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Original Article

Application of Network Pharmacology, Computational Molecular Docking and Experimental Techniques to Study the Anticancer Effects of Ursolic Acid on Oral Squamous Carcinoma Cells

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ABSTRACT

Background: Oral Squamous Cell Carcinoma (OSCC) is a prevalent and aggressive form of cancer with limited effective therapeutic options. This study explores the potential anticancer effects of ursolic acid and its mechanism using a network pharmacology approach, *in silico* molecular docking and various experimental methods to validate these findings. **Materials and Methods:** Oral cancer genes were found using Genecards, Drugbank and Therapeutic Target Database. Swiss Target Prediction and SuperPred provided ursolic acid targets. Out of 94 targets found, only 24 remained after filtering. Using "oral carcinoma" in GeneCards yielded 6108 oral cancer targets. In addition, MTT cell viability, fluorescence microscopy and cell migration assays were used to validate the results *in vitro*. **Results:** Oral cancer and ursolic acid shared 21 common targets, identified via Venn diagram. A Protein-Protein Interaction (PPI) network was constructed using the STRING database to emphasize the strong and torturous relationship among these 21 shared targets. The PPI network analysis using the CytoHubba plug-in revealed that STAT3, RELA and NFKB1 were identified as the primary therapeutic targets of ursolic acid. Molecular docking revealed significant interactions between ursolic acid and protein targets, with binding energies of -8.1, -8.1 and -7.3 kcal/mol for STAT3, RELA and NFKB1 targets, respectively. The experimental validation *in vitro* revealed that ursolic acid-induced apoptosis and inhibited cell migration in oral squamous carcinoma cells in a dose-dependent manner. **Conclusion:** The network pharmacology analysis revealed STAT3, RELA and NFKB1, as key targets of ursolic acid in OSCC which was further supported by both molecular docking and *in vitro* experiments demonstrating its apoptotic and anti-migratory effects.

Keywords: Gene Ontology, Network pharmacology, *In silico* molecular docking, Apoptosis.

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INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the most prevalent and frequent form of oral malignant cancers targeting the buccal mucosa, tongue, palate, alveolar ridge and floor of the mouth. OSCC is responsible for 90% of the morbidity and mortality of neck and head squamous cell carcinoma, which is figured as the sixth most common malignancy in humans. This cancer hampers appearance, swallowing of the food, pronunciation and flavour and fragrance perception. In 2022, 389,485 OSCC cases were reported worldwide. According to the figures issued by the Global Cancer Observatory (GCO), the prevalence of OSCC will increase by almost 40% by 2040, attended by an increase in mortality.[1-4] Tobacco consumption, alcohol abuse, chewing betel leaves remain the main traditional risks of OSCC in the Pacific and Southeast Asia regions leading to high prevalence of this malignancy in these regions. The principal treatment strategy for OSCC remains surgery coupled with chemotherapy and radiotherapy adjuvants.[5,6] However, there are various side-effects associated with chemotherapy and radiotherapy coupled with the fact that tumor invasion, distant metastasis, metastasis of the lymph nodes and higher recurrence rates results in a very low 5-year survival rate in only 50% of the OSCC patients.[7] The lethality of OSCC lies in its vast prevalence, increasing incidences, side-effects in current treatment strategies and poor survival rate

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which necessitates the design and development of novel plant based natural products as potential anticancer drugs with better safety profiles and selectivity towards the cancer cells.

An emerging interdisciplinary field that integrates systems biology, bioinformatics and pharmacology called "network pharmacology" provides a comprehensive knowledge and understanding of the complex interactions between drugs and biological systems, especially in the context of multi-target drugs and diseases like cancer. The network pharmacology approach goes beyond the traditional mono-target paradigm, realising that diseases like cancer involve not one but an intricate array of genes, proteins and biochemical pathways. Network pharmacology maps these complex interactions and helps in the identification of crucial nodes and interactions which can be targeted to achieve desired therapeutic results more effectively. With the use of a molecular target and cancer-related pathway mapping, this strategy helps in the clarification of the mechanisms of action of multi-target phytochemicals such as ursolic acid.[8,9]

Ursolic acid is a pentacyclic triterpenoid with chemical formula of $C_{30}H_{48}O_3$, found in the leaves and fruits of many plant species in high concentrations such as apple, olive tree leaves, rosemary, basil, lavender etc.[10-13] There are evidences that ursolic acid has been reported to exhibit neuroprotective, anti-inflammatory, anti-diabetic and anticancer effects. The anticancer effects of ursolic acid are very well documented from a diverse number of publications targeting a range of human malignancies including prostate cancer, renal, bladder and testicular malignancies. It has been identified as a potential chemopreventive and chemotherapeutic agent in different kinds of cancers affecting a wide array of proinflammatory transcription factors, growth factors, kinases, cell cycle associated proteins, chemokines, cytokines and inflammatory enzymes. Ursolic acid inhibits the initiation, promotion and metastasis of cancer by targeting the above-mentioned multiple targets.[14-16]

To the best of our knowledge, there are no previous reports on the use of network pharmacology combined with *in silico* molecular docking on the anticancer effects of ursolic acid in OSCC, therefore, the current study reports novel findings revealing the complex intricate metabolic pathways, target genes and target proteins through which ursolic acid induces its anticancer effects. The main objective of the present research was to study theoretical and experimental aspects of the anticancer action of ursolic acid in human oral squamous carcinoma cells, mainly involving network pharmacology and *in silico* molecular docking.

MATERIALS AND METHODS

Compound screening, ursolic acid target prediction and oral carcinoma target genes

Drug likeness, bioavailability, Blood Brain Barrier (BBB), Gastrointestinal absorption (GI) are widely used in network pharmacological analysis for compound screening. Druglikeness is an indicator to evaluate the potential of a chemical compound, which takes into consideration its molecular, structural and physiochemical characteristics. A druglikeness score≥0.18 is an indication of a good drug candidate. These parameters were obtained from http://www.swissadme.ch/index.php server by entering the ursolic acid SMILE formula which was obtained from https://pubchem.ncbi.nlm.nih.gov/. Further, the ursolic acid targets were obtained from Super-PRED server (https://prediction.charite.de/index.php), Swiss Target Prediction database (http://www.swisstargetprediction.ch). UniProt (http://www.uniprot.org) was used to acquire the predicted target names and IDs. The target genes associated with oral carcinoma were acquired from Gene Cards database (https://www.genecards.org), with “oral carcinoma” as the key word. The obtained gene names were corrected to their official names (preferred names) using Uniprot data. The obtained ursolic acid and oral carcinoma targets were intersected/overlapped using the Venn diagram obtained from the venny (2.1) online tool. The intersected or overlapping oral carcinoma hit targets with those of the ursolic acid targets were considered as anti-oral carcinoma ursolic acid targets and were further considered for further analysis.

Construction of target genes protein interaction network

The obtained overlapped/intersected genes were then imported into the STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) database (https://string-db.org) to construct Protein-Protein Interaction (PPI) network with selected species of Homo sapiens with a composite score greater than 0.4 as a qualifying condition to acquire protein-protein interaction networks. The PPI network was later analyzed and examined by importing the protein interaction network into Cytoscape 3.10.2 software to acquire the key genes. Using the Cyto Hubba plugin in Cytoscape software, a hub-genes diagram was constructed and analyzed.

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

GO and KEGG pathways were constructed in order to predict the potential targets of ursolic acid against oral cancer. The GO functional enrichment analysis was performed to assess the roles played by target proteins of ursolic acid in gene function with differential gene enrichment. The KEGG pathway enrichment was carried out to get an idea about the genes involved and their pathways. The enrichment analysis of common targets was performed on the ShinyGO 0.80 (http://bioinformatics.sdstate.edu/go/) online tool. The common targets between ursolic acid and oral carcinoma were uploaded and analysed. The enriched pathways were sorted by importance.

In silico molecular docking

The 3D chemical structure of ursolic acid and references to curcumin (for NFKB1 and RELA) and sorafenib (for STAT3), compounds were retrieved from the PubChem database. Curcumin was used as reference against NFKB1and RELA because of its known inhibition activity against NF-κB, while sorafenib against STAT3. The crystal structures of three most important protein targets namely NFKB1, RELA and STAT3 were obtained from the protein data bank (https://www.rcsb.org/) with PDB IDs as 8tqd, 4q3j and 4zia respectively. Ursolic acid and these three target proteins were subjected to molecular docking validation in order to evaluate the molecular interactions between the ligand and the active sites of these proteins. The protein structures were optimised before docking by using PyMOL software in order to eliminate the attached water molecules, heteroatoms and attached ligands. Then AutoDock Vina version 1.2 was used for docking and BIOVIA Discovery Studio 2022 was used for visualization and analysis. CB-DOCK2 (https://cadd.labshare.cn/cb-dock2/index.php) online server was also used to acquire the cartoon surface representations of the target proteins along with locating the active cavities on the proteins.

Cell culture and MTT colorimetric assay for cell viability

The H400-oral squamous cancer cells were cultured in Roswell Park Memorial Institute (RPMI) medium (Sigma) overnight. The medium was supplemented with 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin-streptomycin (Gibco). Cells were maintained in a CO_2 incubator at 37ºC with 5% CO_2 levels, providing optimal conditions for cell growth and viability. The H400 cells were examined for viability after being subjected to different doses of ursolic acid (0, 12, 24, 48 and 96 μM) for 12 and 48 hr using the MTT assay. After drug treatment, cells were cultured with MTT (5 mg/mL) for 4 hr in 96-well plates with 10000 cells/well. A microplate reader was used to capture the absorbance at 540 nm after dissolving the Formazan crystals in DMSO. In relation to control cells treated with 0.1% DMSO, viability was represented as a percentage, with 100% being the reference point. This viability test quantified cytotoxic effects of ursolic acid on H400 OSCC cells at varying doses and time-intervals.

Apoptosis detection using DAPI-staining and fluorescence microscopy

After being seeded into 24-well plates during the exponential phase of development, OSCC cells were subjected to varying concentrations of ursolic acid (0, 24, 48 and 96 μM) for a period of 48 hr. Cells were fixed with 80% ethanol, rinsed and stained with 1 μg/ml DAPI solution for 30 min in the dark for the DAPI staining experiment. After staining, cells were observed under a Nikon fluorescent microscope (Nikon Inc., Japan). Condensed chromatin, which is indicative of apoptotic cells, may be seen when DAPI is attached to DNA.

Cell migration assay

In order to assess the anti-migratory effects of ursolic acid in H400 OSCC cells, transwell migration assay was performed. Transwell chambers with 8 μm pore size membranes, with Matrigel coating were utilized. OSCC cells (5×10^5 cells) in serum-free culture medium were seeded into the upper chambers, supplemented with varying concentrations of ursolic acid (0, 24, 48 and 96 μM). The lower chambers were filled with Dulbecco's Modified Eagle Medium (DMEM) medium containing 25% FBS. After 24 hr of incubation, non-migratory cells were removed from the upper surface of the membrane, while migrated cells were fixed and stained with crystal violet. Subsequently, the stained cells were counted under a microscope.

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 $^{*}p<0.05$, $^{**}p<0.01$.

Table 1: Physicochemical properties and toxicity profile of ursolic acid retrieved from SwissADME and ProTox 3.0 servers.

Molecular property	Value
Molecular weight	456.7
Bioavailability	0.85
GI absorption	Low
BBB permeant	No
Octanol/water partition coefficient (logP)	5.88
Lead likeness violations	2
Lipinski violations	1
Number of hydrogen bond acceptors	3
Number of hydrogen bond donors	2
Topological Polar Surface Area (TPSA)	57.53
Hepatotoxicity	No
Neurotoxicity	No
Nephrotoxicity	No
Immunotoxicity	No
Mutagenicity	No
Cytotoxicity	No

RESULTS

Physicochemical properties and target proteins of ursolic acid

The SwissADME server was used to retrieve the physicochemical properties of ursolic acid, including bioavailability, GI absorption, BBB, druglikeness and partition coefficient. Bioavailability was found to be 0.85, which is very good for a drug candidate, while GI absorption was found to be low. Pro-Tox 3.0 was used to predict potential toxicity of ursolic acid and no major toxicity induced by ursolic acid was seen. Table 1 and Figure 1 show the physicochemical and toxicological profiles of ursolic acid. The physicochemical properties and toxicity profiles of compounds are used as screening criteria in network pharmacology.

The target proteins of ursolic acid were identified by using the Super-PRED server and Swiss Target Prediction database and a total of 94 targets were initially identified, we then removed any duplicate entries and using a probability filter of 75%, the total number of target proteins was reduced to 24 targets.

Identification of oral carcinoma targets using GeneCards website

Data on "oral carcinoma" which was used as the keyword were retrieved from the GeneCards database. GeneCards provides user-friendly and comprehensive information on all annotated and predicted human genes and automatically integrates genomic, proteomic, genetic, transcriptomic, functional and clinical data. A total of 6108 oral carcinoma related targets were identified.

Identification of common targets using venny 2.1 online tool

Out of the ursolic acid and oral carcinoma targets, 21 targets were found to be the common targets between ursolic acid and oral carcinoma, indicating that these 21 targets have very high chances of being involved in the disease initiation and progression and these targets can be modulated using ursolic acid as a drug candidate. These 21 therapeutic targets were further analyzed and examined for constructing the PPI network. The Venn diagram showing the common targets between ursolic acid and oral carcinoma are shown in Figure 2.

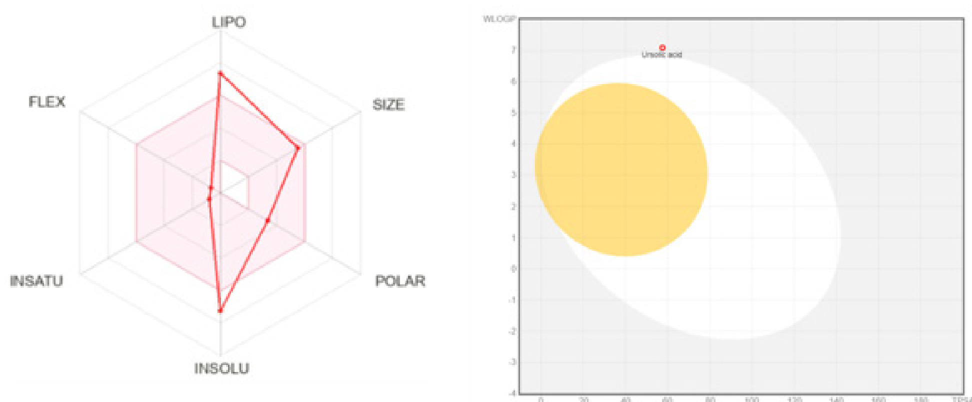

Figure 1: Physicochemical properties of ursolic acid as shown by boiled-egg representation, hinting at its drug likeness, bioavailability and polarity.

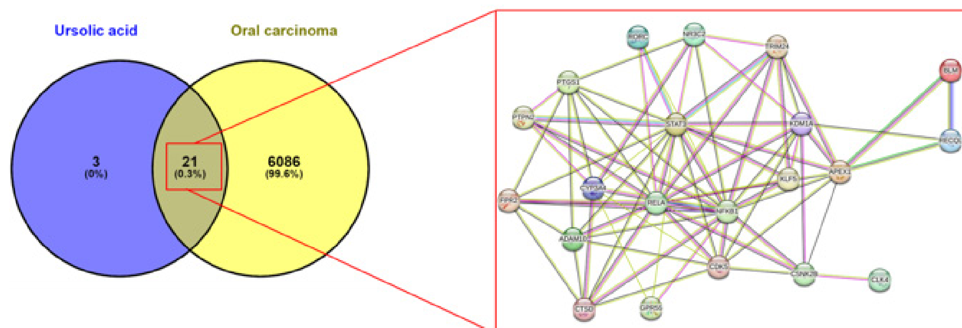

Figure 2: Venn diagram of ursolic acid and oral carcinoma related targets along with the Protein-Protein Interaction (PPI) network.

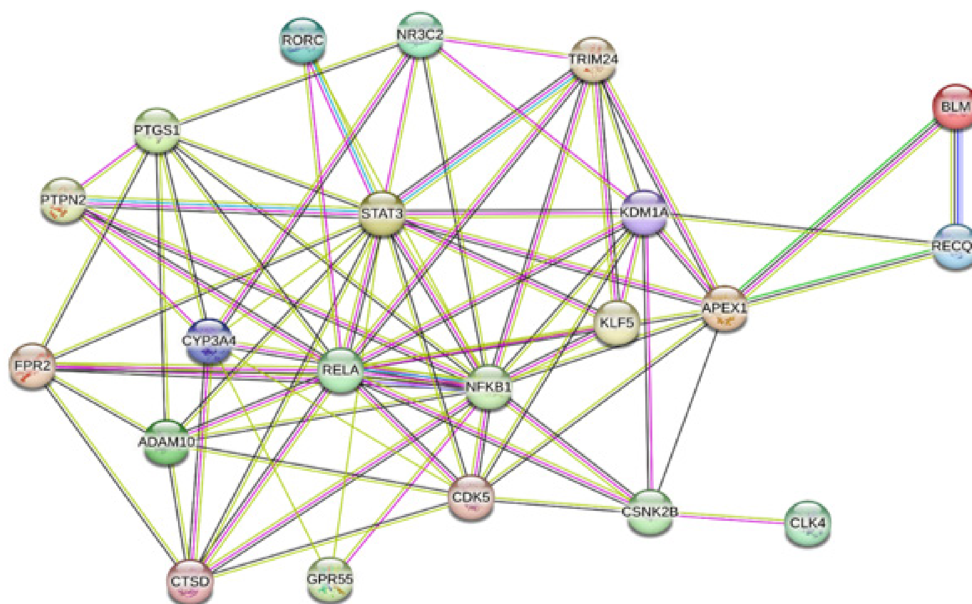

Figure 3: The Protein-Protein Interaction (PPI) network of the 21 common targets between ursolic acid and oral carcinoma, showing the nodes, edges and degree of each protein target. The higher the degree of a node within a network, the greater will be its importance within the network. The number of nodes was 21 and the number of edges were found to be 76 and the average node degree was 7.24 with a PPI enrichment *p*-value of 2.83e-07.

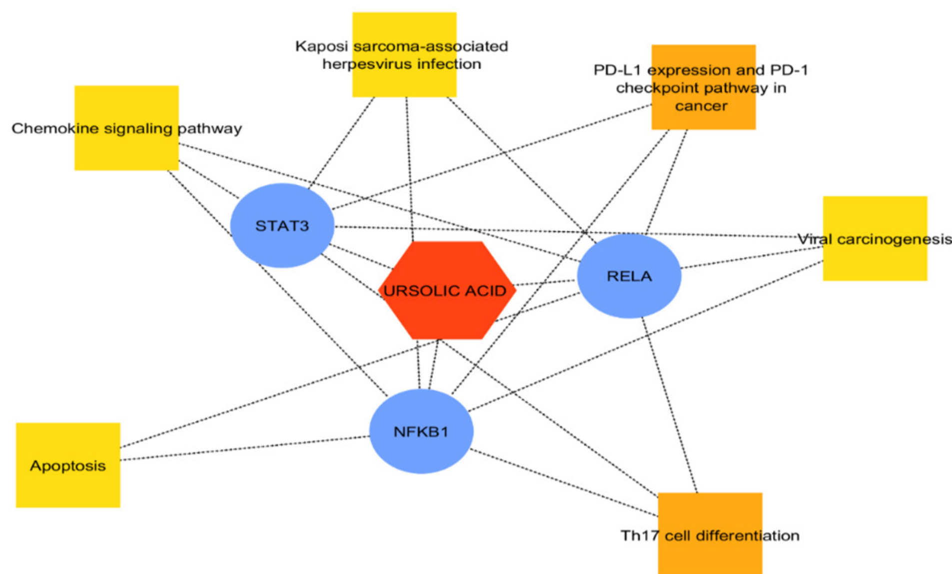

Figure 4: Identification of hub genes and their target pathways involved in oral carcinoma. RELA, NFKB1 and STAT3 are the most significant proteins involved in the therapeutic targeting of oral carcinoma by ursolic acid, these three proteins exhibit the highest degree (interaction) levels.

Protein-Protein Interaction (PPI) and hub genes network visualization and analysis

The STRING database was involved to construct and analyse the PPI network among the 21 common therapeutic targets between ursolic acid and oral carcinoma. Cytoscape 3.10.2 software was utilized for the analysis and visualization of the PPI network. As can be seen from the PPI network (Figure 3), there were 21 nodes and 76 edges and the average node degree was 7.24. The nodes in the PPI represent the target proteins, while the edges represent the interconnections/associations between the proteins. A protein with a higher degree value means that protein

Figure 5-A, B, C: GO enrichment analysis represented by the Bubble plot of the main Biological Process (BP) (A), Molecular Function (MF) (B) and Cellular Component (CC) (C) categories associated with ursolic acid and oral carcinoma. The bubble size and bubble color are an indicator of enrichment gene and p value respectively. A redder color indicates a lower p value and a large bubble indicates higher number of enriched genes within that particular GO term which in turn is an indication of the fact that this particular GO term is much more strongly connected with the treatment of oral carcinoma than other GO terms.

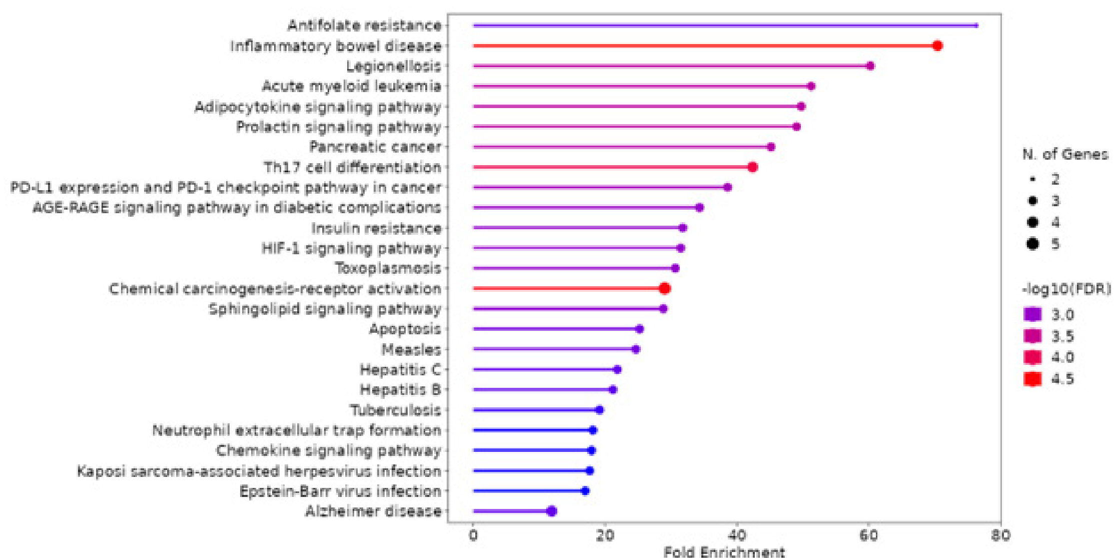

Figure 6: KEGG enrichment analysis-Identification of the potential signalling pathways for the therapeutic targets of the ursolic acid in treating oral carcinoma. The different signalling pathways were sorted by their *p* values and number of enriched genes shown by color and size of the bubble. The bubble size and bubble color are an indicator of enrichment gene and p value respectively.

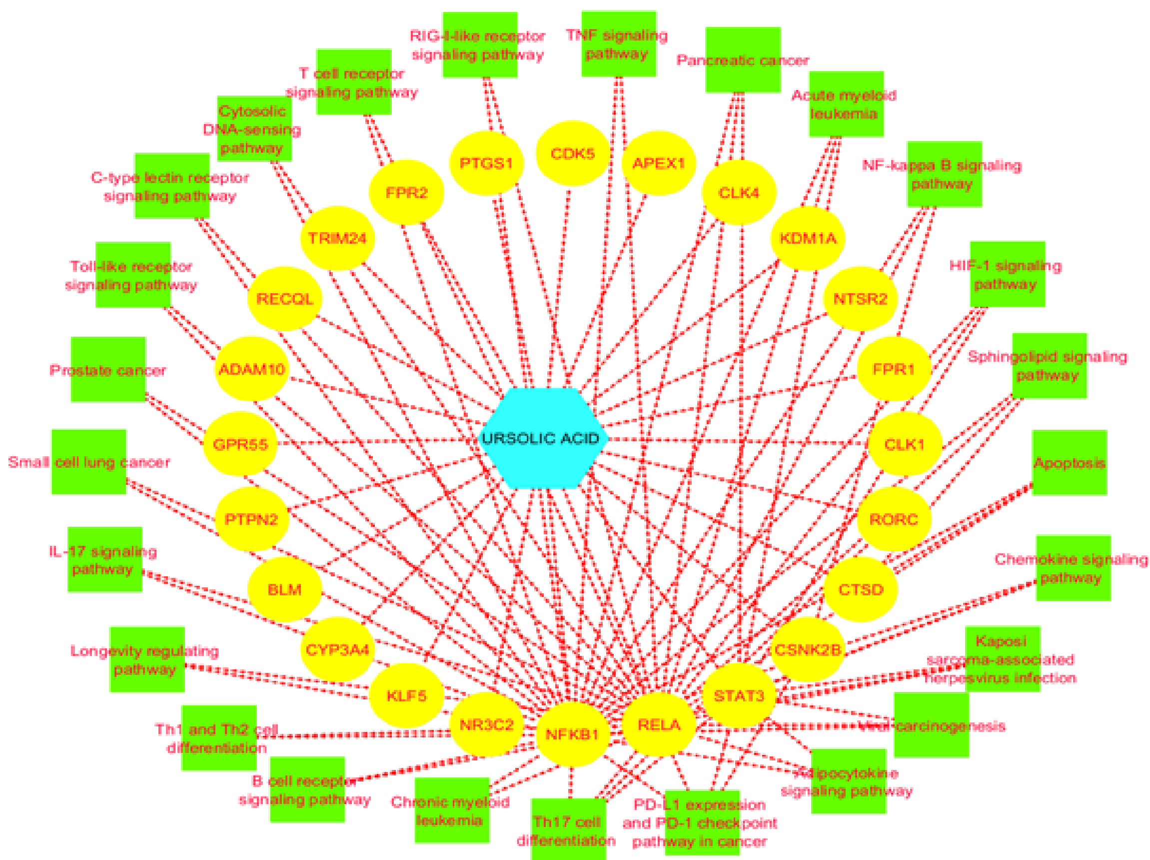

Figure 7: Visualization of the ursolic acid-potential targets-signalling pathways network using Cytoscape 3.10.2: The blue-green (turquoise color) node indicates the bioactive compound-ursolic acid; the yellow nodes indicate the potential targets while as green nodes indicate signalling pathways. The network consists of 50 number of nodes, with 88 number of edges with average number of neighbors as 3.5.

Figure 8: Target genes and the involved biochemical pathway (apoptosis) of anticancer action of ursolic acid in oral carcinoma identified by KEGG analysis.

Figure 9: *In silico* molecular docking analysis showing the molecular interactions between ursolic acid and the active site of STAT3 target protein. A, shows the active site of STAT3 with which ursolic acid gets bound, B and C show cartoon surface representation between the target protein and ursolic acid, while as D, shows 3D-representation and binding interactions between the amino acid residues of the active centre and ursolic acid. A binding energy value of -8.1 kcal/mol was observed indicative of a strong binding between the protein target and ursolic acid.

has a more significant role to play in the PPI. Our PPI network revealed a higher number of interactions than what would be expected from a randomly selected set of proteins of the same size and degree distribution drawn from the genome. This indicates that the proteins within this PPI have a significantly higher level of biological connectivity. Cyto Hubba plugin was used to identify 3 hub genes which included RELA, NFKB1 and STAT3, these genes exhibit better network topology characteristics like degree, BC and CC and are therefore considered to be key targets in the PPI networks (Figure 4). These hub genes are mostly enzymes and cytokines involved in various biological regulation biochemical pathways including signal transcription and protein phosphorylation. Three hub genes, RELA, NFKB1 and STAT3, were the most important proteins and indicate that these three genes significantly influence the anticancer effects of ursolic acid against oral carcinoma.

Gene Ontology (GO) enrichment analysis

In order to understand the biological mode of action of ursolic acid in oral carcinoma, the GO enrichment analysis was carried out on 24 potential therapeutic targets of ursolic acid in the treatment of oral carcinoma. The GO analysis was categorised into three groups: BP (biological process), MF (molecular function) and CC (cellular component) and we obtained 17 BP, 20 MF and 20 CC terms, which are shown as bubble plots in Figure 5. The bubble size and bubble color are an indicator of enrichment gene and *p* value, respectively. A redder color indicates a lower *p* value and a large bubble indicates a higher number of enriched genes within that particular GO term, which in turn is an indication of the fact that this particular GO term is much more strongly connected with the treatment of oral carcinoma than other GO terms. The GO enrichment analysis exhibited that the main enriched BP processes were cellular response to chemical stimulus, cellular response to oxygen containing compounds, cellular response to organonitrogen compounds, cellular response to nitrogen compounds and cellular response to endogenous stimulus. The main enriched MF were DNA-binding transcription factor binding, RNA polymerase ll-specific DNA-binding transcription factor binding, transcription factor binding, sequence specific DNA binding, chromatin binding and p53 binding. The main enriched CC was chromosomes, chromatin, nucleoplasm, nuclear lumen, specific granule and tertiary granule.

KEGG pathway enrichment analysis

KEGG pathway enrichment analysis was carried out in order to identify the potential metabolic pathways of the therapeutic targets of ursolic acid for treating oral carcinoma. Using the DAVID and ShinyGO 0.80 databases, we got 25 signalling pathways; all these signalling pathways were plotted in the form of a bubble plot as shown in Figure 6. The different signalling pathways were sorted by their *p* values and the number of enriched genes shown by the color and size of the bubble. The

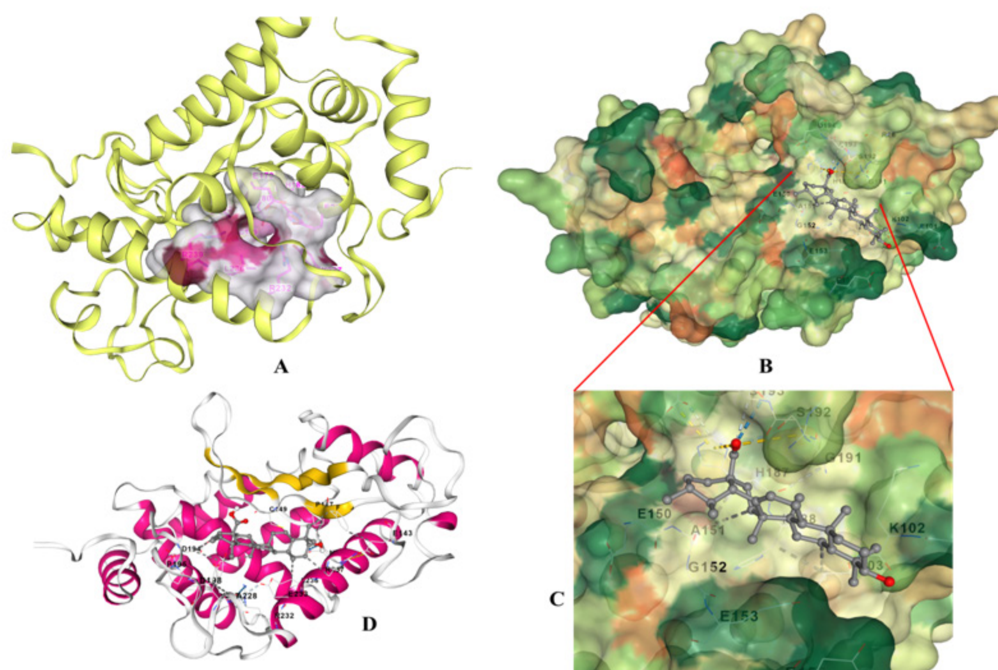

Figure 10: *In silico* molecular docking analysis showing the molecular interactions between ursolic acid and the active site of RELA target protein. A, shows the active site of RELA with which ursolic acid gets bound, B and C show cartoon surface representation between the target protein and ursolic acid, while as D, shows 3D-representation and binding interactions between the amino acid residues of the active center and ursolic acid. A binding energy value of -8.1 kcal/mol was also observed indicative of a strong binding between the protein target and ursolic acid.

bubble size and bubble color are an indicator of enrichment gene and *p* value, respectively. A redder color indicates a lower *p* value and a large bubble indicates a higher number of enriched genes within that particular pathway. The KEGG findings showed that the key targets were enriched in the PD-L1 expression and PD-1 checkpoint pathway in cancer, Th17 cell differentiation, NF-kappa B signalling pathway, HIF-1 signalling pathway, Sphingolipid signalling pathway, apoptosis pathway, Chemokine signalling pathway and Kaposi sarcoma-associated herpesvirus infection (Figure 4).

Construction of the network between ursolic acid-potential therapeutic targets-metabolic pathways using Cytoscape

In order to analyse the relationship between ursolic acid, its potential therapeutic targets and the signalling pathways it targets, we used Cytoscape 3.10.2 software in order to construct and analyse the visual network shown in Figure 7. The network consists of 50 number of nodes, with 88 number of edges, with an average number of neighbours of 3.5. The most important target proteins were found to be RELA, NFKB1 and STAT3, with an edge count of 26, 26 and 10 respectively. These therapeutic targets mainly affected PD-L1 expression and PD-1 checkpoint pathway in cancer, Th17 cell differentiation, NF-kappa B signalling pathway, HIF-1 signalling pathway, Sphingolipid signalling pathway, apoptosis pathway, Chemokine signalling pathway and Kaposi sarcoma-associated herpesvirus infection. Apoptosis was identified as one of the important pathways in the anticancer action of ursolic acid by KEGG enrichment analysis, as shown in Figure 8.

In silico Molecular docking analysis

In order to validate and cross check whether ursolic acid does target RELA, NFKB1 and STAT3 proteins, *in silico* molecular docking was performed and binding affinities between ursolic acid and these target proteins were evaluated. Additionally, for comparison of the binding affinity, reference standard with existing evidences of inhibiting these proteins was used to further strength the outcomes of network pharmacological analysis. As can be seen in Figure 9 A-D, ursolic acid strongly binds to the active centre of the STAT3 target protein with a binding energy value of -8.1 kcal/mol in comparison to -8.6 kcal/mol of the reference. The following amino acid residues between ursolic acid and STAT3: Chain C: TYR22, SER23, ASP24, SER25, PHE26, PRO27, MET28, GLU29, HIS81, ARG84, ARG85, GLN88, PHE89, SER92, ARG93: Chain D: HIS19, GLN20, TYR22, SER23,

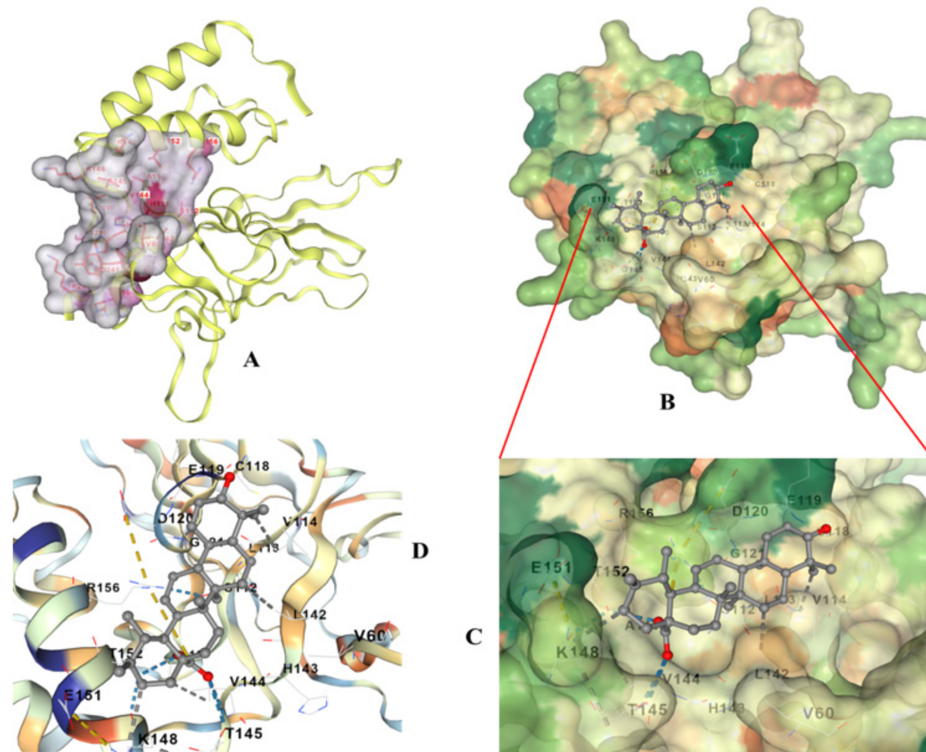

Figure 11: *In silico* molecular docking analysis showing the molecular interactions between ursolic acid and the active site of NFKB1 target protein. A, shows the active site of NFKB1 with which ursolic acid gets bound, B and C show cartoon surface representation between the target protein and ursolic acid, while as D, shows 3D-representation and binding interactions between the amino acid residues of the active center and ursolic acid. A binding energy value of -7.3 kcal/mol was also observed indicative of a strong binding between the protein target and ursolic acid.

ASP24, PRO27, GLU29, HIS81, ASN82, ARG84, ARG85, ILE86, GLN88, PHE89, SER92 and ARG93. Similarly, as can be seen from Figure 10 A-D, ursolic acid also exhibited strong binding interactions with RELA target protein with binding energy value of -8.1 kcal/mol in comparison to -7.1 kcal/mol of the reference. The following amino acids of the active centre being involved in the interaction with ursolic acid: Chain A: ARG26, GLU115, GLU143, PHE146, PRO147, ILE148, CYS149, GLU150, ALA151, PHE163, HIS183, GLU184, HIS187, SER193, ASP194, PRO195, SER196, GLY197, ASP198, TYR227, ALA228, ARG232, GLU233, ALA234, LEU236, ARG237, ARG239, ASN240. Figure 11 A-D, shows various representations of the interaction between ursolic acid and the active centre of NFKB1 target protein, which showed a lesser binding energy value of -7.3 kcal/mol in comparison to -7.0 kcal/mol of the reference. The following amino acids of the target protein being involved in the interaction: Chain A: TYR59, VAL60, CYS61, GLY63, PRO64, SER65, ALA110, HIS111, SER112, LEU113, VAL114, GLY115, CYS118, GLU119, ASP120, GLY121, LEU142, HIS143, VAL144, THR145, LYS146, LYS147, LYS148, GLU151, THR152, ARG156, MET207, ASP208, LEU209, SER210, ASP241, LYS243. This *in silico* molecular docking results validate the network pharmacology findings and these three target proteins were found to be strongly interacting with ursolic acid molecule in comparison to that of reference molecules.

In vitro evaluation of the antiproliferative, apoptotic and anti-migratory effects induced by ursolic acid in H400 OSCC cells

In order to validate the network pharmacology and *in silico* molecular docking results, an *in vitro* MTT assay was set up to assess the antiproliferative effects of ursolic acid in H400 OSCC cells at varying concentrations of ursolic acid and at two-time intervals. The findings of the assay are shown in Figure 12, which indicate that ursolic acid induced potent, dose-dependent as well as time-dependent antiproliferative effects in these cells. Figure 13 shows the apoptotic effects induced by ursolic acid in H400 OSCC cells at four indicated concentrations. As can be seen, as the ursolic acid concentration increased, the cellular morphology and fluorescence intensity also changed, hinting towards the apoptotic induction induced by ursolic acid. Chromatin condensation, membrane blebbing and cell shrinkage are the key indicators of cellular apoptosis induced by ursolic acid. These results validate the network pharmacology findings in which apoptosis was shown to be a key signalling pathway involved in the treatment of oral carcinoma by ursolic acid (Figure 4). Cell migration assay also hinted towards the mode of action of ursolic acid by inducing suppression of cell migration effects in H400 OSCC cells in a dose-dependent manner (Figure 14). This implies that ursolic acid might have significant implications in inhibiting the cancer metastasis that occurs in oral carcinoma and such can be a potential drug candidate.

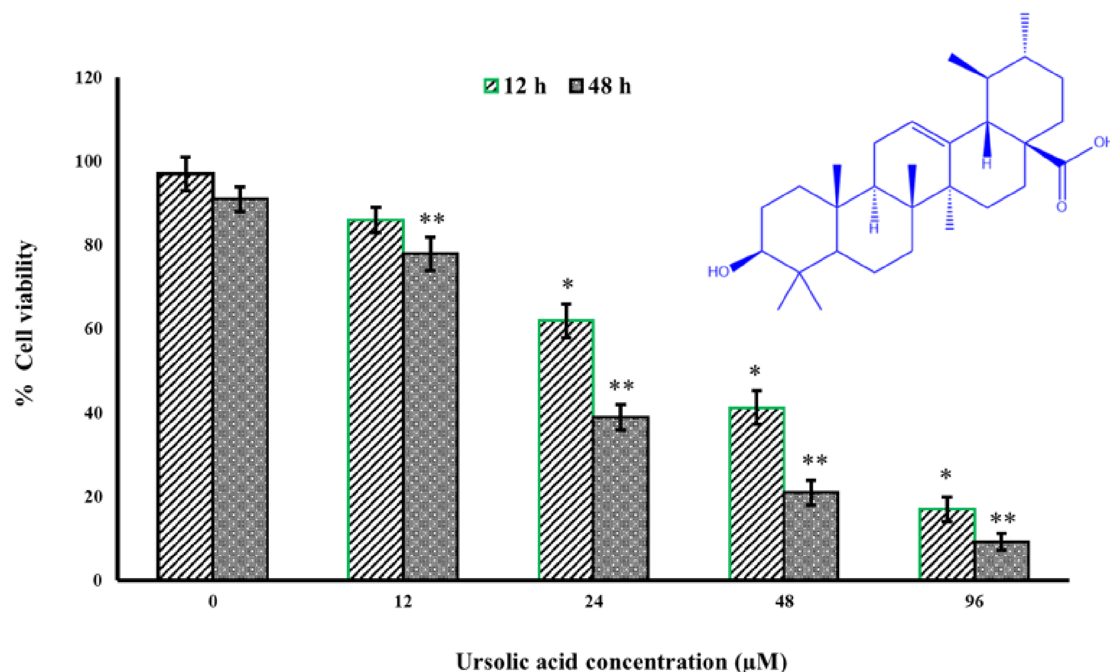

Figure 12: Chemical structure of ursolic acid and MTT assay results indicating the significant, dose-dependent and time-dependent reduction in cell viability induced by ursolic acid in H400 OSCC cells. Data of individual triplicate experiments were presented as mean±SD, * $p<0.05$, ** $p<0.01$ as statistically significant with respect to the control.

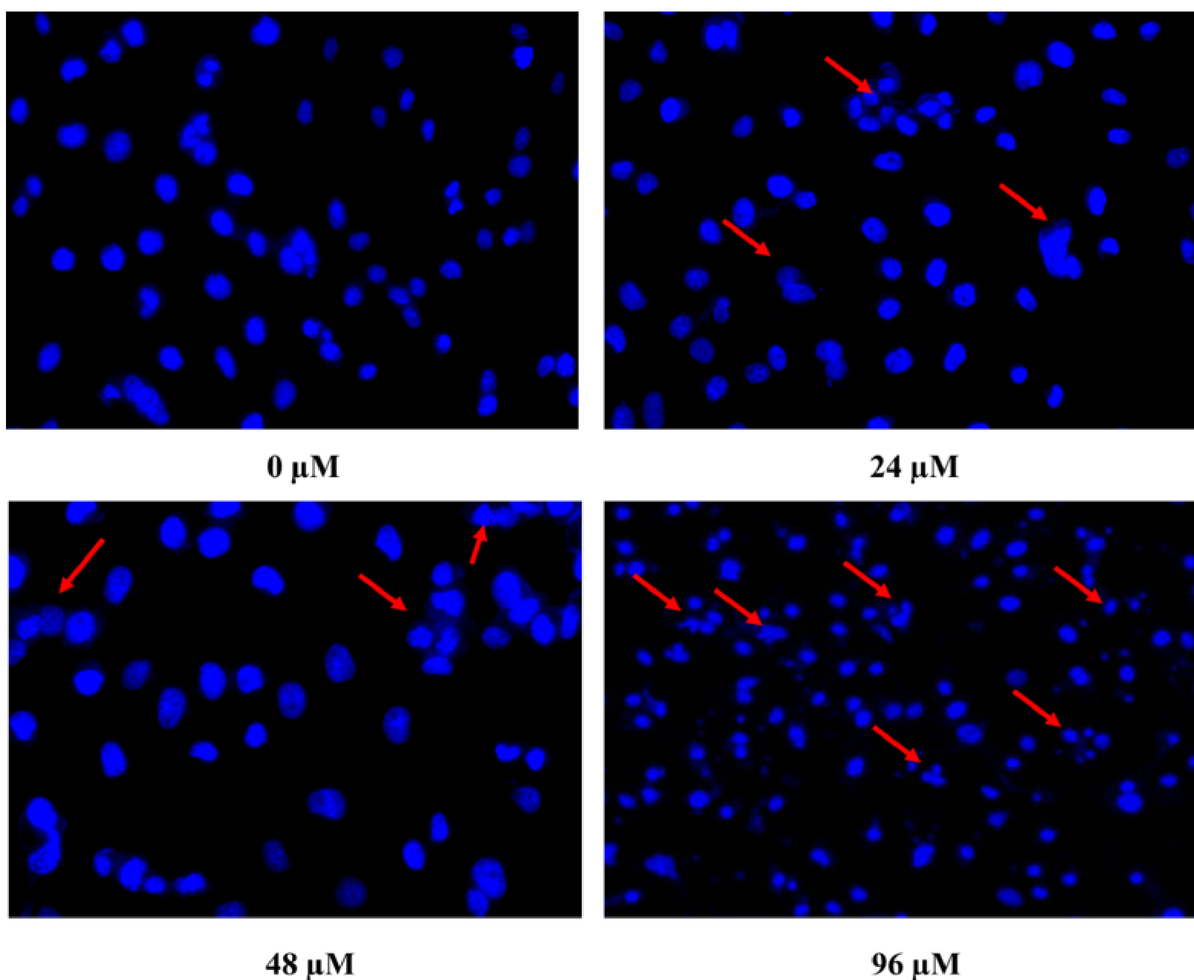

Figure 13: Apoptosis analysis; the DAPI staining assay using fluorescence microscopy indicated that as compared to the untreated control cells which showed normal cell morphology, the ursolic acid treated cells showed signs of dose-dependent cellular apoptosis including chromatin condensation and distorted cell morphology in H400 human oral squamous carcinoma cells.

DISCUSSION

"Network pharmacology," a new approach in drug research and discovery, integrates pharmacology with systems biology to understand drug interactions and activities at the network level. Given the complexity and multifaceted nature of cancer, as well as the shortcomings of conventional single-target therapy, this strategy holds great promise for anticancer research. Due to its complex genetic, epigenetic and environmental origins, cancer is a disease that is difficult to treat with single-agent treatments meant to target specific molecules or pathways. In order to overcome these obstacles, network pharmacology examines how various drug targets interact with biological networks. This analysis helps to identify important nodes and pathways that can be altered to increase therapeutic efficacy and decrease resistance.[17,18] Protein-protein interaction networks, gene regulatory networks and metabolic networks are among the biological networks that form the basis of network pharmacology. These networks are constructed and analysed. These networks provide a thorough schematic of cellular functions, illuminating the ways in which many factors/elements collaborate and advance cancer.[19,20] To the best of our knowledge, there are no previous reports on the anticancer evaluation of ursolic acid in oral carcinoma using network pharmacology approach as well as *in silico* molecular docking. In this study, we explored network pharmacology, *in silico* molecular docking approach as well as experimental studies using MTT assay, fluorescence microscopy and cell migration assay in order to study in detail the mechanism of action of ursolic acid in human oral squamous carcinoma cells. In this study we initially studied the pharmacokinetic and pharmacodynamic properties of ursolic acid using SwissADME server which showed good bioavailability scores, better druglikeness and no toxicity for ursolic acid making it a possible drug candidate. The therapeutic protein targets of ursolic acid were explored through which this molecule exerts its anticancer action. A total of 94 targets were initially identified, which after filtering were reduced to 24 targets only. A total of 6108 oral carcinoma targets were identified from the GeneCards database using the "oral carcinoma" as a keyword. We identified the common targets between oral carcinoma and ursolic acid and a total of 21 common therapeutic targets were identified using Venn diagram (Figure 2). Using STRING

database, a PPI network was constructed among these 21 common targets which shows how closely and tortuously these targets are associated among themselves. There were 21 nodes and 76 edges, the nodes in the PPI represent the target proteins while the edges represent the interconnections/associations between the proteins. A protein with a higher degree value means that protein has more significant role to play in the PPI. Using Cytohubba plugin, we identified 3 hub genes namely RELA, NFKB1 and STAT3, these genes exhibit better network topology characteristics like degree, BC and CC and are therefore considered to be key targets in the PPI networks. RELA, NFKB1 and STAT3 are essential proteins in the development and advancement of OSCC. RELA is the p65 subunit of NF-κB, NFKB1 is the p50 subunit of NF-κB and STAT3 is another crucial protein implicated in the disease's etiology and progression.[21-23] These proteins have crucial functions in controlling inflammation, cell viability, cell division and immunological reactions. RELA and NFKB1 are components of the NF-κB complex, which is often active in OSCC. This activation results in the transcription of genes that facilitate tumour development, invasion and resistance to apoptosis.[24] STAT3, often activated via the JAK-STAT pathway, also promotes carcinogenesis by stimulating cell proliferation, suppressing apoptosis and creating an immunosuppressive environment inside the tumour.[25,26] RELA, NFKB1 and STAT3 play crucial roles in oncogenic processes. Therefore, they are not only significant for comprehending the biology of OSCC but also have the potential to be targeted therapeutically and used as biomarkers for prognosis. These hub genes are mostly enzymes and cytokines involved in various biological regulation biochemical pathways including signal transcription and protein phosphorylation. These hub genes RELA, NFKB1 and STAT3 were the most important proteins and indicate that these three genes significantly influence the anticancer effects of ursolic acid against oral carcinoma. This claim was verified by using these three proteins in *in silico* molecular docking and studying the interactions between their active centres and ursolic acid molecule. The results showed strong binding interactions (Figures 8-10) with binding energy values of -8.1, -8.1 and -7.3 kcal/mol between ursolic acid and STAT3, RELA and NFKB1 respectively. Gene Ontology (GO) and KEGG pathway enrichment analysis revealed various biological processes, molecular functions and cellular components involved in the treatment of oral carcinoma by ursolic acid (Figure 5). The KEGG findings showed that the key targets were mainly affecting the following signalling pathways: PD-L1 expression and PD-1

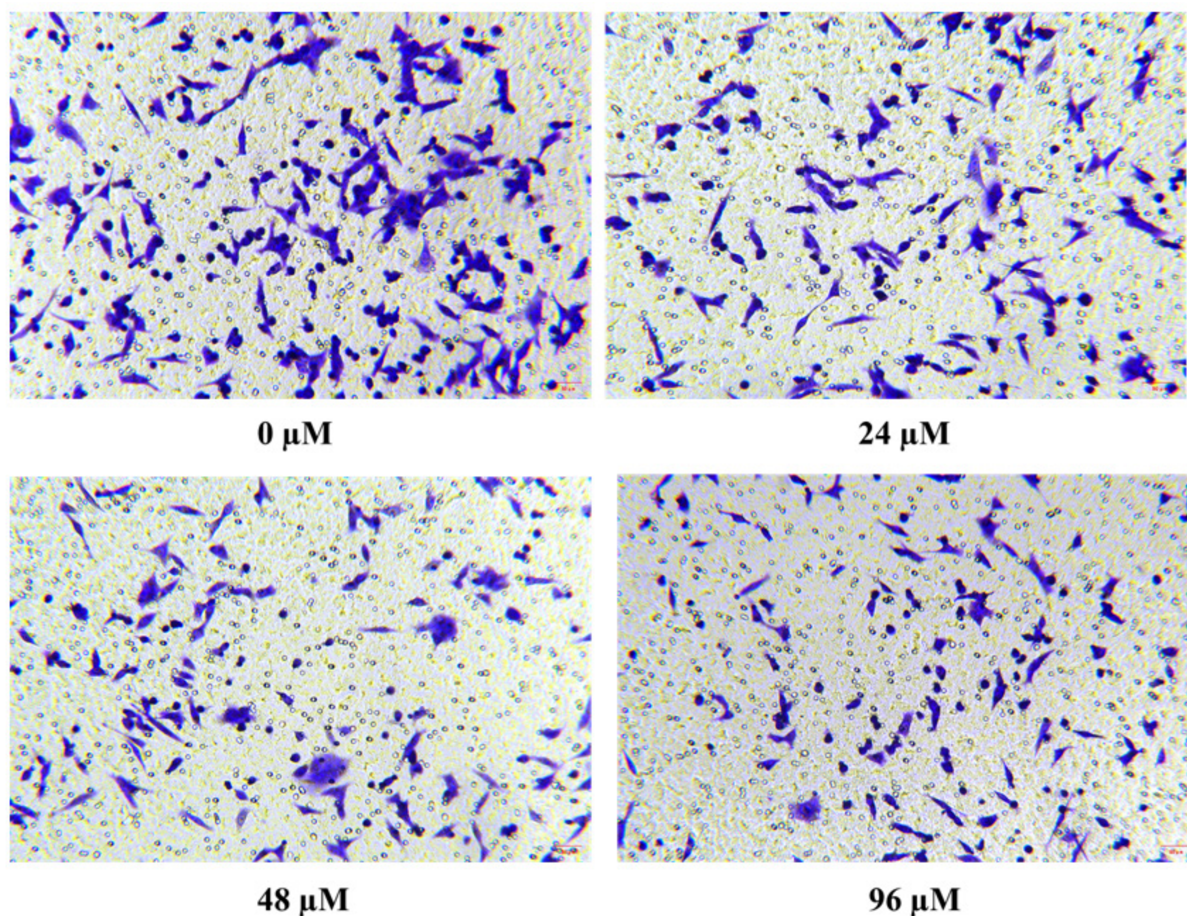

Figure 14: Effect of ursolic acid on the cell migration inhibition in H400 human oral squamous carcinoma cells using a Transwell chambers assay. The cell migration evaluation was tested at various concentrations including 0, 24, 48 and 96 μM and it was witnessed that there was a concentration dependent suppression of cell migration.

checkpoint pathway in cancer, Th17 cell differentiation, NF-kappa B signalling pathway, HIF-1 signalling pathway, Sphingolipid signalling pathway, apoptosis pathway, Chemokine signalling pathway, Kaposi sarcoma-associated herpesvirus infection. KEGG pathway results were confirmed by *in vitro* assays using MTT cell viability assay and fluorescence microscopy assay for evaluating antiproliferative effects and apoptosis induction effects respectively. The *in vitro* results did in fact reveal that ursolic acid significantly led to dose-dependent apoptotic induction in oral carcinoma cells. The final relationship between ursolic acid, its potential therapeutic targets and signalling pathways was finally derived using Cytoscape software version 3.10.2 in the form of a visual network (Figure). This visual network examines in detail all the intricate and complex interactions which exist between the target bioactive compound (ursolic acid), its therapeutic targets and the signalling metabolic pathways which are affected as a result of the modulation on the therapeutic targets.

The absence of *in vivo* investigations to support the conclusions derived from network pharmacology, computational molecular docking and *in vitro* experimental methods limits this work. Although these approaches provide significant evidences on the possible anticancer properties of ursolic acid on oral squamous carcinoma cells, the lack of *in vivo* investigations hinders our capacity to understand the compound's therapeutic effectiveness and safety within a complicated biological system. Future investigations should concentrate on performing *in vivo* studies to validate these results and investigate the pharmacokinetics, bioavailability and general influence of ursolic acid in a living organism, which will be very essential for converting these findings into therapeutic applications.

CONCLUSION

The research effectively showcased the potential of ursolic acid as a possible treatment option for treating oral cancer. The study used network pharmacology, *in silico* molecular docking and *in vitro* experiments to identify RELA, NFKB1 and STAT3 as the primary target proteins responsible for the anticancer effects of ursolic acid. Based on the physicochemical attributes and toxicity profiles, it can be concluded that ursolic acid is a promising therapeutic candidate due to its substantial bioavailability and safety. The Venn analysis revealed the presence of 21 shared targets between ursolic acid and oral cancer, indicating that these targets play a crucial role in the onset and course of the disease. The construction of the PPI network further highlighted the biological importance of these targets. The GO and KEGG pathway enrichment studies highlighted the participation of ursolic acid in important biological processes and pathways, namely apoptosis, which strengthens its relevance in anticancer mechanisms. The compound's antiproliferative, apoptotic and anti-migratory activities were validated by *in vitro* validation, which supports the in-silico predictions and suggests its potential to limit cancer propagation. Further studies are warranted to investigate clinical applications of ursolic acid, with a specific emphasis on its effectiveness and safety in *in vivo* and its potential for development into an oral cancer treatment.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest to indicate.

ABBREVIATIONS

OSCC: Oral squamous cell carcinoma; **MTT:** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **PPI:** Protein-protein interaction; **STRING:** Search Tool for the Retrieval of Interacting Genes/Proteins; **NFKB1:** Nuclear factor NF-kappa-B; **STAT3:** Signal transducer and activator of transcription 3.

SUMMARY

This study aimed at demonstrating the mechanism of the anticancer action of ursolic acid through the involvement of network pharmacology, *in silico* molecular docking and experimental validation. To the best of our knowledge, this is for the first-time network pharmacology coupled with *in silico* molecular docking have been employed in order to study anticancer mechanism at the very gene and protein expression levels. Protein targets of ursolic acid and oral carcinoma were identified and out of these targets, 21 common targets belonging to both ursolic acid and oral carcinoma were identified. These common targets were supposed to be involved in the disease onset and progression and were further fed into string database in order to construct a protein-protein interaction network which describes which targets are associated with which other targets. Further top 3 hub genes were identified for which gene ontology enrichment analysis and KEGG enrichment pathway analysis was carried out. These three top ranked genes were identified using Cyto hubba. These top 3 target proteins were subjected to *in silico* molecular docking and later validated experimentally.

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