Evaluating the Therapeutic Role of Selected Active Compounds in Plumula Nelumbinis on Pulmonary Hypertension via Network Pharmacology and Experimental Analysis

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Abstract

Excessive proliferation of pulmonary artery smooth muscle cells (PASMCs) is one of the most critical factors leading to vascular remodeling in pulmonary hypertension (PH). This study aimed to explore the effect and potential mechanism of *Plumula Nelumbinis* on PH by using network pharmacology and experimental analysis. The potential active components of *Plumula Nelumbinis* and their targets were obtained from TCMSP and PharmMapper databases. The PH-related targets were obtained from GeneCards, OMIM, DisGeNET, and TTD databases. Co-targets between *Plumula Nelumbinis* and PH were obtained by drawing Venn diagrams. The protein-protein interaction networks and compound-target-pathway networks were established using the STRING database and Cytoscape software. Moreover, the binding activities of core targets and essential active compounds were further validated by molecular docking. Finally, we further confirmed in vitro experiments that the main active components of *Plumula Nelumbinis* could inhibit the proliferation of PASMCs induced by hypoxia, as well as the increased expression of p-SRC and PIM1. Based on these observations, we conclude that multi-components (neferine, liensinine, and isoliensinine) in *Plumula Nelumbinis* can inhibit the hypoxia-induced proliferation of PASMCs by regulating the expression of p-SRC and PIM1, thereby delaying the development of PH.

Introduction

Pulmonary hypertension (PH) is a malignant cardiopulmonary vascular disease characterized by persistently elevated pulmonary arterial pressure^{1,2}. Pulmonary vascular remodeling is the most basic pathological feature of PH, involving processes such as the excessive proliferation of pulmonary artery smooth muscle cells (PASMCs), endothelial dysfunction, and extracellular matrix deposition³. Currently approved drugs for the treatment of PH mainly target three molecular pathways related to the pathogenesis of PH: including prostacyclins and prostacyclin-receptor agonists (such as epoprostenol, iloprost, selexipag, and treprostinil), endothelin receptor antagonists (such as ambrisentan, bosentan, and macitentan), nitric oxide pathway Agents (such as sildenafil and tadalafil)². However, the above existing drug treatment usually only leads to an improvement in symptoms and cannot achieve the purpose of a radical cure. Therefore, it is urgent to find more safe and effective drugs to prevent and treat PH.

Network pharmacology is a useful bioinformatics tool to reveal the complex biological network relationship between drugs, targets, and diseases by using high-throughput screening, network visualization, and analysis techniques⁴. In recent years, with the rise of network pharmacology, systematic research on the underlying mechanisms of traditional Chinese medicine (TCM) has been widely carried out, providing a foundation for the modernization of TCM⁵. From the perspective of TCM, the pathological mechanism of PH can be summarized as qi deficiency, blood stasis, and phlegm coagulation. Therefore, PH can be treated by nourishing the qi, invigorating the kidney and benefiting the lung, promoting blood circulation and removing blood stasis. Accumulating evidence suggests that active ingredients derived from TCM, such as magnolol⁶, magnesium lithospermate B⁷, and resveratrol⁸, could

delay the process of pulmonary vascular and right ventricular remodeling during PH^{9–11}, which brings a promising future for the prevention and treatment of PH.

Plumula Nelumbinis, a TCM derived from the dried young leaves and radicles of the mature seeds of the water lily plant *Nelumbo nucifera Gaertn*, could be used for medicine and food. It has multiple pharmacological activities such as cardiovascular protection, anti-oxidation, anti-inflammatory, and anti-tumor¹². Recent studies have shown that alkaloids from *Plumula Nelumbinis* could attenuate vascular remodeling in spontaneously hypertensive rats^{13,14}. However, its effect on vascular remodeling in PH is still unclear.

In this study, we first screened out the main active components and targets of *Plumula Nelumbinis* against PH through a network pharmacology strategy and then performed GO function and KEGG pathway enrichment analysis. Next, the interaction between the core compounds and targets was simulated by molecular docking. Finally, by establishing a hypoxia-induced PASMCs proliferation model, the prediction results were verified by in vitro experiments. The overall design of this study is shown in Fig. 1. The results of this study can further clarify the mechanism of *Plumula Nelumbinis* in the treatment of PH and provide a basis for the development of novel anti-PH drugs.

Methods

Identification of the main active ingredient of *Plumula Nelumbinis*

The active ingredients of *Plumula Nelumbinis* were obtained from the TCMSP database (Traditional Chinese Medicine Systems Pharmacology and Analysis Platform, <https://old.tcmsp-e.com/tcmsp.php>)³⁴ and related literature^{12,35} by using search terms such as “*Plumula Nelumbinis*” or “*Hindu Lotus Plumule*”. The chemical information (such as PubChem CID, 3D conformer, and canonical SMILES) and pharmacokinetic parameters of these active ingredients were collected from the PubChem (<https://pubchem.ncbi.nlm.nih.gov/>) and SwissADME (<http://www.swissadme.ch/>) database. The main active ingredients of *Plumula Nelumbinis* were screened according to the following conditions as described in the previous study³⁶: (1) The gastrointestinal absorption coefficient was set to High, which indicates that these compounds have good oral bioavailability and could be effectively absorbed; (2) The flexibility was evaluated by rotatable bonds: rotatable bonds ≤ 10 ; (3) The polarity was evaluated by topological polar surface area (TPSA): $20 \text{ \AA}^2 \leq \text{TPSA} \leq 140 \text{ \AA}^2$; (4) The drug-likeness ability was evaluated by 5 different rule-based filters (Lipinski, Chose, Veber, Egan, and Muegge filter) and the bioavailability score: the bioavailability score $\geq 10\%$. The potential targets of the main active ingredients of *Plumula Nelumbinis* were predicted through the PharmMapper database (<http://www.lilab-ecust.cn/pharmmapper/submitfile.html>, Normalized Fit Score ≥ 0.70)³⁷. The potential targets were then identified and normalized through the UniProt database (<https://www.uniprot.org/>).

Identification of PH-related targets

The PH-related targets were obtained from GeneCards (<https://www.genecards.org/>), Online Mendelian Inheritance in Man (OMIM, <https://omim.org/>)³⁸, DisGeNET (<https://www.disgenet.org/>)³⁹, and Therapeutic Target Database (TTD, <http://db.idrblab.net/ttd/>)⁴⁰ by using search terms such as “pulmonary hypertension” or “pulmonary arterial hypertension” (accessed on 21 Oct 2021). The above targets were summarized, de-duplicated, and then identified as PH-related targets.

Construction of drug-target-disease and protein-protein interaction (PPI) Network

The intersection between the potential targets of *Plumula Nelumbinis* and the PH-related targets was obtained by the VENNY online tool (<https://bioinfogp.cnb.csic.es/tools/venny/index.html>). The overlapping targets were considered as the potential therapeutic targets of *Plumula Nelumbinis* against PH. The protein interaction relationships of the overlapped targets were acquired through the STRING database (<https://cn.string-db.org/>, Version 11.5)⁴¹. In brief, the overlapped targets were uploaded to the STRING database, and the species was limited to “Homo sapiens”. Finally, the obtained PPI network information of the intersection targets was led to the Cytoscape software for visual analysis (<https://cytoscape.org/>, Version 3.8.2)⁴².

GO and KEGG pathway enrichment analysis

The Metascape online tool (<https://metascape.org/>) was applied for GO and KEGG enrichment analysis⁴³. Briefly, the potential therapeutic targets of *Plumula Nelumbinis* against PH were uploaded to the Metascape platform. The filter thresholds of GO function and KEGG pathway analysis were set as the following conditions: (1) the species was set as “Homo sapiens”; (2) the minimum overlap value was set to 3; (3) the *P* cutoff value was set to 0.01; (4) the minimum enrichment value was set to 1.5. The GO and KEGG enrichment analysis results were visualized through the ggplot2 package (Version 3.3.5) of the R language (Version 4.0.2)⁴⁴.

Construction of the drug-target-pathway network

The main component-target-pathway enrichment network was visualized using the Cytoscape software⁴². The supporting tool “Network Analyzer” of Cytoscape software was used to obtain the network topology parameters (such as degree, betweenness, and closeness) between the effective ingredients and targets. The top-ranked compounds and targets based on degree, betweenness, and closeness were defined as the core compounds and targets. These core compounds and targets were subsequently prepared for molecular docking.

Molecular docking

The binding situation of core compounds and targets was analyzed using AutoDock Vina software (<https://vina.scripps.edu/>, Version 1.1.2). Firstly, the three-dimensional crystal structures of the top 10 core target proteins were obtained from the Protein Data Bank (PDB, <https://www.pdbus.org/>)⁴⁵. The PDB ID of these target proteins are as follows: proto-oncogene tyrosine-protein kinase Src (SRC, PDB ID:

2SRC); serine/threonine-protein kinase PIM-1 (PIM1, PDBID:1XWS); transthyretin (TTR, PDB ID:1BZE); prothrombin (F2, PDB ID:2CN0); estrogen receptor- α (ESR1, PDB ID:5FQV); cyclophilin A (PPIA, PDB ID: 5LUD); mitogen-activated protein kinase 14 (MAPK14, PDB ID:2FST); phenylalanine-4-hydroxylase (PAH, PDB ID:5FII); β -secretase 1 (BACE1, PDB ID: 1TQF); endothelial nitric oxide synthase (NOS3, PDB ID:1M9M). Secondly, the 3D structures of the core compounds were obtained from the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>) and then were converted into PDB file format by using OPEN Babel software (<http://openbabel.org/>, Version 2.4.1) ⁴⁶. Finally, the molecular docking of core compounds and targets was performed using AutoDock Vina software ⁴⁷. The binding energy volcano map was drawn through the pheatmap package (Version 1.0.12) of the R language. At the same time, the molecular docking results were visualized using the PyMol software (Version 2.2.0). The binding affinity value < 0 kcal/mol indicates that the compounds effectively bind to the targets.

Chemicals and reagents

The separation, extraction, identification of neferine, liensinine, isoliensinine, and the total alkaloids from *Plumula Nelumbinis* (TAPN) were performed as described in our previous study ⁴⁸⁻⁵⁰. Dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich (C6164, Darmstadt, Germany). High-glucose Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Thermo Fisher Scientific).

Cell experiments

The isolation and culture of PSMCs from the peripheral small pulmonary arteries of rats were performed as described in our previous study ⁵¹. PSMCs at passages 3 to 6 were used for cell identification and the subsequent experiments. The hypoxia-induced PSMCs proliferation model was conducted as described in our previous study ⁵¹. In order to evaluate the effect of the screened main active components of *Plumula Nelumbinis* on the proliferation of PSMCs induced by hypoxia, the following experiments were carried out: (1) the normoxia group, PSMCs were cultured in a normoxic incubator ($O_2/CO_2/N_2=21/5/74$) for 48 h; (2) the hypoxia group, PSMCs were cultured in a hypoxia incubator ($O_2/CO_2/N_2=3/5/92$) for 48 h; (3) the hypoxia plus drug intervention group, PSMCs were pretreated with different doses of drugs for 1 h and then treated with hypoxia for 48 h, the initial drug doses for liensinine, liensinine, and isoliensinine were set at 1, 2.5, 5, 10, and 20 μ M, respectively, while the initial doses of TAPN were 5, 10, 25, 50 and 100 ng/ml, respectively. (4) the hypoxia plus vehicle group, PSMCs were pretreated with an equal volume of vehicle (DMSO, volume $\leq 1/1000$ of the total medium) for 1 h and then treated with hypoxia for 48 h. At the end of the experiments, the PSMCs were collected for CCK-8 detection and molecular analysis.

Cell counting Kit-8 (CCK-8) detection

Cell proliferation was detected by CCK-8 Assay Kit according to the protocol provided by the manufacturer (BA00208, Bioss, Beijing, China). Briefly, the PSMCs were inoculated into the 96-well

plates and synchronized for 24h after the cell density reached 50%. After drug and hypoxia intervention, the CCK-8 solution was added to each well (10 μ L) and then incubated at 37 °C for 2 h in the dark. The optical density (OD) value was detected at 450 nm with SpectraMax 190 (Molecular Devices, California, USA).

Western blotting

The PSMCs were homogenized in RIPA lysis buffer (Beyotime, Shanghai, China) with a protease and phosphatase inhibitor cocktail (Beyotime, Shanghai, China). Samples containing 30~40 μ g of protein were separated by 10 % SDS-PAGE gel and then transferred to polyvinylidene fluoride (PVDF) membranes (G.E. Healthcare, Germany). After blocking with 5% BSA, the PVDF membranes were incubated overnight on a shaker at 4°C with the following primary antibodies: SRC (AF1831, 1:1000, Beyotime), p-SRC (AF5932, 1:1000, Beyotime), PIM1 (AF1807, 1:1000, Beyotime), eNOS (AF6792, 1:1000, Beyotime), and α -tubulin (sc-5286, 1:500, Santa Cruz). After incubation with horseradish peroxidase (HRP)-linked secondary antibody (Beyotime, Shanghai, China), the protein bands were imaged through Molecular Imager ChemiDoc XRS System (Bio-Rad, Philadelphia, USA). Densitometric quantification was performed by Image J (NIH, USA). The α -tubulin served as a loading control.

Statistical analysis

All quantitative data were presented as the means $\pm$ standard deviation (S.D.) and analyzed using SPSS 20.0 software (SPSS, Chicago, United States). One-way analysis of variance (ANOVA) was used to compare the means among different groups. $P \leq 0.05$ was considered statistically significant.

Data availability

The data supporting this study's findings are available from the corresponding author on reasonable request.

Results

Screening of main active compounds from *Plumula Nelumbinis*

More than 100 compounds have been reported to be isolated and identified from the *Plumula Nelumbinis*, including alkaloids (such as neferine, liensinine, and isoliensinine), flavonoids (such as quercetin, rutin, and kaempferol), volatile oil, and polysaccharides. A total of 92 possible active compounds were initially screened by matching with information from the PubChem database. Then the pharmacokinetic parameters of those compounds were obtained from the SwissADME database. Finally, we conducted a secondary screening of those compounds based on the gastrointestinal absorption rate, flexibility, polarity, and drug-likeness. A total of 46 main active ingredients of *Plumula Nelumbinis* were finally obtained, including 30 alkaloids, 7 flavonoids, and 9 other compounds (volatile oil or polysaccharides). The above 46 compounds were numbered as LZX1-LZX46, and relevant information is shown in Table 1.

Table 1
The main active ingredients of *Plumula Nelumbinis*.

Number	Pubchem name	PubChem ID	Class
LZX1	Neferine	159654	Alkaloids
LZX2	Liensinine	160644	Alkaloids
LZX3	Isoliensinine	5274591	Alkaloids
LZX4	Higenamine	114840	Alkaloids
LZX5	(S)-isococclaurine	138319297	Alkaloids
LZX6	(+)-N-Methylisococclaurine	21817819	Alkaloids
LZX7	Cocclaurine	160487	Alkaloids
LZX8	(R)-N-Methylcocclaurine	440595	Alkaloids
LZX9	Lotusine	5274587	Alkaloids
LZX10	N-Nornuciferine	12313579	Alkaloids
LZX11	Nuciferine	10146	Alkaloids
LZX12	O-Nornuciferine	197017	Alkaloids
LZX13	Nornuciferidine	183520	Alkaloids
LZX14	Nelumboferine	76046684	Alkaloids
LZX15	Dauricine	73400	Alkaloids
LZX16	Pronuciferine	200480	Alkaloids
LZX17	Berberine	2353	Alkaloids
LZX18	(-)-Armepavine	442169	Alkaloids
LZX19	Lysicamine	122691	Alkaloids
LZX20	Anonaine	160597	Alkaloids
LZX21	Roemerine	119204	Alkaloids
LZX22	Liriodenine	10144	Alkaloids
LZX23	Thalifoline	89048	Alkaloids
LZX24	N-Methylcorydaldine	303906	Alkaloids
LZX25	2-Methyl-1H-indole-3-carbaldehyde	73166	Alkaloids
LZX26	(R)-Norcocclaurine	440988	Alkaloids

Number	Pubchem name	PubChem ID	Class
LZX27	1alpha-(4-Methoxybenzyl)-2-methyl-6-methoxy-1,2,3,4-tetrahydroisoquinoline	132579667	Alkaloids
LZX28	N-Methylisosalsoline	40091	Alkaloids
LZX29	O-Methylarmepavine	821338	Alkaloids
LZX30	6,7-Dimethoxy-2-methyl-1,2,3,4-tetrahydroisoquinoline	27694	Alkaloids
LZX31	Quercetin	5280343	Flavonoids
LZX32	Isorhamnetin	5281654	Flavonoids
LZX33	Kaempferol	5280863	Flavonoids
LZX34	Diosmetin	5281612	Flavonoids
LZX35	Naringenin	932	Flavonoids
LZX36	Luteolin	5280445	Flavonoids
LZX37	Tricetin	5281701	Flavonoids
LZX38	5-Hydroxymethylfurfural	237332	Others
LZX39	Linalool	6549	Others
LZX40	(1,7,7-Trimethyl-2-bicyclo[2.2.1]heptanyl) 3-phenylprop-2-enoate	583021	Others
LZX41	Gallic acid	370	Others
LZX42	Dibutyl phthalate	3026	Others
LZX43	Gamabufotalin	259803	Others
LZX44	Loureirin A	5319081	Others
LZX45	Methyl 4-hydroxycinnamate	5319562	Others
LZX46	Propanethial S-oxide	441491	Others

Identification of potential targets of main active components from Plumula Nelumbinis

The potential targets of main active ingredients were obtained from the PharmMapper database. LZX46 (Propanethial S-oxide) was excluded because the predicted target of LZX46 was not available in the PharmMapper database. A total of 173 potential targets for the other 45 compounds were obtained after deduplication. The drug-target network is shown in Fig. 2. The red nodes represent alkaloids, the yellow nodes represent flavonoids, the purple nodes represent volatile oils or polysaccharides, and the green nodes represent the corresponding targets.

Screening of potential targets of *Plumula Nelumbinis* against pulmonary hypertension

After summarizing and de-duplicating the targets, a total of 2326 PH-related targets were collected from GeneCards, OMIM, DisGeNET, and TTD databases. As shown in Fig. 3, a total of 60 common targets were obtained after the intersection of *Plumula Nelumbinis*-related targets and PH-related targets. These common targets were identified as potential therapeutic targets of *Plumula Nelumbinis* against PH.

Construction of PPI network of anti-PH related targets of *Plumula Nelumbinis*

The relationship between 60 intersection targets was obtained from the STRING database. The PPI network between those common targets was visualized using the Cytoscape software. As shown in Fig. 4, albumin (ALB), heat shock protein HSP 90- α (HSP90AA1), proto-oncogene tyrosine-protein kinase Src (SRC), caspase-3 (CASP3), estrogen receptor (ESR1), endothelial nitric oxide synthase (NOS3), vascular endothelial growth factor receptor 2 (KDR), mitogen-activated protein kinase 1 (MAPK1), plasminogen (PLG), peroxisome proliferator-activated receptor γ (PPARG), mitogen-activated protein kinase 14 (MAPK14), and annexin A5 (ANXA5) have high degree values in the PPI network, suggesting that these targets may be the critical targets for the main active components of *Plumula Nelumbinis*.

GO and KEGG enrichment analysis

GO enrichment analyses were conducted to confirm the intersection targets' function. GO function analysis results showed that the anti-PH effect of *Plumula Nelumbinis* might be closely related to a variety of biological processes (BP), including regulation of MAPK cascade (GO:0043408), wound healing (GO:0042060), reproductive structure development (GO:0048608), negative regulation of response to external stimulus (GO:0032102), cellular response to organic cyclic compound (GO:0071407), tube morphogenesis (GO:0035239), and protein phosphorylation (GO:0006468) (Fig. 5A and 5E). GO cellular components (CC) analysis terms mainly include cytoplasmic vesicle lumen (GO:0060205), membrane raft (GO:0045121), collagen-containing extracellular matrix (GO:0062023), lytic vacuole (GO:0000323), and receptor complex (GO:0043235) (Fig. 5B and 5F). GO molecular function (MF) analysis terms mainly include endopeptidase activity (GO:0004175) and protein serine/threonine/tyrosine kinase activity (GO:0004712) (Fig. 5C and 5G). KEGG pathway enrichment analysis indicated that the anti-PH effect of *Plumula Nelumbinis* might be achieved by regulating the following pathways: proteoglycans in cancer (hsa05205) and pathways in cancer (hsa05200) (Fig. 5D and 5H).

Identification of core compounds and targets of *Plumula Nelumbinis*

The compounds-targets-pathways network was constructed to clarify the core compounds and corresponding targets of *Plumula Nelumbinis* in PH treatment (Fig. 6). We first screened out nodes with degrees ≥ 25 in the compound-target-pathway network as core targets, namely SRC, PIM1, TTR, F2, ESR1, PPIA, MAPK14, PAH, BACE1, and NOS3, respectively. Then the top 7 candidate core compounds were screened out according to the network topological parameters with the core targets, namely LZX1 (Neferine), LZX2 (Liensinine), LZX3 (Isoliensinine), LZX6 (N-Methylisococlaurine), LZX8 (N-

Methylcoclaurine), LZX14 (Nelumboferine), and LZX18 (Armepavine). The above core compounds and targets were used for subsequent molecular docking verification.

The molecule docking results for core compounds and targets

In order to determine the possible binding interaction between core compounds and targets, molecular docking analysis was performed by using AutoDock Vina. The molecular docking results showed that the average binding energy between the core compounds and targets is -7.03 kcal/mol, indicating an effective binding between the compounds and targets. As we all know, the lower the binding energy score, the stronger the compound's ability to bind to the targets. By setting the binding energy threshold to -8.00 kcal/mol, we finally obtained three compounds (LZX1, LZX2, and LZX3) and three targets (SRC, PIM1, and NOS3) for subsequent cell experiments verification (Fig. 7A). The molecular docking pattern between the core compounds and targets is shown in Fig. 7B.

Effect of *Plumula Nelumbinis* on hypoxia-induced hyperproliferation of PSMCs

Based on the network pharmacology and molecular docking results, we further explored the effects and underlying mechanisms of the three core compounds on the hypoxia-induced proliferation of PSMCs. Since the core compounds (neferine, liensinine, and isoliensinine) belong to alkaloids, we also observed the effect of total alkaloids from *Plumula Nelumbinis* (TAPN) on cell proliferation. As shown in Fig. 8A-D, the hypoxia-induced proliferation of PSMCs was inhibited by TAPN, neferine, liensinine, and isoliensinine in a dose-dependent manner. Under normoxic conditions, the above drugs did not affect cell proliferation. The drug concentration (TAPN: 50 µg/mL; neferine: 5 µM; liensinine and isoliensinine: 10 µM) for subsequent experiments was determined based on the results of CCK-8.

Finally, we evaluated the underlying mechanisms of the 3 core compounds on PSMCs hyperproliferation. Compared to the control group, the protein expression of p-SRC and PIM1 in the hypoxia group was significantly up-regulated, and these increases were attenuated by TAPN, neferine, liensinine, and isoliensinine (Fig. 8E-G).

Discussion

In this study, the anti-PH effect and potential mechanisms of the main active components of *Plumula Nelumbinis* were evaluated by network pharmacology, molecular docking, and in vitro experimental verification. Firstly, we screened out the main active components of *Plumula Nelumbinis* against PH by using network pharmacology and molecular docking analysis. Then, by constructing a hypoxia-induced PSMCs proliferation model, we further revealed that the anti-PH mechanism of *Plumula Nelumbinis* might be achieved by inhibiting the activation of SRC and PIM1.

PH is a malignant cardiopulmonary vascular disease characterized by pulmonary vascular remodeling. With the continuous improvement of diagnosis and treatment technology in recent years, the five-year

survival rate of patients with PH has reached 53.6%¹⁵. Unfortunately, PH is still an intractable disease for the current therapies can only alleviate patients' symptoms and cannot effectively reverse the process of vascular remodeling. Therefore, it is an urgent clinical problem to seek more precise and effective interventions to prevent or treat PH. TCM has been used to treat PH through a multi-level and multi-target approach¹⁰. The rise of network pharmacology, characterized by "multiple compounds-multiple targets-multiple signaling pathways", provides an effective way to systematically explore the internal mechanism of TCM in the treatment of PH⁴.

Plumula Nelumbinis is composed of various active ingredients, of which alkaloids are one of the main active ingredients. Recent studies showed that TAPN has the protective effect of inhibiting vascular remodeling in spontaneously hypertensive rats¹⁴. In addition, Jun et al. demonstrated that TAPN could inhibit the proliferation and migration of vascular smooth muscle cells, thereby inhibiting the carotid artery restenosis induced by balloon injury¹⁶. The above studies have shown that TAPN plays a cardiovascular protective effect by regulating the proliferation and migration of vascular smooth muscle cells. However, its effect on PH is still unclear. This study preliminarily screened 45 active components of *Plumula Nelumbinis* and 173 potential targets by consulting relevant literature and database information such as TCMSP, PubChem, SwissADME, and PharmMapper. Subsequently, 60 common targets were obtained by intersecting the potential active targets of *Plumula Nelumbinis* with PH-related targets, suggesting that *Plumula Nelumbinis* may delay the progression of PH by regulating multiple targets through multiple compounds.

PH is a complex disease involving multiple targets and pathways. In order to elucidate the functional and pathway enrichment of potential therapeutic targets in *Plumula Nelumbinis*, GO and KEGG enrichment analyses were performed using the Metascape platform. GO enrichment analysis showed that the anti-PH effect of *Plumula Nelumbinis* might be related to multiple biological processes and molecular functions, including regulation of MAPK cascade, wound healing, endopeptidase activity, and protein serine/threonine/tyrosine kinase activity. KEGG enrichment analysis revealed that its anti-PH effect might involve regulating cancer signaling pathways (hsa05205: proteoglycans in cancer; hsa05200: pathways in cancer; hsa05215: prostate cancer). It is worth noting that, comparable to cancer, PH is also a proliferative disease with high mortality and is thus known as the "cancer" of the cardiopulmonary vascular system. However, unlike cancer, which is caused by the excessive proliferation of cancer cells, PH is mainly caused by the excessive proliferation of vascular cells, especially the PSMCs in the media layer. Previous studies by ours and other groups demonstrated that neferine and isoliensinine have protective effects on angiotensin II (Ang II)-induced hyperproliferation of smooth muscle cells¹⁷⁻¹⁹. Based on the above reports and network pharmacology prediction, we speculate that the anti-PH effect of *Plumula Nelumbinis* may be related to the regulation of PSMCs hyperproliferation.

According to the compound-target-pathway network topology parameters, we obtained seven potential core compounds and ten core targets. The binding ability between the above compounds and targets was performed by molecular docking. Finally, three core compounds (LZX1, LZX2, and LZX3) and three core

targets (SRC, PIM1, and NOS3) were identified for subsequent in vitro experiments by setting the binding energy threshold ≤ -8.0 kcal/mol. SRC kinase, a proto-oncogene tyrosine-protein kinase, has been shown to mediate the occurrence and development of hypoxic PH by participating in the regulation of vasoconstriction, cell proliferation, and apoptosis^{20,21}. Recently studies have proved that the effect of SRC in promoting hypoxic PH is closely related to the regulation of the activation of downstream proteins such as HIF-1 α , HIF-2 α , signal transducer and activator of transcription 3 (STAT3) and NADPH oxidase²¹. In addition, inhibition of SRC activation by drug intervention could effectively reverse vascular remodeling in experimental PH^{22,23}, further clarifying the vital role of SRC in the promotion of PH. PIM1, a serine/threonine family kinase, has now been recognized as a novel biomarker and therapeutic target in the development of PH²⁴. A large number of studies have demonstrated that PIM1 is overactivated in patients with PH, preclinical animal models, or cell models²⁵⁻²⁷, and inhibition of PIM1 expression by drug intervention could also effectively delay the progression of PH^{26,28}. NOS3, also known as eNOS, is mainly expressed in endothelial cells and could play a protective role in PH vascular remodeling by regulating nitric oxide synthesis²⁹⁻³¹. The above studies show that SRC and PIM1 may participate in the development of PH by promoting the proliferation of PSMCs. On the contrary, NOS3 may alleviate the PH process by regulating the function of endothelial cells. Combined with the results of KEGG enrichment analysis, we preliminarily inferred that the anti-PH effect of *Plumula Nelumbinis* may be related to the inhibition of PSMCs proliferation by regulating the activation of SRC and PIM1.

Finally, we further clarified the anti-PH effect and potential mechanism of *Plumula Nelumbinis* in vitro cell experiments. Combined with GO enrichment analysis results, the biological process for *Plumula Nelumbinis* against PH involves response to hypoxia. To this end, we constructed a hypoxia-induced PSMCs proliferation model and then observed the effects of the three core compounds and TAPN on cell proliferation. Expectedly, the results from the present study found that TAPN, neferine, liensinine, and isoliensinine have a protective effect on hypoxia-induced PSMCs in a dose-dependent manner. Further results showed that the expression of PIM1 and p-SRC in PSMCs were significantly increased under hypoxic conditions; these phenomena were attenuated in the presence of TAPN, neferine, liensinine, and isoliensinine. These findings are consistent with our network pharmacology and molecular docking predictions, suggesting that the alkaloids of *Plumula Nelumbinis* may inhibit the proliferation of PSMCs by targeting SRC and PIM1, thereby achieving the purpose of treating PH. It is worth noting that we also detected the expression of eNOS in PSMCs after hypoxia treatment. However, the results showed that the basal expression of eNOS in PSMCs was deficient and challenging to detect. Further studies are needed to confirm the role of *Plumula Nelumbinis* on eNOS activity in pulmonary artery endothelial cells.

In addition to the hyperproliferation of PSMCs, hypoxia-induced contraction of PSMCs is also one of the crucial factors leading to PH³². Recently study found that neoliensinine, a novel benzyloquinoline alkaloid extracted from *Plumula Nelumbinis*, has the effect of relaxing vascular smooth muscle cells³³. The above study results show that the anti-PH effect of *Plumula Nelumbinis* may also be related to the inhibition of excessive contraction of PSMCs. However, follow-up studies are still needed to verify the effect of *Plumula Nelumbinis* on pulmonary vasoconstriction.

Conclusion

In summary, this study demonstrated for the first time that multi-components (mainly alkaloids such as neferine, liensinine, and isoliensinine) in *Plumula Nelumbinis* could inhibit the hypoxia-induced proliferation of PSMCs by regulating the expression of multiple targets (SRC and PIM1). The above results can provide an experimental basis for the development of new drugs for PH.

Declarations

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Author contributions

X.H.X. and F.M.L. participated in cell experiments and collected, analyzed, and processed experimental data. X.H.X. wrote the first draft. B.L., M.Y.F., Y.P.J., and S.L. were responsible for the design of the experiments, coordination of the project, and revision of the manuscript. All authors reviewed and approved the final version of the manuscript.

Competing interests

The authors declare no competing interests.

Ethics statement

The isolation and culture of primary PSMCs were derived from Sprague-Dawley (SD) rats. SD rats (~180 g) were provided by Hunan SJA Laboratory Animal Co.Ltd (No. SYXK (Xiang)2019-0004). All experimental animal protocols were approved by the Central South University Veterinary Medicine Animal Care and Use Committee. All animal recruitment, housing, treatment, and procedures were performed following the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The animal studies followed the ARRIVE guidelines.

Additional information

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Figures

Figure 1

The network pharmacology workflow of *Plumula Nelumbinis* against pulmonary hypertension. PH, pulmonary hypertension; PPI, protein-protein interactions; GO, gene ontology; KEGG, Kyoto encyclopedia of genes and genomes.

Figure 2

Potential targets of active ingredients in *Plumula Nelumbinis*. The circles represent targets (173 in total). The squares represent the active compounds from *Plumula Nelumbinis* (45 in total), including 30 alkaloids (marked in red), seven flavonoids (marked in yellow), and nine other compounds (volatile oils or polysaccharides, marked in purple). The importance of the compound or target in the network was evaluated by the degree value. The darker the square/circle color, the higher the degree value of the corresponding compound/ target, indicating that the compound or target is more critical in the network.

Potential targets of *Plumula Nelumbinis*

PH Related Genes

Figure 3

Venn diagram of potential targets of the main active components of *Plumula Nelumbinis* against PH. A total of 173 potential targets of the main active components of *Plumula Nelumbinis* were obtained by using the PharmMapper database. A total of 2326 pulmonary hypertension-related targets were obtained using GeneCards, OMIM, DisGeNET, and TTD databases. The intersection of the potential targets of *Plumula Nelumbinis* and PH-related targets was obtained by using the VENNY 2.1 online tool, which was identified as the potential targets of *Plumula Nelumbinis* against PH.

Figure 4

PPI network diagram of potential targets of *Plumula Nelumbinis* against PH.

Figure 5

GO and KEGG enrichment analysis of the potential targets of *Plumula Nelumbinis* against PH. **(A, E)** GO biological processes analysis. **(B, F)** GO cellular components analysis. **(C, G)** GO molecular functions analysis. **(D, H)** KEGG pathway enrichment analysis.

Figure 6

Compounds-targets-pathways network analysis diagram.

Figure 7

Molecular docking results of core compounds and targets. **(A)** The interaction between 7 candidate core compounds and ten core targets was evaluated by AutoDock Vina. **(B)** The molecular docking pattern between the core compounds and targets.

Figure 8

Plumula Nelumbinis attenuated hypoxia-induced proliferation of PASMCs through inhibition of the activation of SRC or PIM1. **(A)** Effects of TAPN, neferine, liensinine, and isoliensinine on the proliferation of PASMCs induced by hypoxia (n=3). **(B)** The protein expression of p-SRC, SRC, and PIM1 in PASMCs (n=8 per group); Data are expressed as mean $\pm$ SD. +TAPN: hypoxia+ total alkaloids of *Plumula Nelumbinis* (50 μ g/mL); +Nef: hypoxia+ neferine (5 μ M); +Lien: hypoxia+ liensinine (10 μ M); +Iso: hypoxia+ isoliensinine (10 μ M) ** P <0.01 vs Control; # P <0.05, ## P <0.01 vs Hypoxia.