity	Cytotoxicity
1	Piperine	Inactive	Active	Inactive	Inactive
2	Piperlonguminine	Inactive	Inactive	Inactive	Inactive

Identification of targets of piperlonguminine and colorectal cancer

We may expect that piperlonguminine, a bioactive phytoconstituents of EREPL, may also have anti-colorectal cancer capabilities based on the study of alkaloids from prior studies. Understanding how the phytoconstituents affects target proteins will help to further support our prediction. For the retrieval of targets of CRC and piperlonguminine, various databases were used. Piperlonguminine has 100 and 11 protein targets, respectively, according to SwissTargetPrediction and TCMSP. The DisGeNET, GeneCards, and Open Targets Platform databases were used to collect the target genes for colorectal cancer. DisGeNET, GeneCards, and the Open Targets Platform databases yielded 2832, 24607, and 7383 target genes, respectively. They produced 1865 shared target genes when they were overlapped together. The 1865 colorectal cancer target genes were then overlapped with the 111 piperlonguminine target genes (Fig. 2a), to obtain 51 common genes, SOAT1, NTRK1, PRF1, ESR2, MAPK3, IDO1, SRC, NR3C1, KDR, CDK2, HDAC6, TGFBR1, PIK3CD, AURKA, NAMPT, TERT, CDK1, TTK, HDAC1, RET, ABL1, MTOR, PARP1, PFKFB3, JAK2, IKBKB, KCNH2, CCKBR, PRKDC, PIK3CB, HCK, BRAF, EPHB4, EZH2, CDK4, CTSB, MAPK8, JAK3, HDAC3, ADORA2B, CFTR, DDR2, PDGFRA, CHEK2, CTSD, CSNK2A1, PTGER3, LIMK1, CHEK1, RXRA and SAA1 (Fig. 2a).

Construction and analysis of PPI network

To establish the relationships between 51 putative target genes, a PPI network was built with STRING database (Fig. 2b). The top four hub genes, SRC, MTOR, EZH2, and MAPK3 screened out using the PPI network was transferred to Cytoscape 3.2.1 (Fig. 2c). WithcytoNCA, topological variables such as betweenness, degree and closeness were employed to evaluate hub genes. Through a number of signalling pathways, these hub genes may aid in the treatment of colorectal cancer.

Analysis of piperlonguminine –target network

The 51 common targets between piperlonguminine and colorectal cancer were used to build a network for compound-target network analysis. The network showed interactions between 111 piperlonguminine common genes and a variety of colorectal cancer targets. Piperlonguminine may either stimulate or inhibit these target genes, which may have profound effects on colorectal cancer (Fig. 3).

Target enrichment analysis of piperlonguminine

In-depth information of the four hub genes, SRC, MTOR, EZH2, and MAPK3, were acquired from KEGG and GO connections using the DAVID database and ShinyGo 0.76. The analysis comprises of 403 biological processes, 75 molecular functions and 41 cellular components. The top ten biological processes are, cellular response to reactive oxygen species, mesenchymal cell differentiation, post regulation of protein phosphorylation, post-regulation of phosphorus metabolic process, response to organic cyclic compound, and neuron projection development (Fig. 4a). The TORC1 complex, chromatin silencing complex, caveola, pseudopodium, plasma membrane raft, focal adhesion, late endosome, cell-substrate junction, nuclear envelope, and mitochondrion were among the other cellular components regulated by the hub genes (Fig. 4b). The hub genes also control a variety of molecular processes, including RNA polymerase III type 1 promoter sequence-specific DNA binding, 5S rDNA binding, phosphoprotein binding, scaffold protein binding, ribonucleoprotein complex binding, protein kinase activity, phosphotransferase activity (alcohol group as receptor), kinase activity and transferase activity (transferring phosphorus-containing groups) are likewise controlled by hub genes (Fig. 4c).

Target–pathway network analysis

The biological and pharmacological functions of proteins and genes are not carried out independently. They work together inside integrated systems at both the molecular and cell levels (Fig. 5). Results from the target-pathway network showed that 56 KEGG pathways were involved. The colorectal cancer pathway is the most important of the 56 KEGG pathways because, within it, nine other pathways, including cell cycle, apoptosis, Wnt, PI3K-Akt, MAPK, TGF- β , and mTOR signalling pathways, participate and serve an instrumental part in colorectal cancer (Fig. 5). The colorectal cancer pathway involves the two hub genes MTOR and MAPK3. ErbB and mTOR signalling pathways are two of the nine pathways that have been identified in the DAVID database. With SRC, MTOR, and MAPK3 in the ErbB signalling pathway, and MTOR and MAPK3 in the mTOR signalling pathway, which can affect colorectal cancer, the colorectal cancer pathway is noteworthy due to the involvement of the presence of two or more hub genes inside each of the two pathways is seen. The EZH2 hub gene, on the other hand, is not found in any of the pathways that the DAVID database returned. The current study provides fresh insight into the effect of piperlonguminine on EZH2 in addition to its effects on SRC, MTOR, and MAPK3. Therefore, it may be assumed that the effects of piperlonguminine on colorectal cancer are related to its possible targets, SRC, MTOR, MAPK3, and EZH2. Integrative drug target prediction was used to develop the pathway enrichment, GO, and network analysis. Moreover, piperlonguminine may control colorectal cancer throughout the colorectal cancer pathway (Fig. 6), along with ErbB, and mTOR signaling pathways.

Molecular docking

The top four hub genes, SRC, MTOR, MAPK3, and EZH2, were individually docked with piperlonguminine using Autodock 4 for disease-target network analysis. When docked with piperlonguminine, SRC protein displayed the highest binding energy (-7.22 kcal/mol), followed by MAPK3 (-6.42 kcal/mol), EZH2 (-6.3 kcal/mol), and MTOR (-6.14 kcal/mol) (Table 6). The 3D and 2D pictures of the protein-ligand complex (Table 6) produced from docking were then visualized using Discovery Studio. The resultant amino acids of SRC, GLU A:162, TYR B:152, LYS A:198, interact with the piperlonguminine through H-bond with a bond length of 2.89 Å, 2.06 Å and 1.85 Å, respectively. Similar to this, H-bond interactions occur between the following amino acids of MAPK3: [ILE B:190 (3.39 Å)], EZH2: [ASN A: 119 (2.1 Å)], and MTOR: [GLN A: 2194 (3.24 Å), ASP A: 2191 (2.78 Å), TYR A: 2225 (2.96 Å), and ASP A: 2357 (2.47 Å)] (Fig. 7).

Table 6
Results of molecular docking of the top four hub genes of colon cancer with piperlonguminine

Compound	Protein	Binding energy	Number of hydrogen bonds	Hydrogen bond residue and length
Piperlonguminine	SRC	-7.22	3	GLU A:162 (2.89Å), TYR B:152 (2.06 Å), LYS A:198 (1.85 Å)
	MAPK3	-6.42	1	ILE B:190 (3.39 Å)
	EZH2	-6.3	1	ASN A:119(2.1 Å)
	MTOR	-6.14	4	GLN A:2194(3.24 Å), ASP A:2191(2.78 Å), TYR A: 2225 (2.86 Å), ASP A: 2357(2.47 Å)

Protein expression analysis as a means of validating screened hub genes

Human Protein Atlas was used to evaluate the appearance of proteins in both normal and colon cancer cells in order to obtain external confirmation of the influence of the hub gene expression profile on colorectal cancer (Fig. 8). The findings demonstrated that all of the hub genes were highly expressed in the cancerous tissue, causing significant harm (Fig. 8).

Discussion

Because of the existence of various alkaloids in EREPL was confirmed through GC-MS. Plant secondary metabolites are well-classified and acknowledged as bioactive substances for both primary and secondary prevention in the treatment of cancer [12–14]. These substances influence metabolic and signaling pathways, limiting angiogenesis and inhibiting the development of microtubules in cells. The most important groups of secondary metabolites are called flavonoids, terpens, saponins, lignans, steroids, alkaloids, and phenolics. Glucosides are also included in this group. Because these plant-based bioactive substances have the much-needed geno-protective actions, such as protecting healthy cells from DNA damage, they can be used to create customized cancer prevention programs for either individuals or groups. Alkaloids are a promising group of phytochemicals that have anticancer properties [15]. Alkaloids are small organic compounds that often consist of nitrogen in a ring. They are secondary metabolites of plants. Alkaloids make up 20% of plant species. They play a key role in defending plants against diseases and herbivores [16].

For predicting the physicochemical and pharmacokinetic characteristics of piperlonguminine in the current study, Swiss ADME, pkCSM, and ProToxII were employed. For any drug, analysis of ADMET is essential [17, 18]. With regard to the pharmacokinetics and physicochemical characteristics of piperlonguminine found in EREPL, the current study's usage of ADMET revealed a good intestine absorption rate of 94.721%. Additionally, no carcinogenicity, hepatotoxicity, cytotoxicity or mutagenicity were detected by the Protox-II examination. In addition, piperlonguminine complies with the Lipinski's rule, according to ADME characteristics. The rule states that all drugs must meet the five essential physiochemical requirements. The parameters are: HBD should be less than five, HBA should be less than ten, MW should not be above 500 Daltons, lipophilicity (MLOGP) should not be above 5, and TPSA should be under 140 Å [19, 20]. Piperlonguminine met each of the five physiochemical criteria in the current study. The molecular weight is 273.33

g/mol, the number of HBA and HBD were three and one, respectively, the value of MLOGP and TPSA were 2.14 and 47.56 Å respectively.

Any naturally occurring compound must satisfy the druglikeness requirements in order to be classified as a successful drug [17]. Piperlonguminine is a member of benzodioxoles [21]. The benzodioxole ring system is extensively distributed in nature and can be found in a wide range of natural compounds, including safrole, piperonal, and several alkaloids [22]. Regarding piperlonguminine's ability to fight cancer, there is no information available. Using network pharmacology-based research, we hypothesised the effect of piperlonguminine in the EREPL on SRC, MTOR, EZH2, and MAPK3 that participate in the colorectal cancer pathway, ErbB, and mTOR pathway, which play an instrumental part in colorectal cancer. Two alkaloids were found in the ethanolic extract according to the GCMS results. We examined the toxicity, ADMET, and ADME characteristics of both alkaloids. Only piperlonguminine, which had neither toxicity nor any breaches of the Lipinski rule was chosen for further investigation. The canonical smile of piperlonguminine was entered in two online databases, the SwissTargetPrediction and TCMSP, in order to determine the possible targets. There were 100 protein targets from SwissTargetPrediction and 11 targets from TCMSP. DisGeNET, GeneCards, and the Open Targets Platform databases were used to screen the targets of colorectal cancer. DisGeNET, GeneCards, and Open Targets Platform yielded 2832, 24607, and 7383 target genes, respectively. The colorectal cancer target genes from DisGeNET, GeneCards, and Open Targets Platform were overlapped using Venny 2.1.0 to determine the intersection between them. There were 1865 common target genes in all. The 1865 target genes of colorectal cancer and the 111 target genes of piperlonguminine were then overlapped similarly. As a result, 51 common genes, SOAT1, NTRK1, PRF1, ESR2, MAPK3, IDO1, SRC, NR3C1, KDR, CDK2, HDAC6, TGFBR1, PIK3CD, AURKA, NAMPT, TERT, CDK1, TTK, HDAC1, RET, ABL1, MTOR, PARP1, PFKFB3, JAK2, IKBKB, KCNH2, CCKBR, PRKDC, PIK3CB, HCK, BRAF, EPHB4, EZH2, CDK4, CTSB, MAPK8, JAK3, HDAC3, ADORA2B, CFTR, DDR2, PDGFRA, CHEK2, CTSD, CSNK2A1, PTGER3, LIMK1, CHEK1, RXRA and SAA1, were obtained by employing Venny 2.1.0. The relationship between the 51 possible target genes were examined using a PPI network built using the STRING database. Selecting *Homo sapiens* as the organism all 51 targets were submitted to the multiple protein list of STRING. Using CytoNCA application from Cytoscape 3.2.1, the PPI network was provided to Cytoscape 3.2.1 in order to weed out the best four hub genes, SRC, MTOR, EZH2 and MAPK3. Hub genes are characterized by their extensive interconnection with other genes [17]. Topological considerations were used to screen-out hub genes. Supposedly, in light of the outcomes of molecular docking, piperlonguminine showed strong binding affinity with SRC, MTOR, EZH2, and MAPK3, representing that it has good remedial effects in the treatment of colorectal cancer.

Based on the findings of GLOBOCAN 2018, colorectal cancer (also known as CRC) is the third most deadly and the fourth most widespread kind of cancer in the humankind [23]. CRC risk is increased by a variety of factors. People over 50 are more likely to develop CRC, whereas those under 50 have a chance of 4% [24]. People in developing nations who have begun to follow Western diets tend to consume less fiber and more animal proteins and fat [25], therefore, becoming more prone to CRC. In addition to this people with history of CRC polyps [26], are equally prone. The risk of colon cancer can be increased by genetic disorders that have been passed down through the generations within the family. Type II diabetes [27], and a lack of exercise [28] can all raise the risk of colon cancer. Nevertheless the significant amount of work that has been put in over the past few years to develop diagnostics and therapies options, the prevalence of CRC remains extremely high in both women and men [29]. Depending on the stage of the cancer, radiation and/or neoadjuvant chemotherapy may be administered before or after surgery, even though surgery is the primary therapeutic option for patients with possibly curable CRC [4]. Despite these efforts, recurrent disease can develop in up to 30% of patients in stage I–III and more than 65% of patients in stage IV [30]. Due to acquired drug resistance, these traditional treatments are typically linked to high rates of tumor regrowth and recurrence [31]. Natural substances derived from plants, such as flavonoids [32], terpenoids [33], saponins [34], coumarins [35] and xanthenes [36], were described to impede CRC. The compound piperlonguminine was screened in the current investigation

because it did not violate Lipinski's rule and did not cause any toxicity. Subsequent research on piperlonguminine's network-based pharmacology has sought to determine how it affects CRC. The hub genes, SRC, MTOR, EZH2, and MAPK3 can communicate themselves at variable degrees in both healthy and colon cancer cells. More than 80% of human colon tumors have elevated SRC expression and activity. It is a non-receptor protein tyrosine kinase [37]. The upregulation of SRC in colon cancer cells increases the adhesion, invasion, and migration of cells [38]. SRC is required for the mTOR-dependent signaling pathway to function [39]. The often activated mTOR signalling pathway regulates synthesis of protein and transcription to regulate differentiation of immune cell and proliferation of cells. It significantly affects metabolism of tumour as well [40]. Downregulation of SRC is therefore important. In CRC, MTOR affects translational processes, cell adhesion, and metabolism. High tumor mutation burden (TMB) and a number of tumor-infiltrating immune cells (TIICs) are associated with high MTOR expression. MTOR acts as the chief regulator of mTOR signaling pathway [41]. This pathway is one of the most improperly regulated signalling pathways because it regulates transcription and synthesis of protein to control proliferation of cells and differentiation of immune cells, and also has a substantial impact on metabolism of tumour [40]. Therefore, downregulation of MTOR gene is crucial. MAPK3, also called as ERK1, is a crucial cell signaling protein in the ERK/MAPK pathway. MAPK3 causes phosphorylation of the downstream cytoplasmic protein of ERK/MAPK pathway, activating a number of nuclear transcription factors, like, c-fos and c-Jun, which in turn promotes cell expansion and death. The initiation, growth, migration of cancer cells, and resistance to drugs in a variety of carcinomas, including liver, thyroid, lung, and stomach malignancies, have all been linked to the overexpression and/or hyperactivity of MAPK3. Consequently, its downregulation is crucial [42]. EZH2 aids in the growth, invasion, apoptosis, angiogenesis, and metastasis of cancer cells. Numerous neoplasias affecting the ovaries, prostate, bladder and breast have been discovered to overexpressed it in their tumor tissues [43]. Increased expression of EZH2 is linked to CRC development. Early in the development of colorectal tumors, EZH2 expression occurs. Its overexpression has a role in early epigenetic changes that happen as adenoma development progresses from normal epithelial cells [44]. Therefore, making its downregulation necessary.

The ShinyGo 0.76 and DAVID databases were used to show the relationship between GO and KEGG. The target hub genes SRC, MTOR, EZH2 and MAPK3 are involved in a variety of cellular structure, cellular activity, and molecular roles in human development, according to the GO analysis. Using the DAVID web tool, the function of genes was displayed in the KEGG pathway maps. With the aid of the KEGG database, it is simpler to comprehend the fundamental properties and workings of living organisms, including organisms, cells and the environment. KEGG is a computational model of the biological system composed of genes, molecules, proteins, and chemical substances [45]. Results from the target-pathway network showed that 56 KEGG pathways were involved. The colorectal cancer pathway is the most important of the 56 KEGG pathways because, within it, nine other pathways, including cell cycle, apoptosis, Wnt, PI3K-Akt, MAPK, TGF- β , and mTOR signalling pathways, participate and serve an instrumental part in colorectal cancer (Fig. 6) [45]. The colorectal cancer pathway involves the two hub genes MTOR and MAPK3. ErbB and mTOR signalling pathways are two of the nine pathways that have been identified in the DAVID database. With SRC, MTOR, and MAPK3 in the ErbB signalling pathway and MTOR and MAPK3 in the mTOR signalling pathway, which can affect colorectal cancer, the colorectal cancer pathway is noteworthy due to the involvement of two or more of these hub genes in each of the two pathways. The EZH2 hub gene, on the other hand, is not found in any of the pathways that the DAVID database returned. The current study provides fresh insight into the effect of piperlonguminine on EZH2 in addition to its effects on SRC, MTOR, and MAPK3.

The top four hub genes, MTOR, SRC, MAPK3, as well as EZH2, were individually docked with piperlonguminine using Autodock 4 for disease-target network analysis. When docked with piperlonguminine, SRC protein displayed the highest binding energy (-7.22 kcal/mol), followed by MAPK3 (- 6.42 kcal/mol), EZH2 (- 6.3 kcal/mol), and MTOR (- 6.14 kcal/mol) (Table 6). The 3D and 2D pictures of the protein-ligand complex (Table 6) produced from docking were then visualized using Discovery Studio. The resultant amino acids of SRC, GLU A:162, TYR B:152, LYS A:198, interact with

the piperlonguminine through H-bond with a bond length of 2.89Å, 2.06 Å and 1.85 Å, respectively. Similar to this, H-bond interactions occur between the following amino acids of MAPK3: [ILE B:190 (3.39 Å)], EZH2: [ASN A: 119 (2.1 Å)], and MTOR: [GLN A: 2194 (3.24 Å), ASP A: 2191 (2.78 Å), TYR A: 2225 (2.96 Å), and ASP A: 2357 (2.47 Å)] (Fig. 7).

Conclusion

In summarized form, piperlonguminine is a significant alkaloid found in EREPL. On the basis of data of network pharmacology, molecular docking, and protein expression, it reduced CRC. According to the in-silico research, piperlonguminine decreased CRC cell growth and increased cellular death. It also influences migration and invasion by altering colorectal cancer pathway, ErbB and mTOR signaling pathways. To find potential hub genes for piperlonguminine, a network pharmacology technique is applied. SRC, MTOR, EZH2, and MAPK3 are significant hub genes that are connected to CRC. The CRC therapy will benefit from this research. In-silico data will be useful for the expansion of piperlonguminine-based medicines used to treat CRC during in-vivo and in-vitro studies.

Abbreviations

GC-MS	Gas Chromatography-Mass Spectrometry
EREPL	Ethanollic root extract of <i>Piper longum</i>
KEGG	Kyoto encyclopedia of genes and genomes
PPI	Protein–protein interaction
ADME	Absorption, distribution, metabolism, and excretion
HBD	Hydrogen bond donor
HBA	Hydrogen bond acceptor
TPSA	Topological polar surface area
DL	Druglikeness
MR	Molar refractivity
HIA	Human intestinal absorption
BBB	Blood–brain barrier
CYP2D6	CYP2D6 Cytochrome P450 2D6
CYP3A4	Cytochrome P450 3A4
hERG	Human ether-a-go-go related gene
LOAEL	Lowest-observed adverse-effect level
TCMSP	Traditional Chinese medicine systems pharmacology database and analysis platform
STRING	Search tool for the retrieval of interacting genes/proteins

GO	Gene ontology
DAVID	Database for annotation, visualization and integrated discovery
GEPIA	Gene expression profiling interactive analysis
MW	Molecular weight
ADMET	Absorption, distribution, metabolism, excretion, and toxicity

Declarations

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Author's contributions

IS and AK created the idea and layout for the work ,which IS, RD, and AK completed gathering information, analyzing it, and interpreting the findings, fractionation, and preparing the manuscript for submission. The manuscript has been read and approved by all of the authors.

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Data availability

The information that helps to back up the conclusions drawn from this study can be found in public databases.

Declarations

Conflict of interest

The author states unequivocally that they do not have any competing interests.

Ethical approval

Not applicable.

Informed consent

Not applicable.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Research involving human participants and/or animals

There are no experiments with human or animal subjects undertaken by the author in this paper.

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Figures

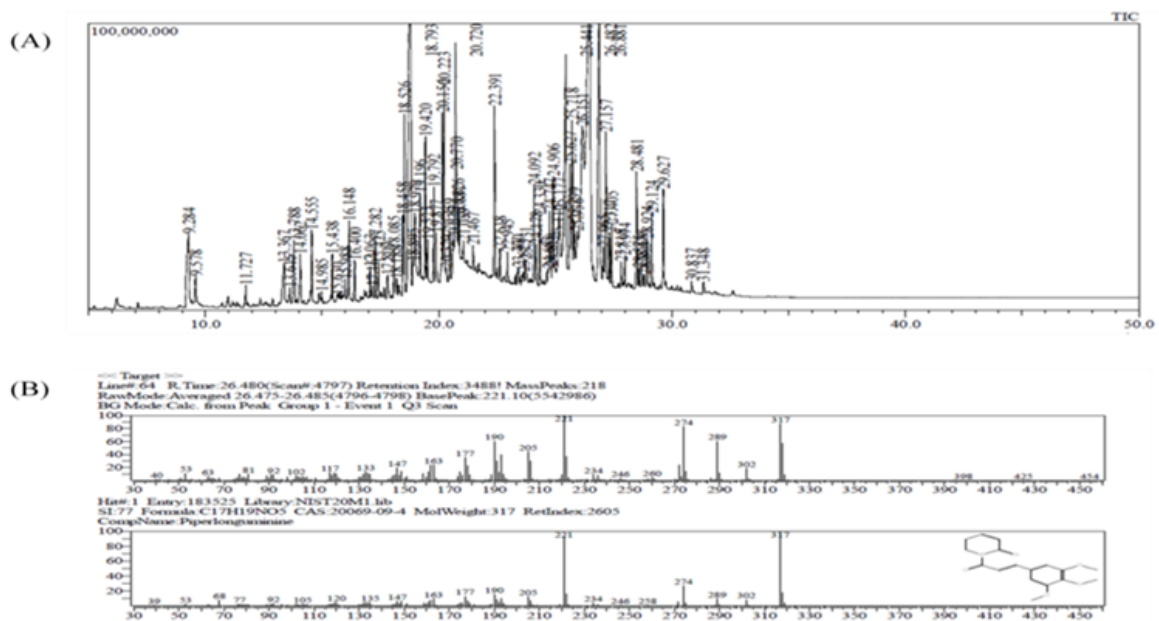


Figure 1

(A) GC-MS Chromatogram of ethanolic extract of *Piper longum* root (B) Piperlonguminine obtained through GC-MS.

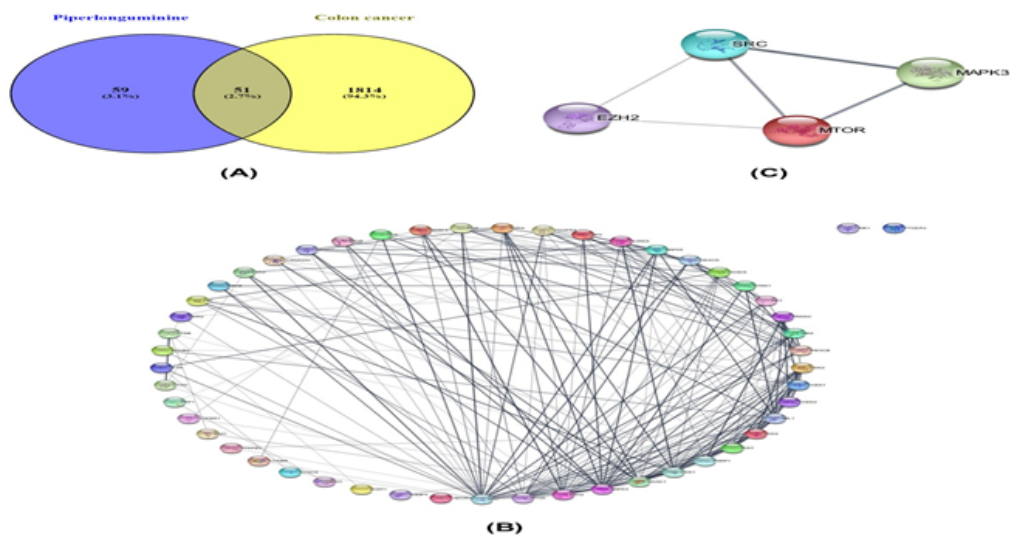


Figure 2

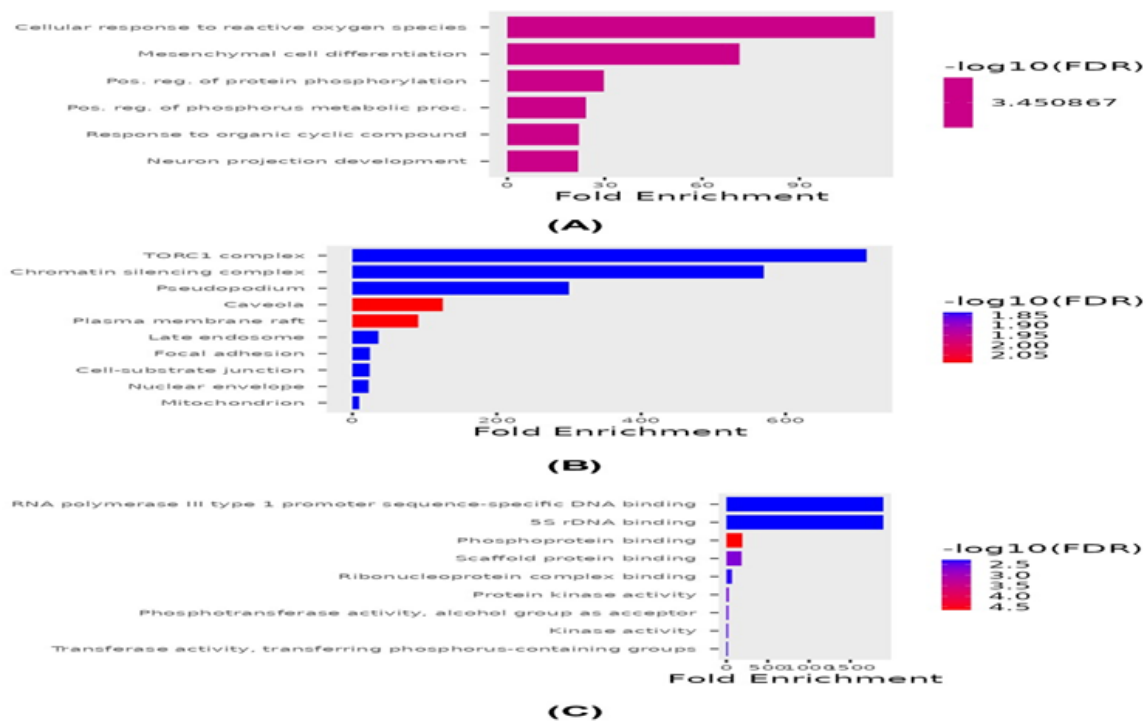


Figure 4

Gene ontology enrichment analysis of the top four hub genes, SRC, MTOR, EZH2 and MAPK3 in (A) Biological process; (B) Cellular component; (C) Molecular function

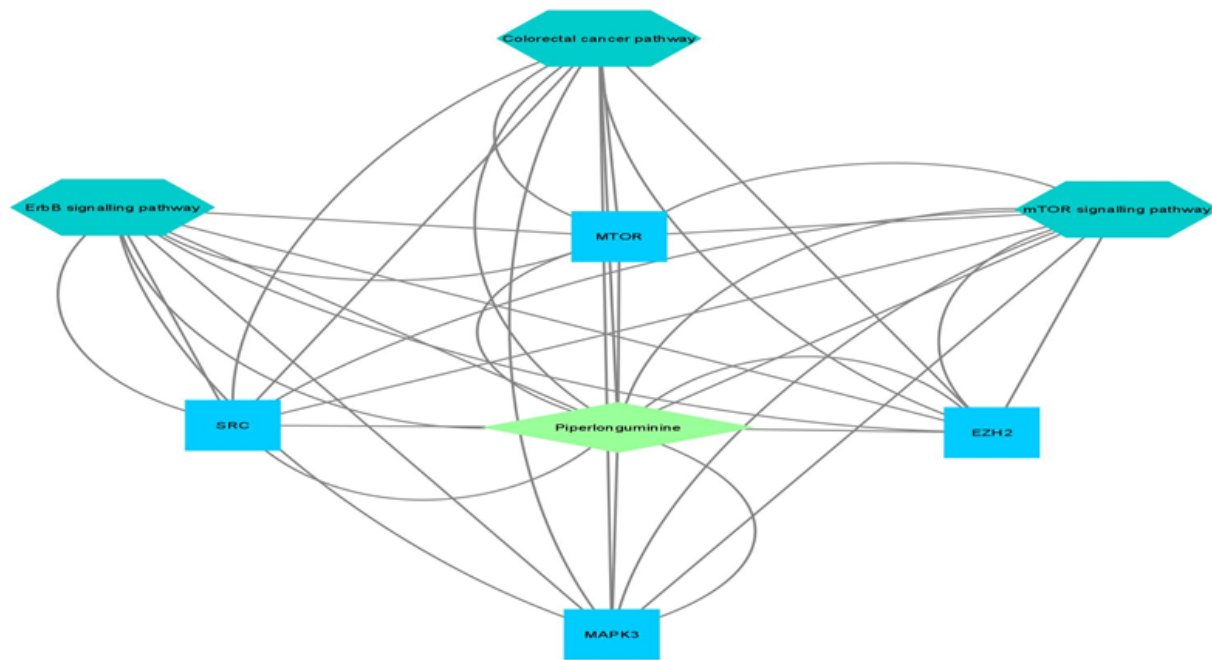


Figure 5

Disease pathway-target-compound network representing the involvement of hub genes, SRC, MTOR, EZH2 and MAPK3, in pathways involved in colorectal cancer

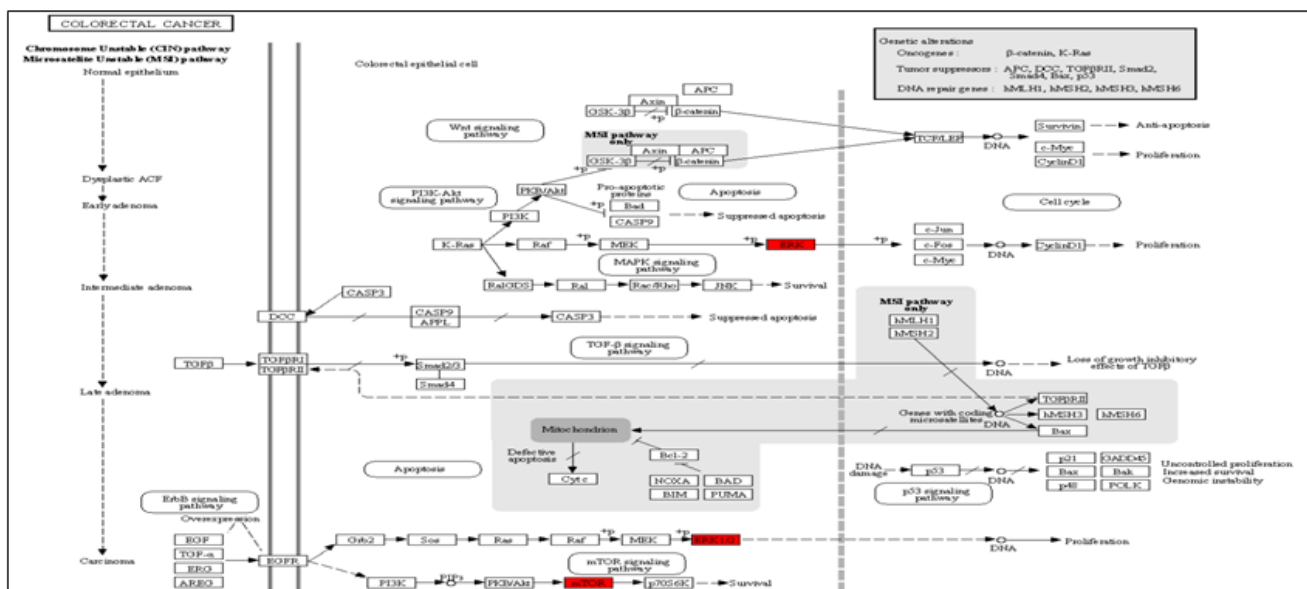


Figure 6

KEGG enrichment network of piperlonguminine representing the colorectal cancer pathway. This pathway contains 2 other pathways, ErbB and mTOR signaling pathways, that are involved in colorectal cancer. The 2 hub genes, MTOR and MAPK3 (in red) are involved in the colorectal cancer pathway

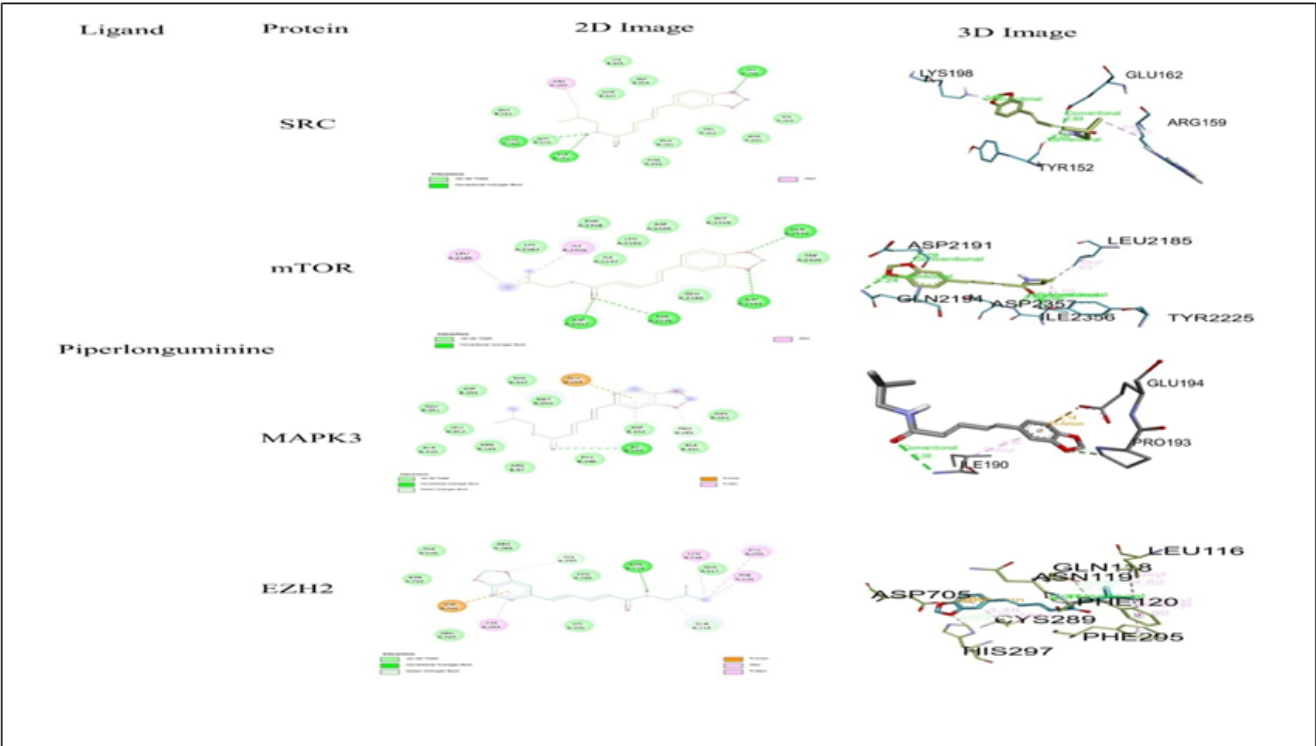


Figure 7

Molecular docking results showing interaction between the top four hub genes, SRC, MTOR, MAPK3, EZH2 (proteins) and piperlonguminine (ligand)

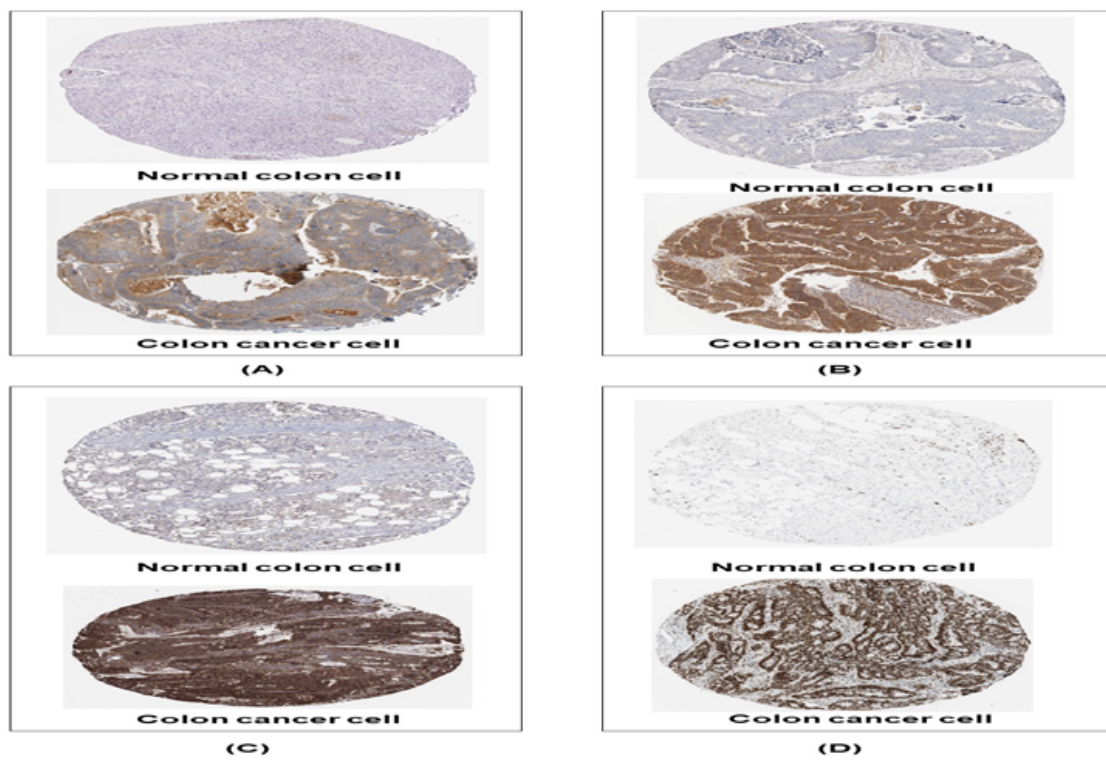


Figure 8

Protein expression of Hub gene in normal cell and cancerous cell of colon (A: SRC; B: MAPK3; C: MTOR; D: EZH2)