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Proteomic Profiling Reveals Differential Mechanisms of Icariin and Epimedin C in Regulating Thymic Immunity

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Abstract

Objective: To systematically compare the differential targets and molecular mechanisms of two active flavonoids from Epimedium, Icariin and Epimedin C, in the thymus using proteomics, and to elucidate the material basis for their immunomodulatory effects. **Methods:** BALB/c mice were randomly divided into Normal, Epimedin C, and Icariin groups. After 3 days of intraperitoneal administration, thymus tissues were collected for Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS). Bioinformatics methods were employed to screen differentially expressed proteins, followed by protein-protein interaction networks construction, and Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses. **Results:** Epimedin C and Icariin regulated 62 and 103 differentially expressed proteins, respectively, with 21 common targets. Through network analysis, Superoxide dismutase 2 (Sod2) and Serine/arginine-rich splicing factor 1 (Srsf1) were identified as the shared core common targets. Functional enrichment analyses results revealed that both Sod2 and Srsf1 significantly affected mRNA splicing and mitochondrial energy metabolism pathways. However, Epimedin C uniquely regulated amino acid biosynthesis and the citric acid cycle (TCA cycle), whereas Icariin was more involved in SNARE interactions in vesicular transport and the JAK-STAT signaling pathway. **Conclusions:** Icariin and Epimedin C collectively maintain mRNA splicing homeostasis through their shared targets, Sod2 and Srsf1. However, due to structural differences, the two tend to regulate thymocyte reprogramming and signal transduction in distinct ways. Ligustrazine and caosetide C ultimately exert their immunomodulatory functions through synergistic and complementary effects on multiple targets and pathways.

1 Introduction

As age increases, the body's immune response ability declines markedly, a phenomenon known as "immunosenescence", accompanied by decreased functions of T cells [1], B cells [2] and innate immunity [3], thymic atrophy [4], chronic inflammation [5], etc. Therefore, exploring effective immunomodulatory methods has become one of the hotspots in current biomedical research. As a key central immune organ for the development and maturation of T lymphocytes, the age-related degeneration of thymus structure and function is a core marker of immunosenescence [6]. Thymus senescence is characterized by a decrease in thymic epithelial cells, weakened T cell output capacity, and tissue fibrosis, ultimately leading to a deficiency in adaptive immune response and increased susceptibility to infections, tumors, and autoimmune diseases [7,8]. Hence, searching for strategies that can delay thymic degeneration and reshape immune homeostasis has become a cutting-edge hotspot in anti-aging research.

Epimedium is the dried leaf of Berberidaceae plants, *Epimedium brevicornum* Maxim., *Epimedium sagittatum* (Sieb. et Zucc.) Maxim., *Epimedium pubescens* Maxim., *Epimedium wushanense* T.S. Ying, or *Epimedium koreanum* Nakai. Epimedium is known in traditional Chinese medicine (TCM) theory for its effects of "tonifying the kidneys and strengthening yang, reinforcing tendons and bones". Modern pharmacological research has revealed that its extracts and flavonoids have great potential in regulating bone metabolism, neuroprotection, and immunoregulation [9,10]. The Chinese Pharmacopoeia lists Epimedin C and Icariin as indicator components for the quality control of epimedium [11], which have previously been confirmed to have antioxidant, anti-inflammatory, and estrogen-like activities, but their specific targets and molecular mechanisms in the immune core organ, thymus, are not yet clear. Particularly, the similarities and differences in their effects need to be systematically elucidated.

Proteomic technology can reveal the expression profile and functional network of proteins in specific physiological or pathological states of organisms from a holistic perspective, and is a powerful tool for dissecting the multi-component and multi-target mechanisms of TCM [12,13]. This study utilizes proteomics technology based on Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS), combined with bioinformatics analysis, to draw the protein expression profile of mouse thymus tissue after intervention with Epimedin C and Icariin, screen and validate its key functional targets, and systematically compare the common and differential molecular mechanisms of two components in regulating thymic immunity. This research will provide a theoretical basis for elucidating the modern scientific connotation of Epimedium's efficacy in "tonifying kidney and nourishing essence" and for developing targeted immunomodulatory drugs.

2 Materials and methods

2.1 Experimental methods

All animal experimental protocols and procedures have been reviewed and approved by the Animal Ethics Committee of Zhejiang University of Traditional Chinese Medicine, with ethics approval number of IACUC-20250331-29. The experiments followed the National Guidelines for the Care and Use of Experimental Animals. 9 healthy female BALB/c mice (6-8 weeks old) were selected, labeled and randomly divided into three groups based on body weight: Normal group, Epimedin C group, and Icariin (ICT) group. Epimedin C (CAS#110642-44-9, Lot#P2382448; 98%, Shanghai Tongtian Biotechnology Co., Ltd.); Icariin (CAS#521-45-9, Lot#24061841; 98%, Shanghai Tongtian Biotechnology Co., Ltd.); dissolved in physiological

saline to a concentration of 5 mg/mL. The treatment group mice were intraperitoneally injected with the corresponding compounds at a dose of 50 mg/kg/day. The control group received an equal volume of physiological saline.

Mice were maintained under specific pathogen-free (SPF) conditions with controlled temperature (22 ± 2 ℃), humidity (50% ± 10%), and a 12/12-hour light-dark cycle. Standard rodent diet and water were freely available. Each group consisted of 3 mice (n = 3). Three days after administration, the mice were euthanized by cervical dislocation , and thymus tissues were collected for subsequent analysis. During the experiment, mice were administrated and weighed daily. General behaviors, including food intake measurement, were observed.

2.2 Proteomics analysis

Thymus tissue proteins were converted into peptides through alkylating pretreatment and trypsin digestion, followed by desalting and purification to obtain concentrated peptide samples for downstream analysis. LC-MS/MS data were acquired in a non-dependent manner using the Evosep One liquid chromatography system coupled with a ZenoTOF 7600 mass spectrometer. The raw data were processed for library search and quantification using DIA-NN software under the following conditions:

(1) Chromatographic condition

System: Evosep One liquid chromatography system coupled with ZenoTOF 7600 mass spectrometer.

Chromatographic column: 150 μm × 15 cm C18 column (1.9 μm packed material, Evosep Biosystems).

Mobile phases: Phase A (0.1% formic acid aqueous solution), Phase B (0.1% formic acid acetonitrile solution).

Gradient protocol: Standard 30 SPD method, flow rate 500 nL/min, total duration 44 min.

(2) Mass Spectrometry Parameters

Ion source: OptiFlow Turbo V, ionization voltage 3200 V (positive ion mode), Gas 120 psi, Curtain Gas 35 psi.

Scan range: MS1 (400–1500 Da, 100 ms), MS2 (140–1750 Da, 25 ms).

Zeno ion trap activation, with 85 Q1 windows.

(3) DA

Software: DIA-NN (v1.8.1).

Database: Uniprot Mouse Database (version 10 March 2023).

Parameters: Trypsin digestion (allowing one missed cleavage), fixed modification (cysteine urea methylation), peptide length 7–30, parent ion charge 1–4, False Discovery Rate(FDR) threshold 1%.

2.3 Bioinformatics analysis

2.3.1 Differential protein analysis

The raw mass spectrometry data were used to calculate p values and log₂Fold Change ($\log_2$FC) values. Genes with $\log_2FC > \log_2(1.5)$ were considered upregulated, and those with $\log_2FC < -\log_2(1.5)$ were considered downregulated. p-value < 0.05 Significant proteins were screened, and heatmaps and volcano plots were generated. Differentially expressed proteins from the Epimedin C and Icariin groups were integrated to obtain the intersection gene. These genes were uploaded to VENNY 2.1 to draw a Venn diagram. All gene symbols adhere to standard naming conventions.

2.3.2 Construction of protein-protein interaction (PPI) network

The differentially expressed proteins were input into the STRING database for prediction, with the species “Mus musculus” selected. After construction, the TSV file was saved and imported into Cytoscape 3.10.0 for visualization. Key proteins were screened based on a

degree value exceeding twice the median. Combined with Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, a component-target-pathway network was plotted.

2.3.3 Gene Ontology (GO) and KEGG enrichment analysis

Common targets were imported into the David database (version 2023q4) for enrichment analysis. Pathways with $p < 0.05$ were selected, and the top ten pathways with the largest Log p values were chosen for GO enrichment analysis and KEGG pathway analysis. GO functions included biological processes (BP), cell composition (CC), and molecular functions (MF). Finally, the results were uploaded to the WeChat platform to create the graphs.

3 Results

3.1 General behaviors of mice

According to Table 1, Table 2, and Figure 1, the body weight of mice in each group was steadily increased. The Epimedin C group had significantly higher food intake in the first two days than the Icariin group, but on the third day, the food intake of both groups was lower than that of the Normal group. The average thymus weight of mice in each group was 0.061 ± 0.006, 0.055 ± 0.007, and 0.047 ± 0.006 (Mean ± SD, n = 3), respectively. Compared with the normal group, the thymus index in the drug group was numerically lower, but there was no statistical difference ($p > 0.05$). The existing data cannot prove that changes in body weight and thymus index were attributed to drugs, necessitating further analysis combined with proteomics.

Table 1 The impact of administration on body weight of mice (Mean ± SD, n = 3) (unit: g).

Group	Day 0	Day 1	Day 2	Day 3
Normal	19.49 ± 0.59	20.06 ± 1.03	20.28 ± 1.16	20.52 ± 1.34
Epimedin C	19.19 ± 1.28	19.48 ± 1.15	19.33 ± 1.05	19.68 ± 1.29
Icariin	19.02 ± 0.44	18.93 ± 0.73	18.88 ± 0.67	19.34 ± 0.67

Table 2 The impact of administration on thymus weight of mice (Mean ± SD, n = 3).

Group	Wet weight of thymus (g)	Thymus index
Normal	0.061 ± 0.006	0.0030 ± 0.0002
Epimedin C	0.055 ± 0.007	0.0028 ± 0.0003
Icariin	0.047 ± 0.006	0.0024 ± 0.0003

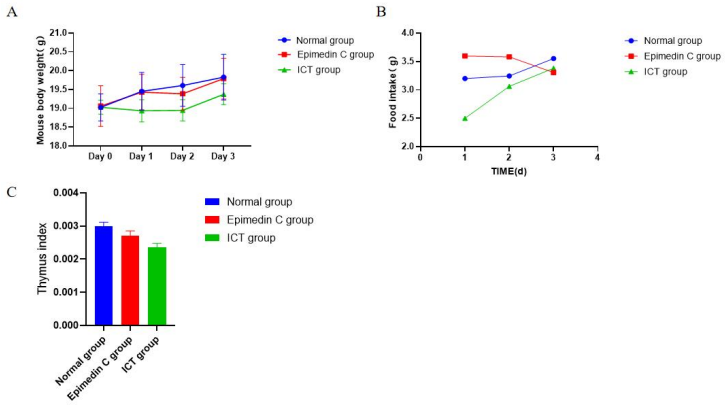

Figure 1 Effects of Epimedin C and Icariin on general physiological indicators in mice. (A) Dynamic body weight changes in mice from the Normal, Epimedin C, and Icariin groups (n = 3). Data are presented as mean ± SD. (B) Comparison of daily average food intake among the groups. Data are presented as mean ± SD. (C) Thymus index

results, calculated as thymus weight (mg) / body weight (g). Values are presented as mean ± SD (n = 3). No statistically significant difference was observed in the thymus index among groups ($p > 0.05$, one-way ANOVA), indicating that short-term drug intervention did not significantly alter this parameter under the present experimental conditions.

3.2 Analysis of differential proteins in the Epimedin C group and the Normal group

As shown in Figure 2 A-B, among the differentially expressed proteins, molecules such as Nsd1 and Ddx5 were closely related to thymus development and immune function. Nsd1 mediates gene expression related to T cell development through methylation and DNA methylation, such as T cell receptor (Tcr) gene expressions [14], while Ddx5, as a key factor in RNA metabolism, may affect T cell receptor signaling pathways via splicing regulation [15]. In addition, upregulated protein Sin3a plays an essential role in maintaining the homeostasis of thymic epithelial cells, and its expression deficiency can cause cell cycle arrest and abnormal differentiation [16]. As a key kinase for chromosome segregation, Sin3a upregulation may promote thymic cell mitosis and be associated with enhanced thymic tissue regeneration ability [17].

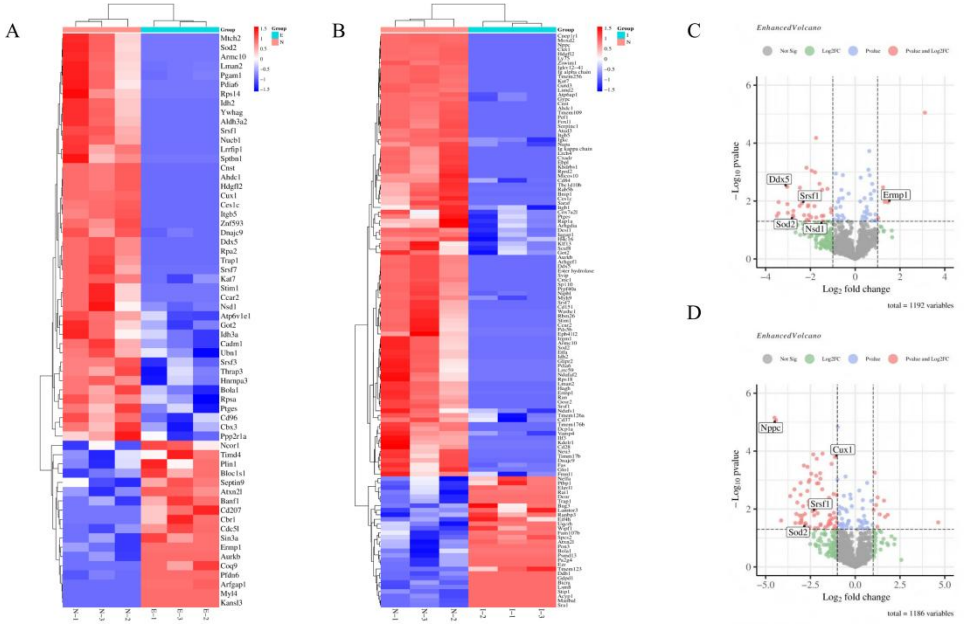

Figure 2 Differential expression analysis of the thymic proteome following Epimedin C and Icariin intervention. (A, B) Hierarchical clustering heatmaps of differentially expressed proteins (DEPs). Display the expression patterns of significant DEPs compared to the Normal group in the (A) Epimedin C group and (B) Icariin group. Each row represents a protein, and each column represents a biological replicate sample (n = 3). The color scale indicates relative expression levels: red denotes upregulation, and blue denotes downregulation. The clustering results show unique protein expression profiles between different treatment groups. (C, D) Volcano plots of differentially expressed proteins. Display the expression changes and statistical significance of all identified proteins compared to the Normal group in the (C) Epimedin C group and (D) Icariin group. The X-axis is $\log_2$ (fold change), and the Y-axis is $-\log_{10}$ (p-value). Some representative key DEPs are labeled. Data points are colored and marked based on the filtering criteria ($|\log_2 FC| > \log_2(1.5)$ and $p < 0.05$): Red dots on the right indicate significantly upregulated proteins, red dots on the left indicate significantly downregulated proteins, and gray dots represent proteins with no significant change.

3.3 Analysis of differential proteins in the Icariin group and the Normal Group

Figure 2 C-D revealed that among the differentially expressed proteins, molecules, such as Ig Alpha Chain c Region, Ig Kappa Chain v-v Region Mopc 149, Cux1, and Nppc, were intimately associated with thymic immune function and microenvironment regulation. The Ig Alpha chain [18] and Ig Kappa chain are important components of immunoglobulin, mainly involved in antibody formation and immune response and indirectly playing a role in the development and maturation of T cells in the thymus. Cux1 is a transcription factor, which may affect T cell maturation during thymic development via regulating cell proliferation and differentiation [19]. Research on the role of Nppc in cardiovascular diseases suggests that it may affect the blood supply and microenvironment of the thymus [20].

3.4 Differential proteins analyses in Epimedin C and Icariin groups

This study identified and quantified 1,237 proteins in thymic tissue. As shown in Figure 3 and Table 3, using thresholds of $|\log_2 FC| > \log_2(1.5)$ and $p < 0.05$, 62 and 103 differentially expressed proteins were screened in the Epimedin C and Icariin groups, respectively. Notably, 21 proteins overlapped between the two groups. Among them, Aurkb and Ermp1 were upregulated in the Epimedin C group but downregulated in the Icariin group, indicating the compound-specific regulatory effects.while the remaining 19 proteins were significantly downregulated. 12 proteins, Cnst, Ahdc1, Itgb5, Ddx5, Srsf1, Srsf7, Idh2, Armc10, Stim1, Ccar2, Sod2, and Pdia6, exhibited identical $\log_2 FC$ values. These proteins may be involved in processes such as metabolic support, protein folding, and RNA processing, thereby influencing T cell development and maturation and indirectly participating in thymic immunomodulation. Although no direct evidence currently linked these 21 overlapping proteins to Epimedin C or Icariin, they were hypothesized to represent common targets of both compounds and warrant further investigation.

These common targets suggested that despite structural differences between the two components, they may exert synergistic effects by regulating certain core biological processes within the thymus.

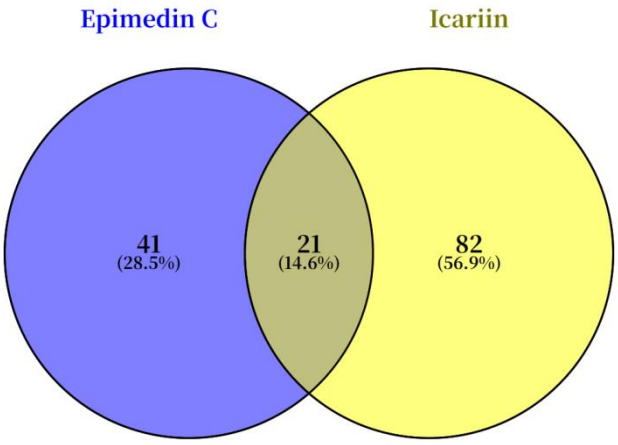

Figure 3 Venn diagram of common and unique differentially expressed proteins. This diagram shows the overlap of DEPs between the Epimedin C intervention group (62 proteins) and the Icariin intervention group (103 proteins). The intersection contains 21 proteins, representing potential common targets that may be involved in the shared immunomodulatory mechanisms of both compounds.

Table 3 Differential proteins coexisting between Epimedin C and Icariin groups.

Genes	Epimedin C		Icariin	
	log_2FC	Regulation	log_2FC	Regulation
Aurkb	0.7174	Up	-1.2074	Down
Ermp1	1.4620	Up	-1.0869	Down
Cux1	-1.7479	Down	-1.1221	Down
Cnst	-2.1764	Down	-2.1764	Down
Ahdc1	-1.9442	Down	-1.9442	Down
Hdgfl2	-0.8671	Down	-2.1461	Down
Itgb5	-3.3907	Down	-3.3907	Down
Kat7	-1.5952	Down	-2.4535	Down
Ddx5	-3.0436	Down	-3.0436	Down
Dnajc9	-1.2376	Down	-0.8746	Down
Srsf1	-2.3807	Down	-2.3807	Down
Srsf7	-2.0981	Down	-2.0981	Down
Got2	-2.1135	Down	-1.9394	Down
Ptges	-1.0685	Down	-0.6570	Down
Idh2	-3.0091	Down	-3.0091	Down
Armc10	-2.7813	Down	-2.7813	Down
Stim1	-1.4170	Down	-1.4170	Down
Ccar2	-1.4934	Down	-1.4934	Down
Sod2	-2.7632	Down	-2.7632	Down
Lman2	-1.7395	Down	-2.8079	Down
Pdia6	-2.0867	Down	-2.0867	Down

3.5 Core targets screening

As depicted in Figure 4, to identify key functional nodes from complex differential proteins, we constructed a PPI network. The results showed that Sod2 and Srsf1 occupy central positions in both networks. Sod2 is a key antioxidant enzyme in mitochondria, and its downregulation may be associated with a potential decrease in ROS levels, indicating an improved redox microenvironment of cells. However, it should be noted that the relationship between Sod2 expression and enzymatic activity may be complex, and future studies directly measuring ROS levels are necessary to validate this hypothesis. More importantly, as a critical splicing factor, the downregulation of Srsf1 strongly suggested that Icariin and Epimedin C may collectively affect the

splicing process of precursor mRNA in thymocytes. The precise regulation of mRNA splicing is crucial for generating T cell receptor diversity and ensuring normal T cell development and differentiation [7]. This study was the first to speculate through proteomic data that regulating mRNA splicing may be a previously underappreciated mechanism by which Epimedium exerts thymus-protective effects.

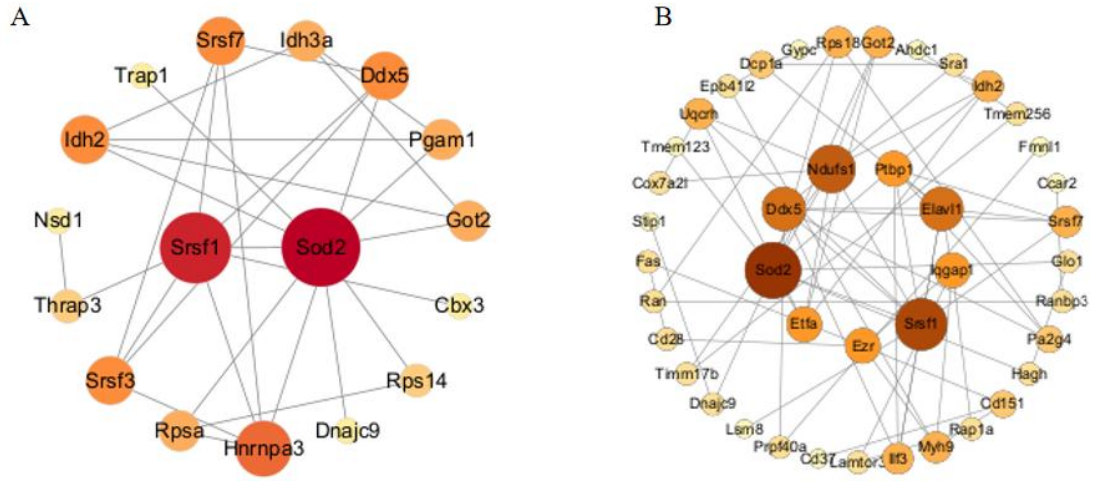

Figure 4 Protein-Protein interaction (PPI) networks of differentially expressed proteins. Networks were constructed using the STRING database and visualized with Cytoscape software. Nodes represent proteins, and connecting lines represent interactions. The color depth and size of the nodes correlate with the number of interactions. (A) PPI network for DEPs in the Epimedin C group. (B) PPI network for DEPs in the Icariin group. In both networks, Sod2 and Srsf1 were identified as core hub nodes, suggesting their potential key roles in the respective regulatory networks.

3.6 GO enrichment analysis

3.6.1 GO enrichment analysis of Epimidin C group

As shown in Figure 5 A, the GO enrichment analysis of differentially expressed genes revealed that proteins in Epimedin C group primarily participated in various biological processes, including cellular respiration, production of precursor metabolites and energy, mRNA splicing, energy derived from the oxidation of organic compounds, regulation of mRNA processing, regulation of RNA splicing, transcription co-regulator activity, and thyroid hormone receptor binding, etc. These processes mainly relate to mitochondrial energy metabolism, mRNA splicing regulation, and nuclear hormone receptor-mediated transcriptional regulation pathways.

GO enrichment analysis revealed that Epimedin C may delay thymic aging through multi-target synergistic effects: (1) mediating mitochondrial oxidative phosphorylation and ROS metabolism balance to maintain energy supply to thymocytes; (2) suppressing excessive activation of spliceosomes and reducing T cell developmental defects caused by abnormal mRNA splicing; (3) maintaining thymic epithelial microenvironment homeostasis through epigenetic modification reprogramming of nuclear receptors. These mechanisms were highly consistent with previous proteomics results (such as differential expressions of Ddx5 and Nsd1), implying that Epimedin C may exert thymus-protective effects via a multi-dimensional regulatory network involving "metabolism-splicing-epigenetics".

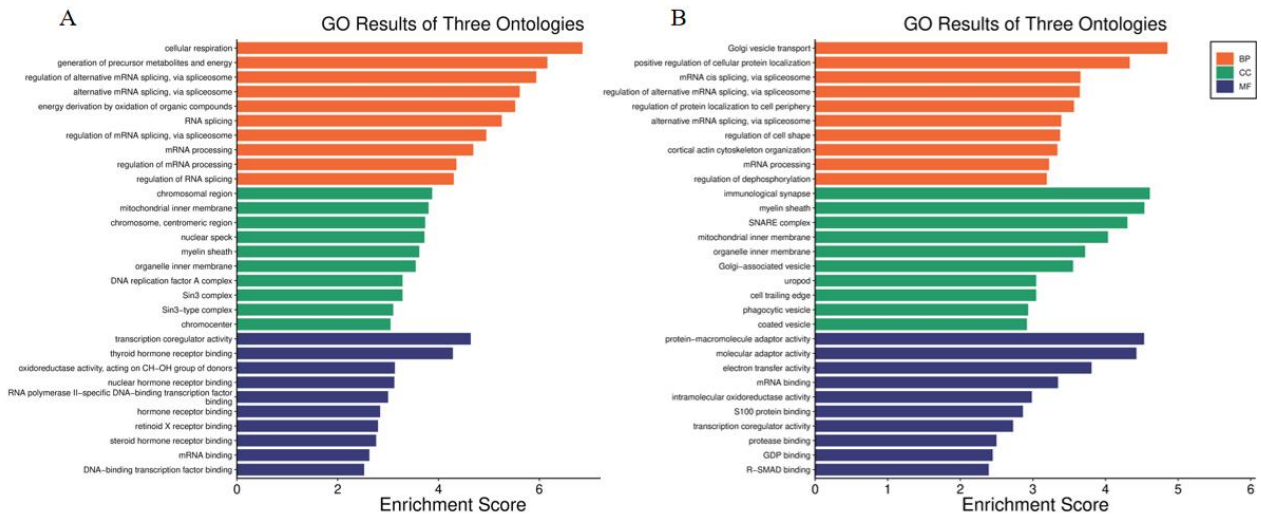

Figure 5 GO functional enrichment analysis of differentially expressed proteins. Bar charts display the top significantly enriched GO terms in the Biological Process, Cellular Component, and Molecular Function categories. The X-axis represents the enrichment factor, and the Y-axis shows the GO terms. The length of the bars corresponds to the statistical significance ($-\log_{10}(p\text{-value})$). (A) GO enrichment for DEPs in the Epimedin C group, showing terms such as "cellular respiration" and "mRNA splicing". (B) GO enrichment for DEPs in the Icariin group, showing terms such as "Golgi vesicle transport" and "SNARE complex". This analysis reveals the distinct biological processes affected by each compound.

3.6.2 GO enrichment analysis of Icariin group

As shown in Figure 5 B, proteins in Icariin group were mainly involved in different biological processes, including Golgi vesicle transport, positive regulation of cellular protein localization, mRNA cis-splicing via the spliceosomes, immune synapses, myelin sheath, SNARE complexes, protein-macromolecule adaptor activity, molecular adaptor activity, and electron transfer activity. These processes are mainly associated with vesicle transport, mRNA splicing regulation, and protein activity modification pathways.

GO enrichment analysis suggested that the mechanism by which icariin counteracted thymic aging may be achieved through multi-dimensional synergistic effects: (1) regulating the transport efficiency of Golgi vesicles and maintaining the secretion function of thymic stromal cells; (2) repressing abnormal mRNA splicing, and ensuring TCR diversity and T cell positive selection; (3) balancing thymocyte proliferation and apoptosis by mediating the activity of oxidoreductase and phosphatase. The above findings were consistent with previous proteomics data (such as differential expressions of Srsf1 and Rab5b), suggesting that Icariin may maintain thymic homeostasis through a dynamic network of “transport-splicing-metabolism”.

3.7 KEGG pathway analysis

3.7.1 KEGG pathway analysis of Epimedin C group

According to Figure 6 A, KEGG pathway enrichment identified 40 signaling pathways, which mainly involved amino acid biosynthesis, 2-oxocarboxylic acid metabolism, spliceosome, carbon metabolism, TCA cycle, Transforming Growth Factor Beta (TGF- β) signaling pathway, arginine and proline metabolism, cell cycle, lysine degradation, glycolysis/gluconeogenesis, etc.

Epimedin C synergistically protected thymic function through multi-dimensional metabolism and signaling pathways: (1) regulating amino acid metabolism to

maintain thymic matrix structure and balance cell proliferation; (2) dampening TCA cycle flux and delaying cell senescence through SIRT1 activation; (3) activating TGF- β /SMAD3 signaling to suppress excessive immune response and promote Treg differentiation. The above mechanisms showed high consistency with previous proteomics data (such as differential expressions of Idh2 and Smad3) and GO enrichment results of " mitochondrial energy metabolism ", suggesting that Epimedin C delayed age-related thymic degeneration through a "metabolism-immunity-epigenetics" network.

3.7.2 KEGG pathway analysis of Icariin group

In light of Figure 6 B, the KEGG pathway enrichment identified 55 signaling pathways, primarily involving SNARE interactions in vesicle transport, proteoglycans in cancer, non-alcoholic fatty liver disease, spliceosomes, 2-oxocarboxylic acid metabolism, pyruvic acid metabolism, chemical carcinogenesis-reactive oxygen species, regulation of actin cytoskeleton, oxidative phosphorylation, graft rejection, etc.

Icariin protected thymus function through multi-pathway synergistic effects: (1) inhibiting SNARE-mediated vesicle transport and reducing microenvironmental disorders induced by abnormal protein secretion; (2) reprogramming the pyruvic acid metabolic flux and decreasing oxidative phosphorylation-dependent Reactive Oxygen Species(ROS) generation; (3) mediating cell cycle checkpoints and apoptosis pathways to eliminate senescent thymocytes. These mechanisms complemented the previous GO enrichment analysis of " mitochondrial energy metabolism and spliceosome regulation", hinting that Icariin delayed thymic aging via a multi-dimensional network of "metabolism-transport-apoptosis".

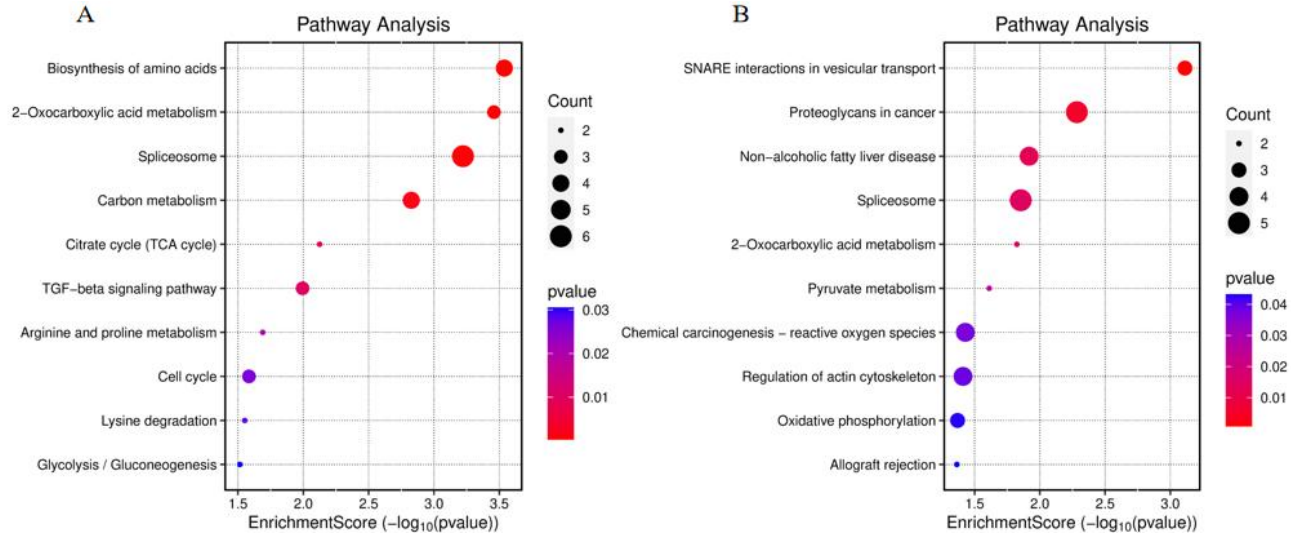

Figure 6 KEGG pathway enrichment analysis of differentially expressed proteins. Bubble charts of the top significantly enriched KEGG pathways. The Y-axis represents the pathway names, and the X-axis represents the enrichment factor. The bubble size indicates the number of DEPs enriched in the pathway. The color represents the range of -$\log_{10}$(p-value), with redder colors indicating higher significance. (A) Enriched pathways for the Epimedin C group, including "Amino acid biosynthesis" and "TGF-β signaling pathway". (B) Enriched pathways for the Icariin group, including "SNARE interactions in vesicular transport" and "JAK-STAT signaling pathway". This highlights the divergent signaling mechanisms modulated by Epimedin C (metabolism-focused) and Icariin (signaling-focused).

3.8 Screening of pathway-related proteins

The pathway-related proteins obtained from KEGG enrichment analysis were integrated with the enriched

pathways into a document. The document was then imported into Cytoscape software to construct a network diagram (Figure 7). In the Epimedin C group, the spliceosome, amino acid biosynthesis, carbon metabolism, 2-oxocarboxylic acid metabolism, and the TGF- β signaling pathway were influenced by modulating pathway-related proteins such as Ddx5, Hnrnpa3, Srsf1, Cdc5l, Srsf3, Srsf7, Pgam, Idh2, Got2, Idh3a, Ncor1, Ppp2r1a, and Sin3a. In the Icariin group, SNARE interactions in vesicular transport, proteoglycans in cancer, spliceosomes, and non-alcoholic fatty liver disease were affected by modulating pathway-related proteins such as Gosr2, Bnip1, Vamp4, Ddx5, Itgb5, Fas, Iqgap1, Ezr, Lsm8, Srsf1, Prpf40a, Srsf7, Cox7a2l, Ndufs1, and Uqcrh.

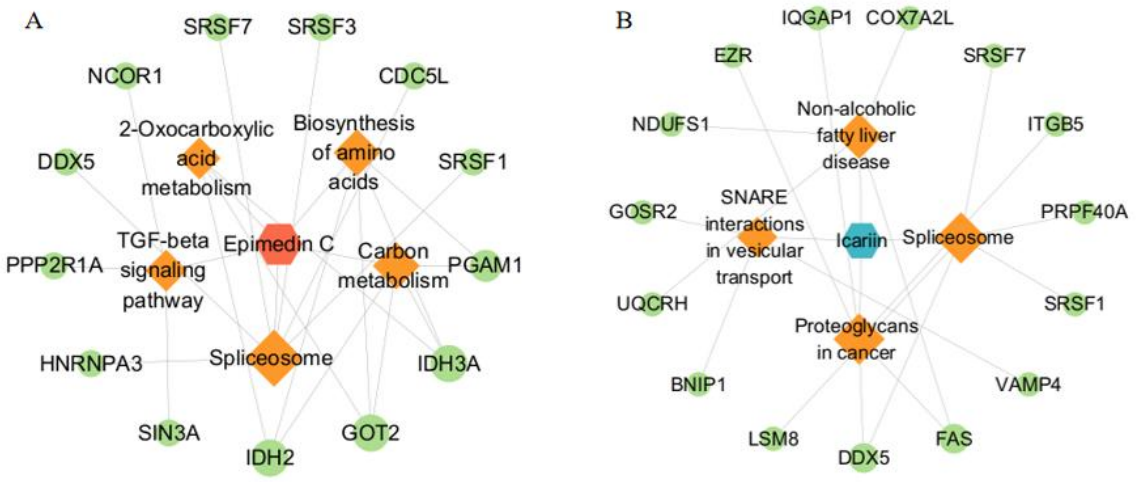

Figure 7 Component-Target-Pathway network diagrams. Network diagrams visualize the connections between the component (Epimedin C or Icariin), the DEPs, and the significantly enriched KEGG pathways. Rectangular nodes represent components or pathways, circular nodes represent DEPs, and connecting lines represent regulatory relationships. (A) Network for the Epimedin C group, showing its association with pathways like the spliceosome and TGF-β signaling through targets like Ddx5 and Smad3. (B) Network for the Icariin group, showing its association with pathways like SNARE interactions and proteoglycans in cancer through targets like Gosr2 and Itgb5. These networks provide a systems-level view of the multi-target, multi-pathway mechanisms.

4 Discussion

Epimedin C and Icariin are both flavonoids, with their parent nucleus being 8-prenylflavonoid aglycone. The functional differences between them stem from subtle variations in their chemical structures [21]. Epimedin C has a larger molecular weight and higher glycosylation. These properties may cause strong hydrophilicity and poor membrane permeability. Consequently, it may interact more easily with metabolic enzymes and signaling molecules in the extracellular matrix or cytoplasm [22]. On the contrary, the smaller molecular weight and different substituents of Icariin may endow it with superior membrane penetration ability, enabling easier access to the nucleus or direct binding with intracellular signal transduction complexes and JAK-STAT pathway components [23]. Through in-depth analysis of the KEGG pathway enrichment results, we found that two monomers act on different signaling axes at the system level: the action profile of Epimedin C was strongly enriched in basal substances and energy metabolism. Epimedin C significantly regulated pathways such as amino acid biosynthesis, carbon metabolism, and the citric acid cycle [24], indicating that its core function lies in providing sufficient raw materials and energy support for the high turnover

proliferation of thymocytes and the maintenance of thymic matrix homeostasis. In addition, its intervention in the TGF-β signaling pathway suggests that it may be involved in regulating the formation and maintenance of immune tolerance in the thymus [25]. The action profile of Icariin clearly leans towards cellular communication and signal transduction. Its specificity affects SNARE interactions during vesicle transport, implies a potential regulatory role in antigen presentation or cytokine secretion processes of thymic epithelial cells [26]. More notably, the enrichment of Icariin in the JAK-STAT signaling pathway directly reflects its potential central role in affecting cytokine signaling, lymphocyte activation and differentiation [27].

In this study, through quantitative proteomics analysis, we found that Icariin and Epimedin C jointly mediated a PPI network with Sod2 and Srsf1 as the core nodes, suggesting their potential synergistic role in maintaining thymic immune microenvironment homeostasis by coordinately regulating two critical biological processes: redox homeostasis and post-transcriptional regulation. Epimedin C has significant antioxidant effects and can protect cells from oxidative stress damage by free radical scavenging. Sod2, as an antioxidant enzyme, can directly scavenge superoxide radicals. Epimedin C may exert its antioxidant effect by enhancing the expression or activity of Sod2. Research has proved that moderate changes in redox status can activate cellular defense pathways such as Nrf2/ARE, leading to subsequent upregulation of antioxidant enzymes and the formation of a new redox balance [28]. In this study, the expression changes of Sod2 were accompanied by alterations in the expression of multiple key enzymes in the tricarboxylic acid cycle and oxidative phosphorylation pathway, indicating that the reorganization of entire energy metabolism network. This metabolic remodeling may provide optimal energy supply and redox environment for thymocytes during differentiation and development, which is crucial for maintaining the function of thymic epithelial cells and the selection process of thymocytes [29]. Srsf1 plays a central regulatory role in alternative splicing of precursor mRNA [30]. In the thymus, precise mRNA splicing is pivotal for generating diversity in T-cell receptor variable regions, and any splicing abnormalities may cause autoimmune reactions or immune deficiencies [31]. We speculated that drug-induced moderate downregulation of Srsf1 may affect the developmental trajectory of thymocytes by altering the splicing patterns of specific genes. This mechanism was consistent with the significant enrichment of "mRNA splicing regulation" and "RNA metabolism processes" observed in our GO analysis.

Of note, Sod2 and Srsf1 may form a functional synergistic unit. On the one hand, changes in the redox state can directly affect the activity of splicing factors via regulating the sulfydryl modification state [32]. On the other hand, splicing factors can indirectly influence cellular metabolic status by mediating alternative splicing of metabolism-related genes. This metabolic-epitranscriptomic crosstalk may form a sophisticated regulatory loop, ensuring that thymocytes maintain an appropriate balance between proliferation rate and apoptosis during development. This discovery provides a new perspective for understanding the characteristics of the multi-component, multi-target effects of TCM.

This study reveals the multi-target interaction network through which Icariin and Epimedin C regulate thymic immune function at the system level, particularly identifying the crucial role of Sod2 and Srsf1 as core nodes in integrating metabolic regulation and post-transcriptional regulation. This discovery provides a modern scientific basis for elucidating the traditional efficacy of Epimedium in supporting vital energy and consolidating the foundation.

It should be noted that the conclusions drawn from this proteomics study, particularly regarding the core functions of Sod2 and Srsf1, are primarily based on bioinformatics inferences. Future research should focus on validating expression changes of these key targets using techniques such as Western blotting or immunohistochemistry. Additionally, functional studies, including gene knockdown or overexpression experiments in thymic cell lines or primary thymic cells, are crucial for elucidating the causal relationship between these targets and the observed immunomodulatory effects. Finally, investigating the effects of these compounds in aging or immunodeficient mouse models will provide more physiologically relevant insights into their potential to combat immune aging.

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Conflicts of Interest

The authors declare that there is no conflict of interests.

Author Contributions

H.W.: Methodology, Software, Validation, Data curation, Writing–original draft, Formal analysis. H.W.: Validation, Data curation. B.H.: Validation, Data curation. H.W.: Writing – original draft. B.H.: Data curation, Investigation. B.H.: Data curation, Visualization. B.H.: Data curation, Investigation. B.H.: Investigation. H.W.: Conceptualization, Writing – review & editing. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

All animal experimental protocols and procedures have been reviewed and approved by the Animal Ethics Committee of Zhejiang University of Traditional Chinese Medicine, with ethics approval number of IACUC-20250331-29.

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Availability of Data and Materials

The data presented in this study are available on request from the corresponding author.

Supplementary

Not applicable.

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