Research Article

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Integrating network pharmacology, in silico molecular docking and experimental validation to explain the anticancer, apoptotic, and anti-metastatic effects of cosmosiin natural product against human lung carcinoma

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Abstract

Objectives: This research aimed to examine the anticancer properties of cosmosiin, a natural flavonoid, on human lung carcinoma cells by in silico molecular docking, network pharmacology, and *in vitro* experiments.

Methods: The targets of cosmosiin and targets related to lung cancer were retrieved from various databases. The common targets between cosmosiin and lung cancer were identified by venny online server followed by construction of the protein-protein interaction (PPI) network. Further, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment were performed to identify main genes involved along with the signalling pathways affected. The hub genes were used for in silico molecular docking to identify molecular interaction between these targets and cosmosiin. *In vitro* experiments which consisted of MTT cell viability, clonogenic, cell apoptosis and cell migration assays validated the network pharmacology results.

Results: Network pharmacology identified 63 common targets between cosmosiin and lung cancer and out of these 63, eight protein targets were found to be most important based on their involvement in numerous signalling pathways in lung cancer. Two (NFKB1 and PIK3R1) out of eight targets showed highest degree values and were subjected to in silico molecular docking which showed cosmosiin showing strong binding the active sites of these two target proteins with PIK3R1 showing higher binding energy value (−9.7 kcal/ml) than NFKB1 (−7.5 kcal/mol). GO and KEGG enrichment analysis revealed key gene functions, molecular functions, cellular components as well as key signalling pathways involved in the treatment of lung cancer by cosmosiin. MTT and apoptotic assays indicated that cosmosiin induced concentration-dependent cytotoxic and apoptotic induction effects in A-549 human lung cancer cells respectively. Cell migration assay exhibited that cosmosiin treatment at varying doses led to a concentration-dependent suppression of cell migration hinting towards the anti-metastatic potency of cosmosiin against lung carcinoma.

Conclusions: In conclusion, the present study provides strong theoretical and experimental evidence of the anticancer, apoptotic and anti-metastatic potential of cosmosiin natural product against lung carcinoma along with the detailed mechanism of action involving various biological targets, cellular components and signalling pathways.

Keywords: cosmosiin; lung cancer; in silico molecular docking; network pharmacology; apoptosis

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Introduction

Globally, lung cancer continues to be the primary cause of cancer-related fatalities with a significant impact on the global disease burden; the most common subtype being non-small cell lung carcinoma (NSCLC). It is one of the most

prevalent and lethal forms of malignancy worldwide. According to a report, lung cancer was the most frequently diagnosed cancer in 2022 accounting for about 2.5 million new cases which makes it 12.4 % of all cancers worldwide [1]. China bears a disproportionately high burden of lung cancer, with roughly one-third of cases worldwide. High smoking rates, industrial pollution, and pervasive indoor air pollution from the use of coal and biomass fuels are the main causes of the high prevalence in China. Strict anti-smoking laws and public health initiatives are among the measures used to fight this, but difficulties still exist because smoking rates are rising among women and men [2, 3]. The treatment of lung cancer involves a wide spectrum of approaches depending on the type and stage of the disease and health status of the patient. Surgery still remains a principal approach for localized cancer followed by chemotherapy and radiotherapy in case surgical procedure is not a viable option. The latter two methods are often utilised both before and after the surgery to enhance the efficacy of the treatment and control metastasis. The development of immunotherapy, which uses drugs like nivolumab and pembrolizumab to increase the immune system's capacity to combat cancer, has completely changed the way advanced lung cancer is treated. This is especially true for patients with high tumour mutational burden or high PD-L1 expression. However, due to the high cost and unwanted side-effects, adverse reaction events associated with the above-mentioned treatment modalities, there is a pressing need for novel and cost-effective treatment regimens needed for this deadly cancer [4–6].

Natural products have always played a crucial role in anticancer drug discovery with majority of the chemotherapeutic agents clinically used against various cancers derived from natural products or their derivatives. Flavonoids comprises of a huge and structurally diverse group of polyphenolic compounds found in many vegetables, fruits and nuts. This group of natural products has received significant attention in lung cancer research due to their ability to inhibit cell proliferation, induction of apoptosis and targeting of many signalling pathways in cancer. Some flavonoids also have shown promise in improving chemotherapy treatment as an adjuvant therapy by increasing the efficacy of the clinical drugs [7–11]. Cosmosiin, also known as apigetrin, has been shown to exhibit anticancer effects in many cancers targeting diverse biochemical pathways including Improving T-Cell Activity by blocking PD-1/PD-L1 Interaction in humanized PD-1 Mouse Model, promoting TNFα-induced apoptosis, necroptosis, ROS generation, induction of cell cycle arrest, inhibition of NF-kB pathway, inhibition of AKT pathway [12–14]. However, use of network pharmacology, in silico molecular docking and experimental methods for evaluating anticancer effects of cosmosiin in human lung cancer is not reported so far to the best of our knowledge. Therefore, the main objective of the current study was to investigate the anticancer effects of cosmosiin in human lung cancer cells by using systems biology-based network pharmacology approach which reveals relationship between the drug and its biological targets making it a significant tool in identifying the anticancer mechanism of action.

Network pharmacology has emerged as a powerful tool in anticancer research, particularly in the exploration of natural products. Network pharmacology has revolutionized the area of Traditional Chinese Medicine (TCM) by discovering intricate relationships between multi-component herbal formulas and their molecular targets, thus allowing a systems-level understanding of their effects on therapy. It represents a way of integrating modern bioinformatics and pharmacology into evidence-based medicine from traditional practice. Network pharmacology maps the complex interactions occurring between the drug and its biological targets elaborating its crucial protein targets and pathways involved. Network pharmacology constructs interaction network between the drug, its biological protein targets and the pathways involved which for instance helps to understand how these drugs modulate various biological process like cell cycle, apoptosis, metastasis, necrosis etc. Network also helps in identifying target genes and constructs network between the genes and pathways which involves gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis. Network pharmacology, by integrating biological data and computational methods, is especially helpful in identifying the multiple targets of natural products and complex plant extracts which so far has been a daunting task. Network pharmacology is a significant milestone in the anticancer research of natural products because these exhibit anticancer action by targeting a number of biological targets simultaneously and all these complex and intricate interactions between the anticancer biological targets and natural product can be mapped in great detail by using network pharmacology. This holistic approach is very significant in cancer research because cancer is a very complex disease driven by multifaceted genetic and epigenetic alterations [15–18]. In addition to carrying out network pharmacology analysis and in silico molecular docking, we also verified these findings with experimental analysis involving demonstrating inhibition of cancer cell viability by MTT assay, apoptosis induction by annexin-V FITC assay using flow cytometry and inhibition of cell migration by transwell migration assay.

Materials and methods

Identification of cosmosiin and lung cancer targets

Bioavailability, druglikeness, blood brain barrier (BBB) and gastrointestinal absorption parameters were obtained by entering the cosmosiin SMILE formula, which was obtained from https://pubchem.ncbi.nlm.nih.gov/, into the https://www.swissadme.ch/index.php server. Additionally, the Swiss Target Prediction database (https://www.swisstargetprediction.ch) and the Super-PRED server (https://prediction.charite.de/index.php) furnished the biological targets of cosmosiin. Utilising UniProt (https://www.uniprot.org), the anticipated target names and IDs were obtained. We acquired the target genes linked to lung cancer from the Gene Cards database (https://www.genecards.org) with lung cancer as "keyword". Using Uniprot data, the obtained gene names were changed to their official names (preferred names). The Venn diagram from the venny (2.1) online tool was used to intersect/overlap the obtained cosmosiin and lung cancer targets. The lung carcinoma targets that overlapped or intersected with the cosmosiin targets were identified as anti-lung carcinoma cosmosiin targets and were subjected to further network analysis.

Construction of target genes/protein interaction network

The overlapping/intersected genes were subsequently imported into the STRING database (https://string-db.org), which is a search tool for the retrieval of interacting genes/proteins. This allowed for the construction of protein-protein interaction (PPI) networks that met the qualifying condition of having a composite score greater than 0.4 in order to obtain protein-protein interaction networks. Importing the protein interaction network into Cytoscape 3.10.2 software allowed the identification of the key genes. We created and examined a hub-genes diagram using the Cyto Hubba plugin in Cytoscape software.

Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis

GO and KEGG pathways were identified to predict the potential targets of cosmosiin against lung cancer. The GO functional enrichment analysis was carried out to determine the roles played by target genes of cosmosiin along with differential gene enrichment analysis. The KEGG pathway enrichment was performed for determining genes involved and their biological pathways. The enrichment analysis of common targets was performed on the ShinyGO 0.80 (https://bioinformatics.sdstate.edu/go/) online tool. The common targets between cosmosiin and lung cancer were uploaded and analysed. The enriched pathways were sorted based on their importance.

In silico molecular docking analysis

The 3D chemical structure of cosmosiin was obtained from PubChem database. The crystal structures of two most significant protein targets namely NFKB1 and PI3K were obtained from the protein data bank (https://www.rcsb.org/) with PDB IDs as 1svc and 4jps respectively. Cosmosiin was docked onto these two protein targets for determining the molecular interactions between cosmosiin and the target proteins along with evaluating their binding energy values. PyMOL software was used to optimise the protein structures prior to docking in order to remove any associated ligands, heteroatoms, and water molecules. Then, for docking, AutoDock Vina version 1.2 was utilised, and for analysis and visualisation, BIOVIA Discovery Studio 2022 was employed. In addition to identifying the active cavities on the target proteins, the cartoon surface representations were obtained using the CB-DOCK2 (https://cadd.labshare.cn/cb-dock2/index.php) internet server. The significant advantage of CB-DOCK2 is that it supports blind docking, identifying the binding pockets without any knowledge about the active site. The structure-based and template-based docking methods are combined for increased accuracy and efficiency. It features rapid cavity detection, interactive 3D visualizations, and customizable input/output interfaces, which are user-friendly and adaptable to complex tasks in drug discovery.

Chemicals and reagents

The cosmosiin (98 % purity by HPLC) molecule under investigation was sourced from BiobioPha, Kunming, Yunnan, China. MTT dye, DMSO, RPMI medium, DMEM, and FBS were procured from Sigma-Aldrich, St Louis, USA. All additional chemicals and reagents were obtained from Sigma-Aldrich, St. Louis, USA, or as otherwise specified.

MTT colorimetric assay for evaluating cell viability

The A-549 human non-small cell lung cancer cells were cultured in RPMI medium (Sigma) overnight. The medium was supplemented with 10 % fetal bovine serum (FBS) and 1 % penicillin-streptomycin (Gibco). Cells were maintained in a CO_2 incubator at 37 °C with 5 % CO_2 levels. The A-549 cells were observed for viability after being exposed to varying concentrations of cosmosiin (0, 10, 20, 40 and 80 μM) for 24 and 72 h using the MTT assay. After cosmosiin treatment, cells were cultured with MTT (10 mg/mL) for 6 h in 96-well plates with 10^4 cells/well. After dissolving the Formazan crystals in DMSO, the absorbance at 540 nm was measured using a microplate reader (Varioskan™ LUX, Thermo Scientific™, Waltham, MA, USA). Viability was expressed as a percentage with 100 % serving as the reference point for control cells treated with 0.1 % DMSO.

Apoptosis assay using annexin V and flow cytometry

In order to determine the number of apoptotic cells after cosmosiin treatment, human lung carcinoma cells were analyzed by flow cytometry using annexin V-FITC/PI apoptosis detection kit (KeyGen Biotech Co. Ltd. Najing, China). After treatment with increasing concentrations of cosmosiin viz 0, 20, 40 and 80 μM, staining of cell cultures was performed using annexin V-FITC and PI (10 μL each) in a dark room at room temperature for about 10 min. Finally, using (FACSCalibur, BD Biosciences, San Diego, CA, USA) each sample was analyzed to quantify apoptosis in these cancer cells.

Transwell assay for cell migration evaluation

The transwell migration assay was used to evaluate the anti-migratory effects of cosmosiin in A-549 human lung cancer cells. Matrigel-coated transwell chambers with membranes with an 8 μm pore size were used. The upper chambers were seeded with A549 cells (1×10^6 cells) in serum-free culture media, and the cells were subjected to different concentrations of cosmosiin treatment (0, 20, 40, 80 μM). Dulbecco's Modified Eagle Medium (DMEM) medium with 25 % FBS was poured into the lower chambers. Non-migratory cells were removed from the membrane's upper surface after a 24 h incubation period, and migrated cells were fixed and stained with crystal violet. The stained cells were then counted under a light microscope (Zeiss, Germany).

Western blotting

Lung cancer cells (A549) were treated with varying concentrations of cosmosiin (0, 20 μM, 40 and 80 μM) for 24 h. Total protein lysates were extracted using RIPA buffer with protease and phosphatase inhibitors. Equal amounts of protein (30 μg) were separated by SDS-PAGE and transferred onto PVDF membranes. The membranes were blocked with 5 % non-fat milk and incubated overnight at 4 °C with primary antibodies against PIK3R1 and NFKB1, followed by HRP-conjugated secondary antibodies. Protein bands were visualized using enhanced chemiluminescence (ECL) and quantified using ImageJ software, with β-actin as the loading control.

Statistical analysis

The data were analyzed by GraphPad Prism 5 (San Diego, USA) and represented as mean ± SD, the statistical analysis was performed by one-way ANOVA followed by Turkey's comparison test. Statistical differences between different groups were defined at $*p<0.05$, $**p<0.01$.

Results

ADME properties and target proteins of cosmosiin

The physicochemical properties (ADME) of cosmosiin, such as its bioavailability, GI absorption, BBB, druglikeness, and partition coefficient, were obtained using the SwissADME online server. While GI absorption was found to be poor, bioavailability was found to be 0.55, which is good for a drug candidate. Pro-Tox 3.0 online server predicted no major toxicity associated with cosmosiin hinting at its safe nature. Table 1 and Figure 1A–C represent the ADME properties and toxicity of cosmosiin molecule respectively. In network pharmacology, the physicochemical characteristics and toxicity profiles of drugs are utilised as screening criteria. Super-PRED and Swiss Target Prediction databases were used for identifying the target proteins of cosmosiin and a total of 103 targets were initially identified.

Identification of lung cancer targets

Data on "lung carcinoma" which was used as the keyword were acquired from GeneCards database. GeneCards provides intelligible and in-depth information on all annotated

Table 1: Physicochemical properties (ADME) and toxicity of cosmosiin were calculated using SwissADME and ProTox 3.0 servers respectively.

Molecular property	Value
Molecular weight	432.38 g/mol
Bioavailability	0.55
GI absorption	Low
BBB permeant	No
Octanol/water partition coefficient (logP)	5.88
Lead likeness violations	1, MW>350
Lipinski violations	1, yes; 1 violation: NH or OH>5
Number of hydrogen bond acceptors	10
Number of hydrogen bond donors	4
Topological polar surface area, TPSA	170.05 Å^2
Hepatotoxicity	No
Neurotoxicity	No
Nephrotoxicity	Mild active
Immunotoxicity	No
Mutagenicity	No
Cytotoxicity	No

and predicted human genes and automatically integrates genomic, proteomic, genetic, transcriptomic, functional and clinical data. A total of 25,000 lung carcinoma related targets were identified which after applying 55 % gift score filter was reduced to 1,357.

Identifying common targets of cosmosiin and lung carcinoma using venny 2.1 online tool

The individual targets of cosmosiin and lung carcinoma were fed into venny online tool in order to find the common targets between the two and it was found that 63 targets were found to be common between the two which accounts for 4.5 % of the total targets. These common targets have a high probability of being involved in the lung cancer initiation and progression and using cosmosiin as a drug, these targets can be modulated in order to bring a therapeutic effect. Further analysis of these 63 common targets was performed in order to construct a protein-protein interaction (PPI) network. Figure 2A shows the Venn diagram displaying the common targets between cosmosiin and lung cancer along with displaying the PPI network.

Construction of protein-protein interaction (PPI) network and identification of hub genes

In order to construct and analyse the PPI network, the 63 common genes were fed into the STRING database. As is

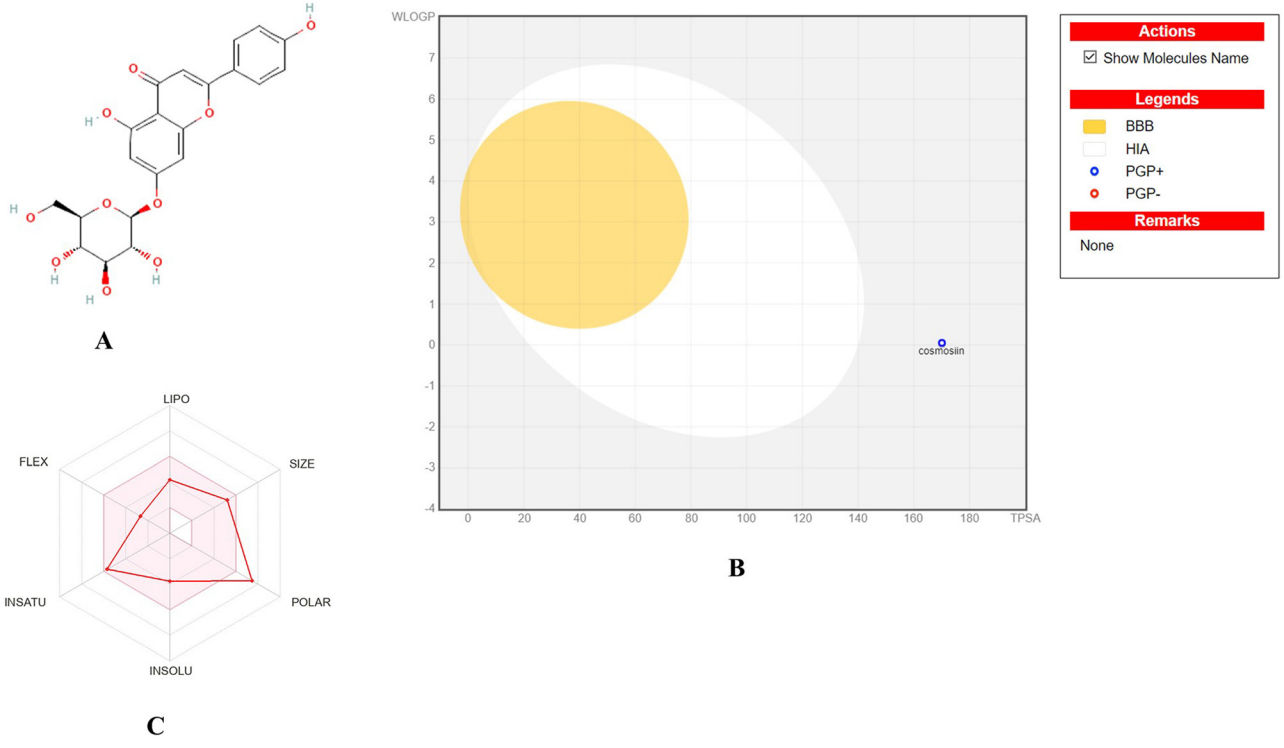

Figure 1: Prediction of physicochemical properties of cosmosiin using SwissADME server. (A) Shows the molecular structure of cosmosiin, (B) shows the BIOLED-Egg model and (C) shows the radar of physicochemical properties of cosmosiin.

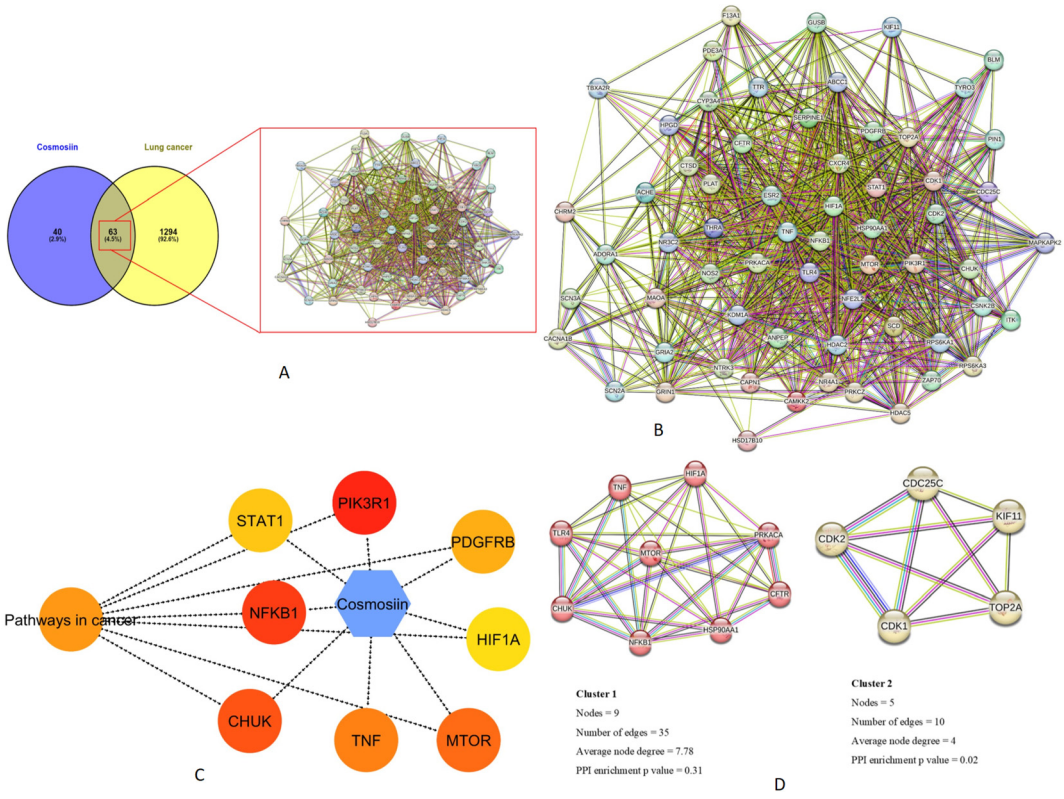

Figure 2: Network pharmacological analysis of cosmosiin molecule. (A) Representation of the common targets between cosmosiin and lung cancer using a venn diagram along with network showing protein-protein interaction (PPI). (B) The protein-protein interaction (PPI) network of the 63 common targets between cosmosiin and lung carcinoma, displaying the nodes, edges and degree of each protein target. The higher the degree of a node within a network, the greater will be its significance within that network. The number of nodes was 63 and the number of edges were found to be 879, and the average node degree was 27.9 with a PPI enrichment p value of 1.0e-16. (C) Construction of hub gene network and identification of top eight hub genes and their target pathway involved in lung carcinoma. PIK3R1 with node degree of 33, NFKB1 (node degree of 30), CHUK (node degree of 24), MTOR (node degree 22), TNF (node degree 16), PDGFRB (node degree 13), STAT1 (node degree 12), HIF1A (node degree 11). (D) Cluster analysis of the protein modules within the protein-protein interaction (PPI) network. Two cluster modules were identified which consisted of 14 important protein targets including NFKB1, TNF, MTOR, HIF1A, CHUK etc.

evident from the figure (Figure 2B), there were 63 nodes, 879 edges and the average node degree was found to be 27.9. While as nodes in a PPI network represent target proteins, the edges show relationships/connectivity between the proteins. Target proteins with higher degree value means that protein is more important in that PPI network. This network exhibited unusually higher number of nodes than expected for a random set of proteins of the same size and degree distribution. This holds importance as the target proteins in such network are expected to be much more connected biologically. Hub genes show high degree values indicating that these are connected with many other genes and as such play a key role in the biological process and gene regulation. This is why hub genes are considered to be most closely related with the disease. Cyto Hubba plugin was used to recognize the hub genes and the top eight hub genes were found to be PIK3R1 with node degree of 33, NFKB1 (node degree of 30), CHUK (node degree of 24), MTOR (node degree 22), TNF (node degree 16), PDGFRB (node degree 13), STAT1 (node degree 12), HIF1A (node degree 11) (Figure 2C). Hub genes display better network topological characteristics like degree, BC and CC and that is why these genes are considered to be crucial targets in any PPI network. These eight hub genes will have a defining contribution in the anticancer action of cosmosiin. As can be seen from Figure 2C, all these eight genes are having a definite contribution in the cancer pathways modulated by cosmosiin in lung cancer. Figure 2D shows the cluster analysis of the protein modules within the PPI network and shows two important protein clusters nine and five nodes respectively. Cluster modules within the PPI network indicate most important protein targets.

Gene Ontology (GO) enrichment analysis

The Gene Ontology (GO) enrichment analysis was performed in order to understand the mechanism of action of cosmosiin in human lung carcinoma cells and to identify which genes

are involved in there. This enrichment analysis was done using 63 potential biological targets of cosmosiin in the lung carcinoma treatment. The GO enrichment analysis comprises of three groups including BP (biological process), MF (molecular function) and CC (cellular component) and our findings reveal that we identified 15 each of BP, MF and CC shown in Supplementary Figure 1A–C as a bubble plot. The bubble size and color determine number of enriched genes and p value respectively. A larger bubble within a given GO term implies a higher number of enriched genes, and a redder colour suggests a lower p value. These findings suggest that the treatment of lung cancer is significantly more strongly associated with that specific GO term than it is with other GO terms. The main BP processes were response to oxygen containing compounds, cellular response to oxygen containing compound, response to organic substance. The main enriched MF were small molecule binding, nucleotide binding, nucleoside phosphate binding, kinase activity, anion binding, tyrosine kinase activity etc. The main enriched CC were intracellular vesicle, cytoplasmic vesicle, cell junction, nuclear lumen, receptor complex among others.

KEGG enrichment analysis

In order to identify the potential metabolic pathways associated with the therapeutic targets of cosmosiin in treating lung cancer, KEGG pathway enrichment analysis was carried out using DAVID and ShinyGO 0.80 databases. We identified 25 signalling pathways and were plotted in the form of a bubble plot as depicted in Supplementary Figure 2. The sorting of the pathways was done on the basis of p value and number of enriched genes indicated by color and size of the bubble, redder color meaning lower p value and larger bubble size depicting higher number of enriched genes. The main enriched signalling pathways were pathways in cancer, PD-L1 Expression and PD-1 checkpoint pathway in cancer, prostate cancer, Th17 cell differentiation, HIF-1 signalling pathway, small cell lung cancer, PI3K/Akt signalling pathway, CAMP signalling pathway, calcium signalling pathway, viral carcinogenesis, chemical carcinogenesis-receptor activation etc. The gene mapping diagrams of the key targets involved in the two most important signalling pathway viz., PD-L1 Expression and PD-1 checkpoint pathway in cancer and pathways in cancer are shown in Supplementary Figure 3A and B.

Network construction between cosmosiin-potential biological targets-metabolic pathways using cytoscape

Cytoscape 3.10.2 software was used to construct a comprehensive network depicting the relationship between cosmosiin, its biological targets and signalling pathways affected. This visual network is shown in Figure 3A and shows the central yellow colored hexagon depicting cosmosiin, green-colored circles represent the biological targets of cosmosiin and the yellow outer circles represent the biological pathways affected through these biological targets of cosmosiin. The network consists of 153 number of nodes, with 376 number of edges with average number of neighbors as 4.91. Top eight targets were found to be PIK3R1 with node degree of 33, NFKB1 (node degree of 30), CHUK (node degree of 24), MTOR (node degree 22), TNF (node degree 16), PDGFRB (node degree 13), STAT1 (node degree 12), HIF1A (node degree 11) and the most affected biological pathways included pathways in cancer, PD-L1 Expression and PD-1 checkpoint pathway in cancer, prostate cancer, Th17 cell differentiation, HIF-1 signalling pathway, small cell lung cancer, PI3K/Akt signalling pathway, CAMP signalling pathway, calcium signalling pathway, viral carcinogenesis, chemical carcinogenesis-receptor activation. The biological targets of cosmosiin are shown in Figure 3B.

In silico molecular docking analysis

The two main biological targets of cosmosiin identified by network pharmacology procedure viz., NFKB1 and PIK3R1 were subjected to in silico molecular docking using cosmosiin as a target ligand for binding and the binding affinities were evaluated. As shown in Supplementary Figure 4(A–E), cosmosiin binds strongly with the active centre of the NFKB1 target protein exhibiting a binding energy value of −7.5 kcal/mol indicating a very strong binding between the target protein and the ligand. The following amino acid residues of the target protein were involved in interaction with the cosmosiin molecule: ARG57 PHE58 ARG59 TYR60 GLU63 HIS67 HIS144 LYS147 GLU207 MET208 ASP209 LEU210 SER211 VAL212 TYR241 ASP242 SER243 LYS244 ALA245 PRO246 ASN247 SER249 ASN250 ASP274 LYS275 GLN277 ARG308 GLN309 PHE310 SER338. Supplementary Figure 4A shows the active cavity site of the target protein NFKB1 in ribbon model, B and D show the protein surface along with its grooves and cavities as cartoon representation exhibiting

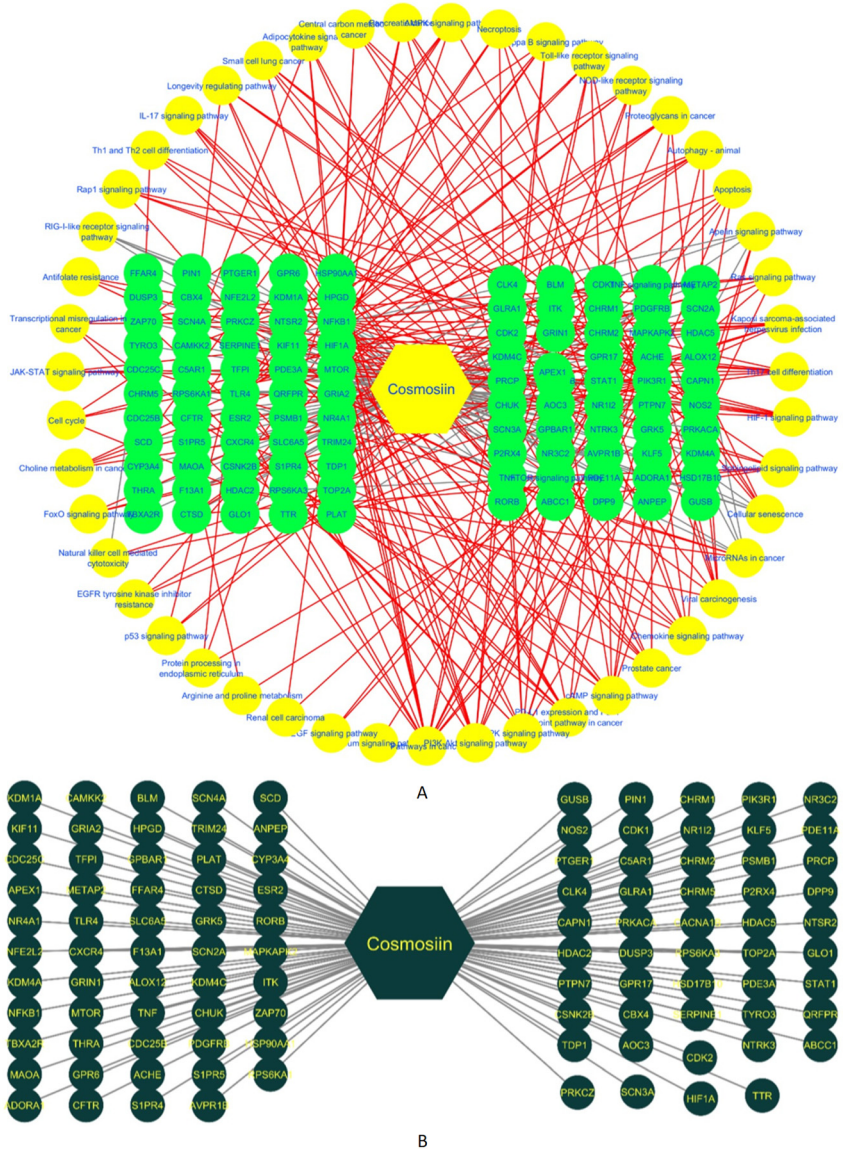

Figure 3: Cytoscape analysis of the main compound-biological targets-signalling pathways. (A) Comprehensive network depicting the relationship between cosmosiin, biological targets and signalling pathways affected, the network was constructed by using Cytoscape 3.10.2. The central yellow colored hexagon shows cosmosiin, green colored circles represent the biological targets of cosmosiin and the yellow outer circles represent the biological pathways affected through these biological targets of cosmosiin. The network consists of 153 number of nodes, with 376 number of edges with average number of neighbors as 4.91. (B) Network construction between the cosmosiin and its biological targets using Cytoscape 3.10.2.

cosmosiin being embedded in the active site, C shows 2D-representation showing key molecular interactions between the amino acid residues of the active site and the cosmosiin ligand, E shows 3D-representation of the target protein with bound cosmosiin ligand exhibiting various interactions. Similarly, Supplementary Figure 5(A–E) show different perspectives of the binding interaction between cosmosiin and the target protein (PIK3R1) exhibiting a binding energy value of −9.7 kcal/mol indicating a very strong binding and hence better inhibition of this target protein. The following amino acid residues of the target protein were involved in interaction with the cosmosiin molecule: ASN170, GLU172, GLU259, LYS271, TYR272, SER275, MET278, ASP626, LYS627, LEU628, SER629, GLN630, LEU632, ILE633, ARG662, ILE663, PHE666, HIS670, LEU755, ASN756, PRO757, ALA758, HIS759, ASP810, MET811, LEU814, GLN815, ILE816, ARG818, GLU821, ASN822, GLN825, PRO835, TYR836, GLY837, CYS838, LEU839, GLU849. Supplementary Figure 5A displays the cavity position of the target protein PIK3R1 in ribbon model, B and D show the cartoon representation of the protein surface with clearly visible grooves and cavities along with showing bound cosmosiin ligand, C shows 2-D while E shows 3D-representation of the target protein along with bound cosmosiin ligand. As can be clearly seen from Supplementary Figure 5, a higher number of hydrogen bond interactions can be seen between the ligand and the target protein leading to higher binding energy value of −9.7 kcal/mol. Thus cosmosiin binds much more strongly with PI3KR1 as compared to NFKB1. The binding efficacy of cosmosiin with both NF-KB1 and PI3KRI was compared with

two known reference inhibitors which are known to inhibit these protein targets. These two inhibitors included tribromsalan which is a known inhibitor of NFKB1 while as umbralisib is a known inhibitor of PIK3R1. Tribromsalan showed a binding score of −10.5 kcal/mol with NFKB1 while as umbralisib exhibited a binding score of −9.7 kcal/mol with PIK3R1 indicating that cosmosiin can also bind strongly with these two protein targets since it also shows comparable docking scores.

Experimental validation

MTT cell viability and apoptosis assays

The network pharmacology and in silico molecular docking results were validated through the intervention of MTT cell viability and apoptosis assays. MTT assay using different concentrations of cosmosiin and different time intervals viz., 24 and 72 h was performed and results are shown in Supplementary Figure 6. The results indicate that cosmosiin induced concentration-dependent as well as time-dependent cell viability inhibition in A-549 human lung carcinoma cells. Apoptosis assay using annexin V and flow cytometry indicated that cosmosiin has the ability to induce dose-dependent early and late apoptotic events in these cell lines. The results of this assay are shown in Supplementary Figure 7 and reveal that cosmosiin led to a dose-dependent increase in the early and late apoptotic cells. The effect was much more pronounced at 80 μM dose where it was seen that the apoptotic cell population in both early and late apoptotic phases increased significantly. Thus, this assay coupled with MTT assay indicate that the cell inhibition effect induced by cosmosiin was due to the induction of apoptosis which is basically a programmed cell death. These findings further substantiate the results obtained through network pharmacology, which indicated that apoptotic induction is a key cellular process affected by cosmosiin.

Cosmosiin inhibited cell migration in lung carcinoma cells

Transwell migration assay was performed to evaluate the anti-migratory effects of cosmosiin on the migration tendency of A-549 lung cancer cells and it was observed that cosmosiin also induced a significant and concentration-dependent suppression of cell migration in these cells. Supplementary Figure 8A shows the impact of increasing concentrations of cosmosiin on the cell migration in these lung cancer cells. Supplementary Figure 8B shows the graphical representation of the cell migration inhibition effect of cosmosiin in A-549 lung cancer cells. The effect was observed to be much more pronounced at 40 and 80 μM concentration. This signifies that cosmosiin can be a potential drug candidate for inhibiting cancer cell metastasis in lung cancer provided further studies are carried out.

Cosmosiin led to the downregulation of two key hub proteins (NFKB1 and PIK3R1)

In order to experimentally validate the involvement of the hub protein targets in lung cancer cells, and the role played by cosmosiin treatment in modulating their expression, we performed western blot assay which indicated that cosmosiin treatment led to a concentration-dependent downregulation of these two key target proteins (Supplementary Figure 9). The decrease in the protein expression was much more pronounced linear in case of PIK3R1 than NFKB1. Beta actin was used as a normalisation control.

Discussion

Network pharmacology has become a paradigm-shifting methodology in the study of different cancers because it offers a thorough framework for comprehending the complex relationships that exist within biological systems. Through the integration of network analysis and pharmacological data, this technique facilitates the holistic explanation of drug action mechanisms and the identification of novel therapeutic targets. The ability of network pharmacology to map the complex and varied molecular interactions involved in cancer is one of its main advantages. Traditional drug research frequently concentrates on single targets which is not the case in case of cancer, because it is a very complex disease involving various biological targets and varied types of signalling pathways. Owing to the intricate interdependencies among the molecular constituents of a human cell, abnormalities in a single gene seldom result in a disease; instead, it is indicative of disruptions within the intricate intracellular network. A more accurate depiction of the cancer network is offered by network pharmacology, which takes into account the connections between several genes, proteins, and metabolites. Researchers can find important nodes and pathways that are essential for the progression of cancer and may be used as possible treatment targets by building and examining these networks [19–21].

The present study underlines the therapeutic potential of cosmosiin in lung cancer by utilizing network pharmacology and molecular docking approaches to clarify the mechanisms of action. Cosmosiin is a natural flavonoid that

has been reported to have anticancer properties, showing excellent bioavailability but poor gastrointestinal absorption according to SwissADME screening. Nonetheless, because of its safety profile and minimal toxicity, its bioactive potential demands further investigation. The findings demonstrate a promising therapeutic role in the targeting of lung cancer-associated pathways and gene networks via cosmosiin.

Network pharmacology insights into the mechanism of action of cosmosiin include identification of 63 cosmosiin-related targets and 1,357 lung carcinoma-related targets for further extraction of 63 overlapping targets, which depicts specificity and potential in modulating lung cancer-associated processes. These shared targets are likely to be involved in the pathogenesis and progression of lung cancer and are therefore critical candidates for therapeutic intervention. The PPI network analysis is quite complex, showing two hub genes, NFKB1 and PI3KR1, to be central players in the network.

NFKB1 is an important transcription factor in the NF-κB pathway and mediates both inflammatory responses and cell survival and proliferation [22]. NF-κB aberrant activation has also been associated with lung carcinoma in relation to the features of tumor progression, immune evasion, and resistance to apoptosis [23]. In addition, PI3KR1 is a part of the signaling complex of PI3K/Akt that controls cell growth, metabolism, and survival [24]. It has an inherent feature of being over-expressed in cancer that contributes to inappropriate cell proliferation and evasion from apoptosis [25]. These genes' identification as hub targets thus underscores their pivotal positions in lung cancer biology and the tremendous therapeutic potential.

The molecular docking studies showed a strong binding of cosmosiin both to NFKB1 and to PI3KR1 by −7.5 kcal/mol and −9.7 kcal/mol, respectively. A stronger interaction with PI3KR1 indicates a potential higher affinity, which could offer more effective modulation of the PI3K/Akt signaling pathway. This high binding efficiency clearly points to the ability of cosmosiin to interfere with essential oncogenic pathways, thus opening up its application as a targeted therapeutic agent.

GO and KEGG pathway enrichment analyses further explained the biological significance of cosmosiin in lung cancer therapy. Involvement of crucial pathways such as PD-L1 expression and PD-1 checkpoint regulation, PI3K/Akt signaling, and Th17 cell differentiation point to the complex mechanisms by which cosmosiin exerts its anticancer effects. Notably, the PI3K/Akt pathway is central to both tumor cell survival and immune evasion, aligning with the molecular docking results and emphasizing cosmosiin's potential to disrupt these processes.

Experimental bioassays, including MTT, annexin V, and transwell migration assays, confirmed the anticancer properties of cosmosiin that induce apoptosis and inhibit cell migration. These effects support its mechanistic role, modulating pathways associated with the survival and metastasis of cancer cells, validating the in silico predictions.

This research introduces a new type of network pharmacology approach, coupling systems biology, computational tools, and experimental validation to investigate therapeutic potential of cosmosiin. Although previous studies have reported anticancer effects of cosmosiin against colorectal cancer and colon adenocarcinoma, its applicability and mechanistic insights in the context of lung cancer have been untouched until now [12, 26]. Subsequent research should be aimed toward the optimization of cosmosiin bioavailability and studying its *in vivo* effects toward transitioning from preclinical findings toward clinical applications. This brings enough novelty and a different network pharmacology approach which actually integrates systems biology, computational methods and pharmacology together.

Conclusions

In conclusion, our study reveals through the involvement of computational, systems biology, pharmacology, in silico molecular docking and experimental techniques that cosmosiin is a potent anticancer agent in lung cancer affecting various key biological targets and modulating a range of signalling pathways involved in lung cancer. Thus, this study provides both theoretical and experimental validations of these claims.

Research ethics: The local Institutional Review B deemed the study exempt from review.
Informed consent: Informed consent was obtained from all individuals included in this study.
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