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Research Article

Pomegranate Peel in the Amelioration of High-Altitude Disease: A Network Pharmacology and Molecular Docking Study of Underlying Mechanisms

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High-altitude disease (HAD) describes the failure to adapt to the lack of oxygen found at high altitudes and therapeutic antioxidant effects have been attributed to pomegranate peel (PP) extract. Network pharmacology, molecular docking, and experimental validation were used to study mechanisms responsible for the alleviation of HAD by PP. The aim was to establish a reference for future research and aid technological development, particularly in clinical settings. Network pharmacology analysis showed that PP affected many targets in HAD via the active ingredients, luteolin 7-O-glycoside, punicalagin, and ellagic acid. HNRNPA1, HSPA1B, HSPA1A, CUL4B, CLTC, PPP1CA, PARP1, RACK1, NEDD8, and MAP3K1 were all targets, responsible for effects on ribosomes, apoptosis, cell cycle, mRNA surveillance pathway, and the MAPK signaling pathway. PP had an antiapoptosis effect on H9c2 cells damaged by hypoxia, as shown by annexinV-FITC/PI double staining. *Practical Applications*. HAD comprises a group of diseases caused by failure to adapt to a low-oxygen environment. PP extract has previously been shown to have antioxidant effects. PP attenuated damage to H9c2 cells and reduced the apoptosis rate. The current results lay the foundation for further experimental investigations.

1. Introduction

HAD or mountain sickness describes a group of diseases caused by failure to adapt to the low oxygen environment found at high altitudes. It affects those who migrate from the plain to altitudes 3000 meters above sea level and those staying at high altitudes for short periods [1]. The high-altitude environment is characterized by thin air, low atmospheric pressure, low partial pressure of oxygen, a cold and dry climate, and strong ultraviolet radiation. High-altitude pulmonary or cerebral edema may occur with a threat to life [2]. China's evolving tourism industry and economy have resulted in increasing communication between the mainland and high-altitude areas and highlighted the importance of a study of HAD. The pathogenesis of HAD is poorly understood and few drugs exist for its treatment. The current study demonstrated the alleviation of hypoxic damage to H9c2 cells by PP with a view to exploring whether this agent may be a suitable drug for HAD.

PP is a by-product of the processing of pomegranates and is usually discarded as waste. Numerous biological activities have been attributed to PP, such as antioxidant, antitumor, anti-inflammatory, neuroprotective, antiviral, and antibacterial effects [3]. Ahmadipour et al. found that PP reduced pulmonary hypertension and blood pressure in broiler chickens due to its antioxidant and

hypolipidemic effects [4] and it was also shown to reduce serum malondialdehyde in broilers [5]. The phenolic and flavonoid compounds present in PP scavenged reactive oxygen species to produce antihyperglycemic and antihyperlipidemic actions. PP also enhanced liver and kidney function by comparison with glibenclamide and atorvastatin in diabetic and hyperlipidemic rats [6]. Some active components of PP have been shown to have antioxidant effects and pretreatment with luteolin 7-O-glycoside mitigated the cell apoptosis induced by hypoxia/reoxygenation in the H9c2 cells model via an effect on the MAPK pathway [7]. In addition, punicalagin prevented endothelial dysfunction and pulmonary hypertension in rats through antioxidant effects [8]. Pulmonary hypertension is a component of HAD pathogenesis and punicalagin might be expected to have an ameliorating impact on the disorder. Moreover, ellagic acid antagonized the mitochondrial damage caused by Bnip3, reducing cellular oxidative damage [9]. Despite the demonstrations of the antioxidant effects of PP, little information is available regarding mechanisms whereby hypoxic damage is alleviated.

"Network pharmacology" is a tool that enables the analysis of large amounts of data to contribute to theoretical knowledge and is of particular utility at the intersection of artificial intelligence and Chinese and Western medicine [10]. Mechanistic correlations of drugs and diseases may be achieved and drug mechanisms quantitatively evaluated [11]. Molecular docking allows the prediction of the affinity and binding patterns of proteins and ligands. Functions and mechanisms of action of a given protein and ligand may be approached from predictions of binding mode and free energy of binding. Virtual screening by molecular docking has become a vital step in the development of drugs targeting specific proteins [12].

2. Materials and Methods

2.1. Screening of PP Components and Targets. A search for PP components was conducted using the TCMSP (https://old.tcmsp-e.com/tcmsp.php) database [13]. Absorbability and drug-like properties were adopted as the selection index, and chemical components with $OB \geq 30$ and $DL \geq 0.18$ were selected as the main active components of PP. Compounds were included if relevant pharmacological effects had been reported previously. Molecular structures of components were obtained from the PubChem (https://pubchem.ncbi.nlm.nih.gov/) database [14] and stored in mol2 format from the TCMSP database. Potential targets were identified from the PharmMapper (https://www.lilab-ecust.cn/pharmmapper/index.html) database [15–17] using pharmacophore matching. Target proteins with a Normalized Fit Score (NF) ≥ 0.7 were selected. Gene symbols were assigned to potential targets using the UniProt (https://www.uniprot.org/) protein database [18]. A compound-target network was constructed by Cytoscape 3.8.2 software [19].

2.2. Screening of PP Targets. The GeneCards (https://www.genecards.org/) [20], DisGeNet (https://www.disgenet.org/home/) [21], and OMIM (https://www.omim.Org) [22] databases were searched using the terms: "plateau disease, high-altitude disease, altitude sickness, mountain sickness, high-altitude sickness, and high-altitude pulmonary hypertension" as keywords. The resulting core HAD targets were merged after the removal of duplicates. Gene symbols were assigned to potential HAD targets using the UniProt protein database. The intersection of drug and disease targets was found using the draw Venn diagram data processing site.

2.3. PPI Network Construction and Key Target Screening. Two separate protein-protein interaction (PPI) networks of PP and HAD targets were constructed using the BisoGenet plug-in [23] and the intersection between the two PPIs identified by Cytoscape 3.8.2 software and filtered by the CytoNCA plug-in. Nodes with a degree value greater than the median of 2 times the degree value of all nodes were selected as key nodes in the "big hubs" filtering network. Other nodes with values greater than the median of all nodes were also filtered and constituted the key genes. The above selection process was considered to result in the identification of the chemical components and targets constituting the direct or indirect actions of PP on HAD.

2.4. GO and KEGG Pathway Enrichment Analysis of Target Sites. Key targets identified in 2.3 were imported into the metascape (https://metascape.org/gp/index.html#/main/Step1) data analysis website [24] for gene ontology (GO) enrichment analysis according to biological process (BP), molecular function (MF), cellular components (CC), and Kyoto Encyclopedia of Gene and Genomes (KEGG) pathway analysis. Results were sorted by p value and the top 15 GO results and top 30 KEGG pathways with $p \leq 0.01$ were selected.

2.5. Molecular Docking of Key Targets and Components. Key targets identified in 2.3 were ranked in descending order of degree value and protein structures searched in the RCSB (https://www.rcsb.org/) database [25]. Human proteins were screened with a resolution ≤ 2.5 Å. The following methods were used: X-ray diffraction and with ligands and PDBID and 3D structures of the top 10 proteins were downloaded and saved in PDB format. Water was removed and hydrogenation was performed by AutodockTools 1.5.7 (https://autodock.scripps.edu/resources/tools) software [26] and results were stored as PDBQT format files. Structures of potential core components of PP were imported into Chem3D software for energy minimization, assignment of ligand atom types and ingredients screened according to docking score affinity. A docking score affinity $< -4.25\ \text{kcal·mol}^{-1}$ was considered to indicate binding of ligand to the target site, a score $< -5.0\ \text{kcal·mol}^{-1}$ indicates stronger binding, and a score $< -7.0\ \text{kcal·mol}^{-1}$ suggests a strong affinity between the two.

2.6. Cell Experimental Validation

2.6.1. Cell Culture. H9c2 cells (donated by Xinjiang Medical University) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, HyClone), supplemented with 10% fetal bovine serum (Gibco, USA) under 5% CO_2 at 37°C.

2.6.2. Measurements of Apoptosis. H9c2 cells in the logarithmic growth phase was inoculated into two 6-well plates at a density of 1×10^5 cells/mL in a volume of 2 ml per well and cultured overnight. One 6-well plate was added PP (Xian Ruihe Biotechnology Co., Ltd, Xian, China) at concentrations of 0 μg/L (hypoxic injury model group), 300 μg/L, and 600 μg/L and cells cultured for an additional 24 h in a hypoxic chamber and other 6-well plates were cultured in the chamber in a 5% CO_2 thermostatic incubator (HERA cell; Thermo Fisher Scientific, Inc.). Apoptosis was measured by AnnexinV-FITC/PI apoptosis double staining kit (BD Biosciences, USA). Cells were harvested by trypsinization, lightly pipetted with 2 mL of PBS (Hyclone, Utah, USA), centrifuged at 1000 rev/minutes for 5 minutes, washed 3x with PBS and a blank (no dye), 5 μl FITC single staining, a 5 μl PI single staining, and a double staining treatment group (5 μl FITC and 5 μl PI) created. Cells were resuspended at a density of 1×10^6 cells/ml in 1 × Binding Buffer and 5 μl each of FITC and PI were added to 100 μl of cell suspension, followed by 15 minutes incubation in darkness and the addition of 400 μl Binding Buffer. Cells were filtered through nylon mesh and assessed by flow cytometry (BD Biosciences). A protocol flow chart is shown in Figure 1.

3. Results and Discussion

3.1. Screening of Active PP Compounds and Targets. A total of 27 PP components were identified from the TCMSP database and 7 active ingredients satisfied the criteria of potential core compounds (Section 2.1). Luteolin 7-O-glycoside and punicalagin did not meet the screening criteria but were included on the basis of previous literature reports.

The final 9 active PP ingredients were luteolin 7-O-glycoside, punicalagin, ellagic acid, (+)-catechin, beta-sitosterol, quercetin, luteolin, fritillaziebinol, and kaempfero (Table 1). Targets with NF ≥ 0.7 were predicted using PharmMapper, and after the removal of duplicates, gene symbols were assigned by the UniProt protein database. A final total of 43 potential targets of PP components were identified. The resulting compound-target network constructed by Cytoscape 3.8.2 software contained 49 nodes and 142 edges (Figure 2(a)). The top 3 active components predicted from degree values were the phenolic compounds, luteolin 7-O-glycoside, punicalagin, and ellagic acid (Table 2).

Actions in counteracting hypoxic damage have been reported for all 3 compounds and ellagic acid, luteolin, and fritillaziebinol showed the strongest binding to key targets of HAD. From the combined results of degree value and molecular docking, ellagic acid emerged as a significant active component of PP with the potential for HAD treatment. Network pharmacology also identified (+)-catechin, beta-sitosterol, quercetin, and kaempferol as potential anti-HAD components with antioxidant and antihypoxia effects. Quercetin has previously been reported to reduce oxidative stress in the lungs under hypoxic conditions and to restore the integrity of the alveolar epithelium [27]. Hypoxia is the most important predisposing factor for HAD and agents with antihypoxia actions show promise for HAD prevention and treatment. Molecular docking demonstrated the strongest binding of potential active PP ingredients to the proteins 4G55, 6HX1, and 5V86, and binding with affinity < -5 kcal·mol^{-1} was shown by 71 out of 90 (78.9%) receptor-ligand combinations. The highest molecular docking score was shown by beta-sitosterol, confirming the reliability of network prediction results.

3.2. Screening of HAD Targets. Searches of GeneCards, DisGeNet, and OMIM databases yielded 2132 targets after the removal of duplicates and merging of HAD targets. The intersection of 43 PP targets with HAD targets produced 14 common targets (Figure 2(b)).

3.3. PPI Construction and Key Target Screening

3.3.1. PPl Network. The intersection of PP and HAD targets is shown in Figure 2(c).

3.3.2. HAD Targets of PP. 142 key nodes were screened for direct or indirect effects of PP in the treatment of HAD by the median of betweenness, closeness, LAC, and neighborhood connectivity (Figure 2(d)), and a network diagram of target interactions was constructed (Figure 3). Higher target degree values are represented by darker colors indicating larger nodes and increased significance to the network. PP components interacted with the highest frequencies with the HAD targets, HNRNPA1, EEF1A1, FUS, TUBB, HSPA1B, and HSPA1A.

HNRNP-A1 has been associated with apoptosis [28], and the MAPK family of tyrosine kinases is known to be linked to hypoxic damage to cells [29]. Thus, these targets, which may be associated with the inflammatory and apoptosis responses to oxidative stress [30], may be PP targets for the prevention and treatment of HAD.

3.4. GO and KEGG Analyses. GO and KEGG enrichment analyses were performed on 142 key nodes imported into the Metascape database (Figure 4). Biological processes of cytoplasmic translation, peptide biosynthetic process, amide biosynthetic process, and peptide metabolic process; cell components, ribonucleoprotein complex, cell-substrate junction, focal adhesion, ribosome, ribosomal subunit, molecular functions, structural molecule activity, structural constituent of ribosome, nucleoside-triphosphatase activity, pyrophosphatase activity, hydrolase activity, acting on acid anhydrides, and ATP-dependent activity were all enriched according to GO analysis. KEGG analysis showed that ribosomes, apoptosis, mRNA monitoring pathway, MAPK signaling pathway, cell cycle, RNA degradation, DNA

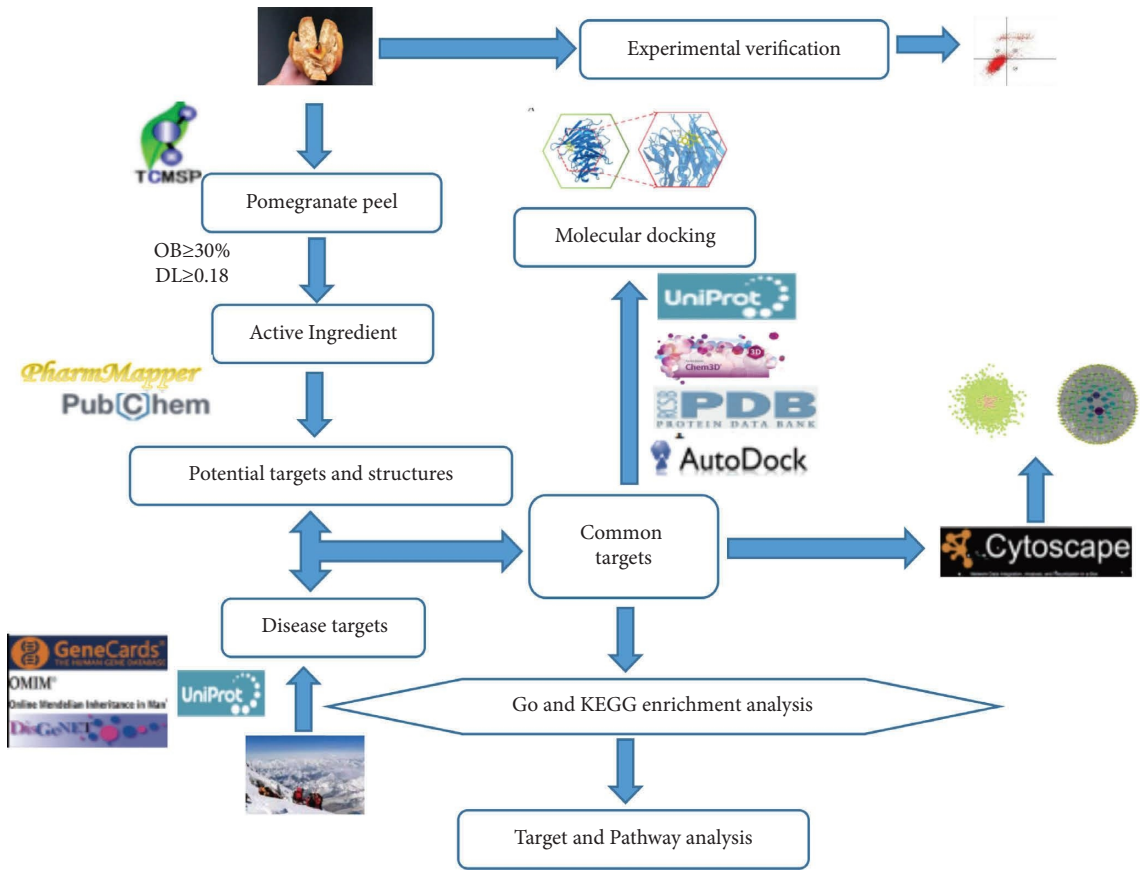


Figure 1: Flow chart.

replication, and gap junctions were enriched. These results may give insights into potential mechanisms of PP action on HAD. Further work on pharmacodynamics and mechanisms of action are required to confirm the pathways involved.

3.5. Molecular Docking Simulation of Compound-Target Interactions. Nine active ingredients of PP were shown to interact with 10 key targets, HNRNPA1 (PDBID : 1U1N), HSPA1B (PDBID : 3ATV), HSPA1A (PDBID : 6G3R), CUL4B (PDBID : 5FQD), CLTC (PDBID : 4G55), PPP1CA (PDBID : 3E7B), PARP1 (PDBID : 4HHY), RACK1 (PDBID : 6HX1), NEDD8 (PDBID : 5V86), and MAP3K1 (PDBID : 6GES) by molecular docking. 90 combinations of receptor-ligand docking interactions were examined and 71 combinations (78.9%) had an affinity < -5 kcal·mol^{-1} and 26 (28.9%) had an affinity < -7 kcal·mol^{-1}. Beta-sitosterol had the lowest binding energy of -10.45 kcal·mol^{-1} with CLTC. The top three active ingredients with affinities < -5 kcal·mol^{-1} were ellagic acid, luteolin, and quercetin, indicating strong interaction with core targets. Docking patterns for some compounds are shown in Figure 5 and molecular docking results in Figures 6(a)–6(c).

3.6. Cell Experimental Validation

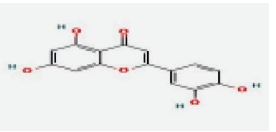
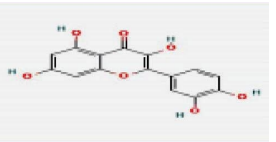
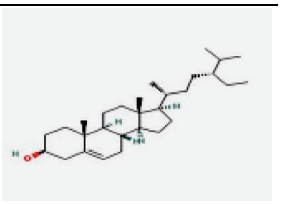
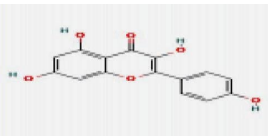
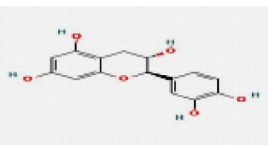
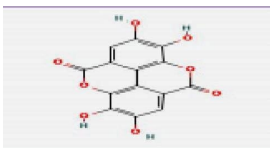
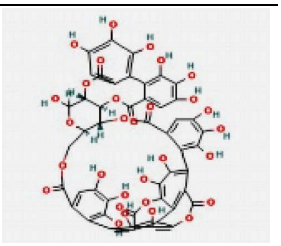
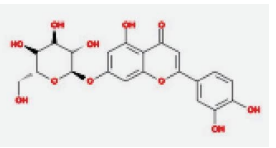
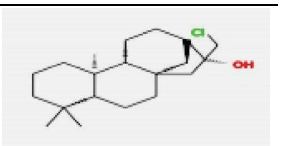
3.6.1. Application of H9c2 Cells. At high altitudes, low arterial PO_2 is a sustained feature, even after allowing adequate time for acclimatization [31]. Acute and chronic exposure to hypobaric hypoxia increase the levels of oxidative stress biomarkers [32], and the heart is a target organ for oxidative stress-related injuries [33].

H9c2 cells are rat embryonic myoblast cells that maintain several cardiac-like characteristics and have been widely used in vitro models to study cardiac hypoxia [34]. Therefore, in the present investigation, H9c2 cells were selected to study the protective effect of PP on hypoxic injury.

3.6.2. PP Reduced the Apoptosis of H9c2 Cells under Hypoxia. Hypoxic damage is a key contributor to the occurrence and development of HAD. Figure 7 shows dead cells in quadrant $Q1$, normal cells in quadrant $Q3$, cells undergoing early apoptosis in quadrant $Q2$, and cells undergoing late apoptosis in quadrant $Q4$. PP was shown to enhance the resistance of H9c2 cells to hypoxia-induced apoptosis. The total apoptosis rate of control group H9c2 cells ($Q2 + Q4$) was 15.2% and that of hypoxic injury model group H9c2 cells ($Q2 + Q4$) was 28.9%. With the addition of 300 μg/L or 600 μg/L PP, total apoptosis rates declined to 17.3% and 14.8%, respectively.

As a kind of flow cytometry, to quantify and characterize cell death in its various forms, AnnexinV-FITC/PI double staining remains a powerful methodology of choice. Flow cytometry represents a solid technique and allows robust data reproducibility [35]. Thus, the current study has

Table 1: Nine ingredients with the highest activity in pomegranate peel.

No	Ingredients	CAS ID	Molecular formula	Chemical structure
1	Luteolin	491-70-3	C15H10O6	
2	Quercetin	117-39-5	C15H10O7	
3	Beta-sitosterol	83-46-5	C29H50O	
4	Kaempferol	520-18-3	C15H10O6	
5	(+)-catechin	154-23-4	C15H14O6	
6	Ellagic acid	276-66-4	C14H6O8	
7	Punicalagin	65995-63-3	C48H28O30	
8	Luteolin 7-O-glycoside	—	—	
9	Fritillaziebinol	—	—	

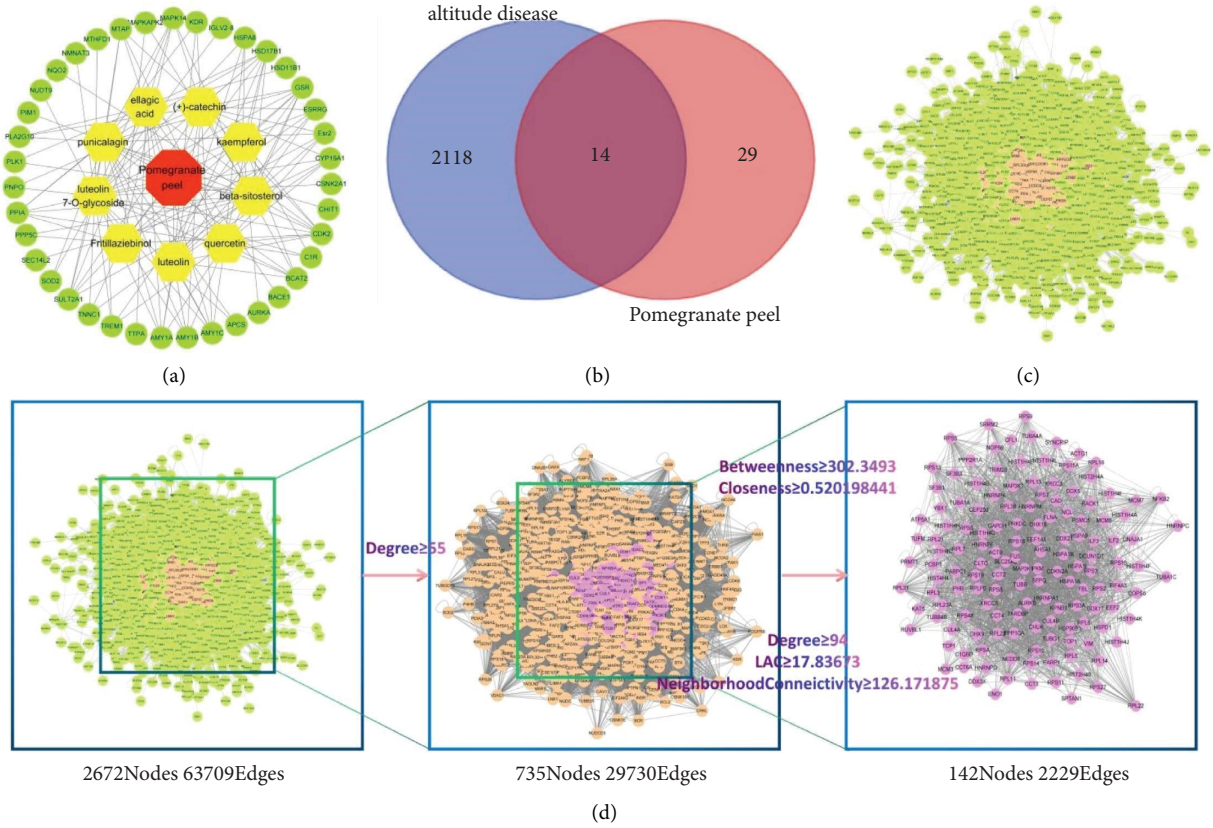


Figure 2: (a) Pomegranate peel active ingredient target network. (b) Venn map of drug target and disease target. (c) Pomegranate peel-disease intersection network. (d) Target screening strategy map of pomegranate peel intervention on key targets of high-altitude disease.

Table 2: Network node characteristic parameters of main active ingredients in pomegranate peel.

Ingredients	Degree	Betweennesscentrality	Closenesscentrality
Luteolin 7-O-glycoside	25	0.325209802	0.558139535
Punicalagin	19	0.14245289	0.489795918
Ellagic acid	16	0.176794744	0.461538462
(+)-Catechin	16	0.069025156	0.461538462
Beta-sitosterol	16	0.193772099	0.461538462
Quercetin	15	0.05078314	0.452830189
Luteolin	13	0.037554261	0.436363636
Fritillaziebinol	12	0.054754251	0.428571429
Kaempferol	10	0.05869621	0.413793103

demonstrated that PP enhances the resistance of H9c2 cells to apoptosis which is induced by hypoxic damage. The pathogenesis of HAD is known to involve hypoxic damage and PP may ameliorate this aspect of the disease. The current findings may inform future investigations into PP mechanisms of anti-HAD effects.

Previous literature reports, experimental validation, and the current findings have been combined to indicate that inflammation, hypoxic damage, ribosomes, apoptosis, ROS, and ATP-dependent activity are involved in HAD and these pathways constitute potential targets for the drug action of PP. PP may prevent and control HAD through

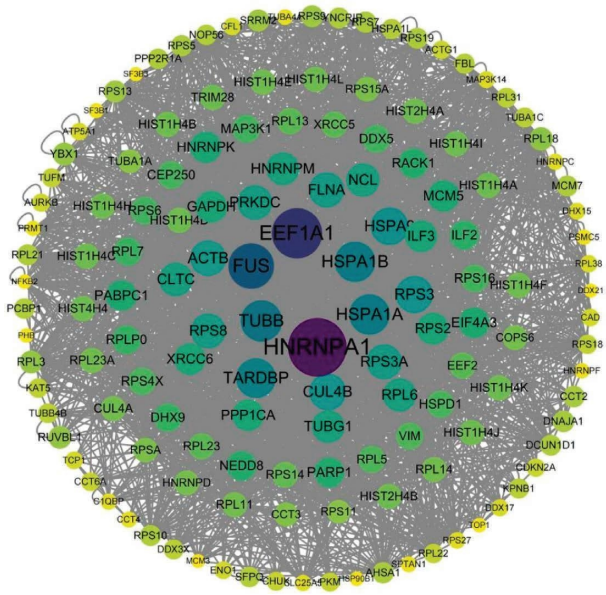


Figure 3: Analysis of the key targets of pomegranate peel in the treatment of high-altitude diseases.

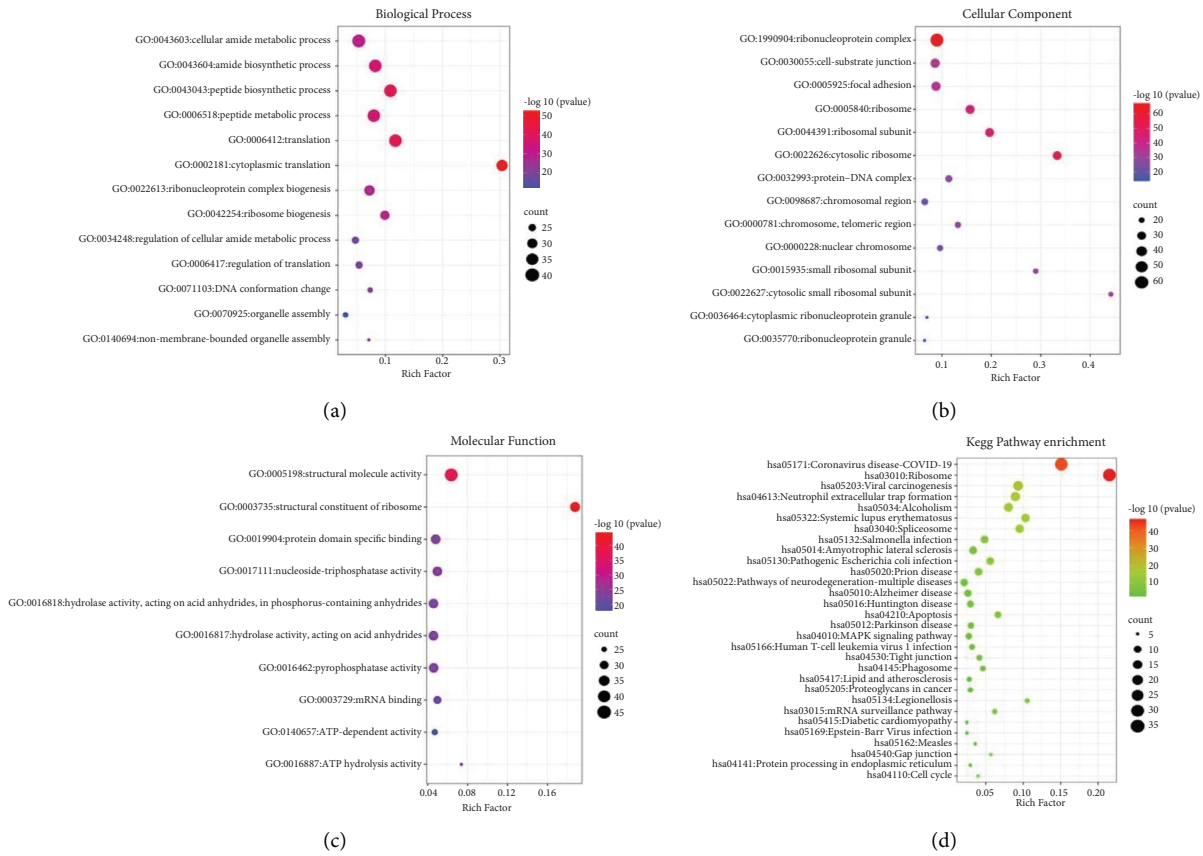


Figure 4: Enrichment analysis of key targets of pomegranate peel for the intervention of high-altitude disease.

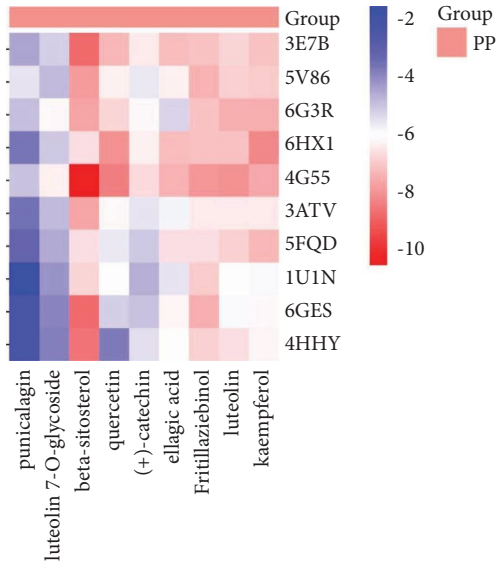
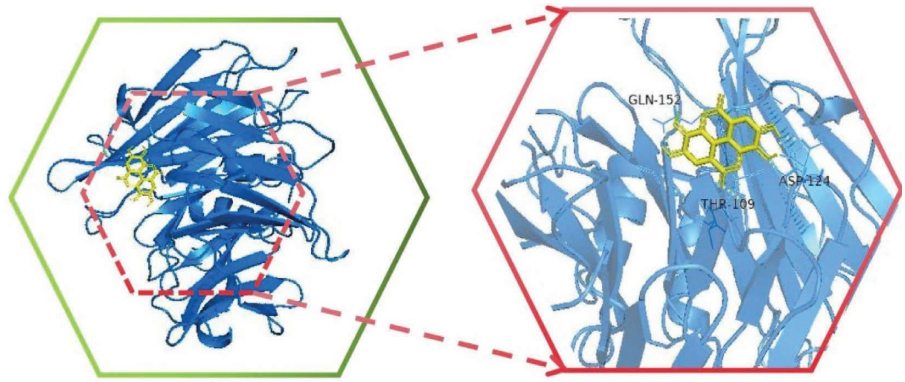
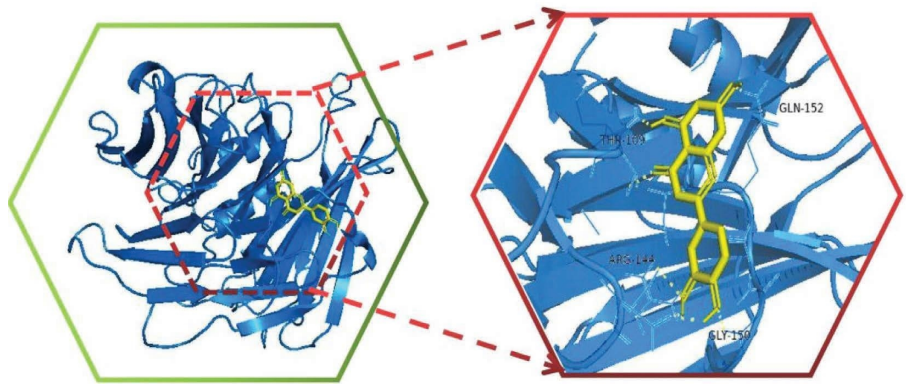


Figure 5: Result of molecular docking.

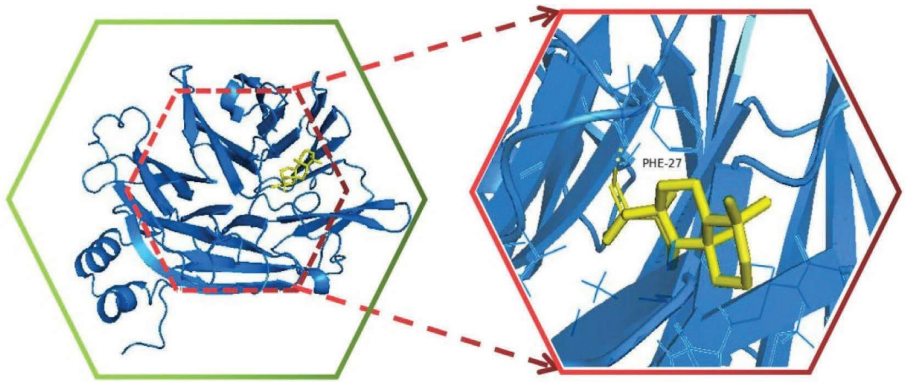


(a)



(b)

Figure 6: Continued.



(c)

Figure 6: Visualization of molecular docking (a) 4G55-ellagic acid. (b) 4G55-luteolin. (c) 4G55-fritillaziebinol.

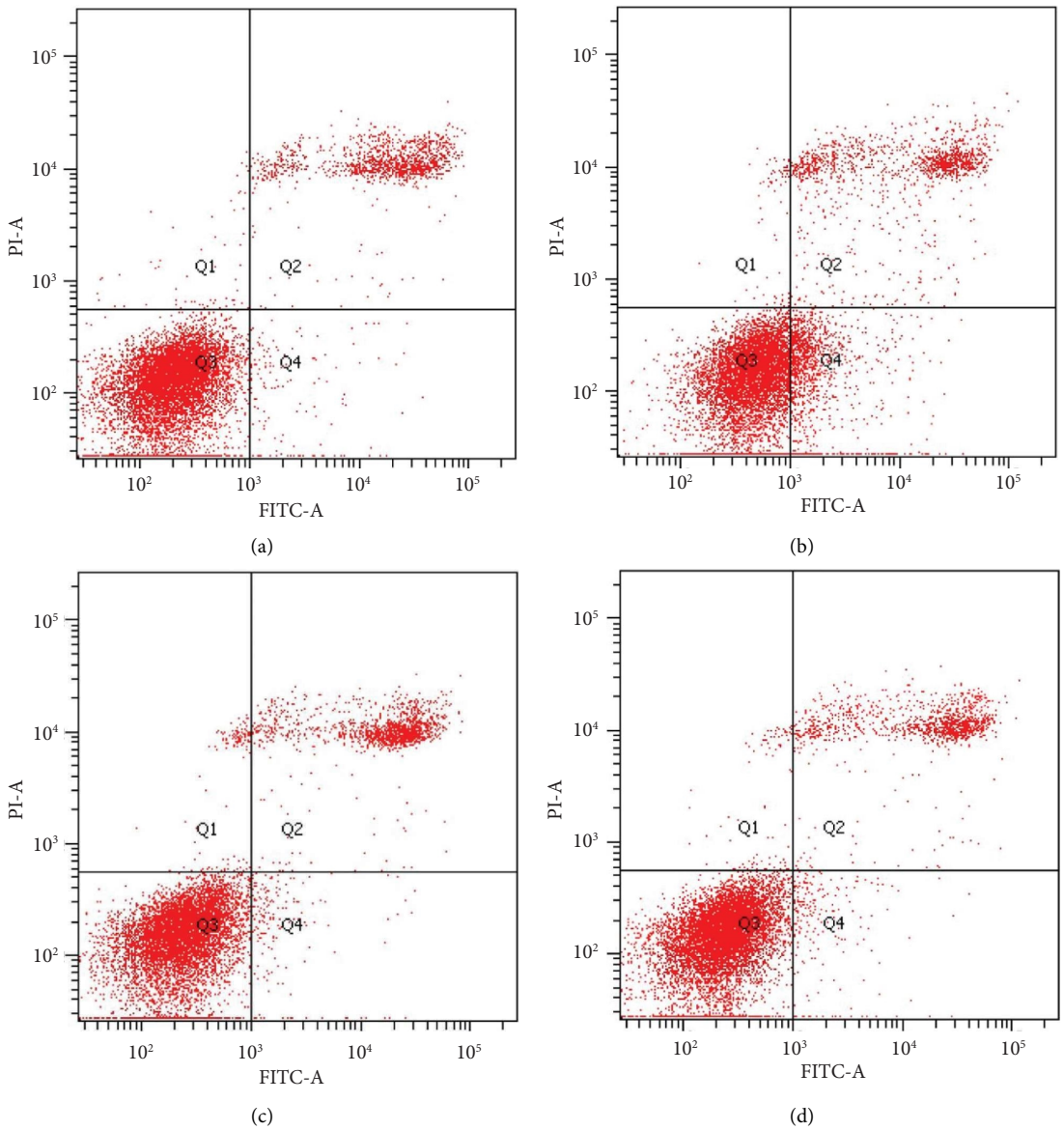


Figure 7: AnnexinV-FITC/PI double staining: (a) control group; (b) hypoxic injury model group; (c) concentrations of 300 μg/L; (d) concentrations of 600 μg/L.

impacts on the mRNA monitoring pathway, MAPK signaling pathway, cell cycle, RNA degradation, and DNA replication.

4. Conclusions

The current study utilized a combined approach of network pharmacology, molecular docking, target prediction, PPI network, GO enrichment analysis and KEGG pathway enrichment analysis to show that PP can improve the damage caused by HAD through multi-component-multi-target-multi-pathway, PP had an antiapoptosis effect on H9c2 cells damaged by hypoxia.

The current results may inform future in-depth studies. Further work is planned on PP pharmacology and animal and cell-based experiments to reveal more targets and mechanisms of PP intervention in HAD.

Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Alimire Yeerjiang and Dilinuer Maimaitiyiming contributed equally to this work.

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