

Exploring the Role of Radix Polygalae in Melanogenesis Related to Vitiligo: A Network Pharmacology Analysis with in vitro Validation

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Objective: Radix Polygalae is a traditional Chinese herbal medicine with various pharmacological effects, including improvement of cognitive ability, anti-neurodegenerative property, and antiviral property, among others. Radix Polygalae was also utilized in the treatment of vitiligo in traditional folk clinics, although its mechanism of action remains unclear. This study investigates the potential active ingredients of Radix Polygalae in the treatment of vitiligo.

Methods: This study screened the potential active ingredients and core target genes of Radix Polygalae in the treatment of vitiligo through network pharmacology, and validated the mechanism of Tenuifolin, the key active ingredient, through the in vitro experiments.

Results: We finally identified 10 core target genes: TNF, CASP3, IL-1 β , IL-6, ESR1, STAT3, VEGFA, PPARG, ALB, and IL-2. Additionally, we found 7 core active ingredients: Tenuifolin (TEN), N-Acetyl-D-Glucosamine, 1,6-Dihydroxy-3,7-dimethoxyxanthone, 1-Hydroxy-3,7-dimethoxyxanthone, aristolactam a, carvacrol, and cordarine. TEN, which contains the highest number of core target genes, was selected for further investigation of its mechanism. According to our findings, TEN could promote melanin production in pigment cells and skin, as well as induce browning of apple slices. Further investigation showed that TEN reduced the expression of CXCL10 and increased the activity of tyrosinase (TYR), without affecting the pH value or the expression of melanogenesis-related genes (MITF, TYR, TYRP1, and DCT), as well as paracrine factors of ET-1 and VEGF.

Conclusion: TEN is one of the active ingredients of Radix Polygalae that shows potential in the treatment of vitiligo. It may regulate TYR to promote skin pigmentation.

Keywords: network Pharmacology, radix polygalae, tenuifolin, vitiligo, melanogenesis

Introduction

Melanin is widely found in human skin, which can protect the skin from ultraviolet rays.¹ Melanin is synthesized within melanosomes, which are distributed within melanocytes² and then transferred to adjacent keratinocytes through the dendrites of melanocytes.³ Melanin is divided into eumelanin and pheomelanin,⁴ and its production involves complex genes and signaling pathways. The Microphthalmia-associated transcription factor (MITF) is the most important transcription factor in melanogenesis,⁵ which can activate and regulate the expression of several key genes related to melanogenesis, such as tyrosinase, tyrosinase-related protein 1 (TYRP1), and dopachrome tautomerase or tyrosinase-related protein 2 (DCT/TYRP2), thus promoting melanogenesis. TYR, the rate-limiting enzyme of melanogenesis,

catalyzes the oxidation of tyrosine to dopaquinone,⁶ DCT catalyzes the conversion of dopaquinone to 5,6-dihydroxyindole-2-carboxylic acid (DHICA),⁷ and then TYRP1 catalyzes DHICA to eumelanin.⁸

Moreover, tyrosinase also exists in plants and is known as polyphenol oxidase (PPO). PPO is a type 3 copper-containing enzyme that is widely distributed in plants and plays a crucial role in melanin production and enzymatic browning.⁹ PPO is also commonly found in fruits¹⁰ and can cause browning of fruits, vegetables, and seafood.¹¹ During browning, PPO can hydroxylate monophenol to ortho-diphenol and oxidize ortho-diphenol to ortho-quinone. This ortho-quinone then polymerizes with amino acids or proteins to form melanin.¹² Kojic acid is a widely used anti-browning agent and is often used as a food additive and preservative,^{13–15} which can inhibit blackening by suppressing the activity of tyrosinase.¹⁶ Vitamin C (VC), also known as ascorbic acid, is a potent antioxidant frequently used as an additive in whitening products.¹⁷ Previous studies have shown that ascorbic acid can induce a conformational change in PPO and form chelates with copper ions located in the enzyme's active center, and inhibit the browning of fresh-cut apples by inhibiting the activity of PPO and peroxidase (POD).¹⁸

The pH value in melanocytes is another crucial factor in regulating tyrosinase function and melanosome maturation. At a pH range of 5.8 to 6.3, tyrosinase produces the most pheomelanin, while the production of eumelanin is inhibited at pH 5.8.¹⁹ One study demonstrated that VC could dose-dependently inhibit tyrosinase activity and melanin content, but it had no effect on the expression levels of TYR and MITF. Additionally, treatment with VC led to significant acidification in melanocytes, and the inhibitory effect on tyrosinase activity disappeared after neutralizing with ammonium chloride (NH₄Cl).²⁰

Decreased melanin production or destruction of melanocytes can be characterized by decolorizing diseases, such as vitiligo.²¹ Vitiligo is the most common skin depigmentation disease, with an incidence rate of 0.5%–1% in the general population,²² which has a great impact on the appearance and psychology of patients.^{23,24} Similar to many autoimmune diseases, a combination of factors (genetic, environmental, etc.) contributes to the development of vitiligo.²⁵ However, its pathogenesis is still unclear. Recently, accumulating evidence suggests that immune-mediated melanocyte destruction, particularly driven by cytotoxic CD8⁺ T cells and inflammatory cytokines, plays an important role in disease progression.^{26,27} Among these pathways, interferon- γ (IFN- γ)-induced chemokines, such as CXCL10, have been implicated in amplifying T-cell recruitment to the skin and sustaining local inflammation.^{28–30}

Currently, the treatment of vitiligo is primarily categorized into two aspects: immune suppression and stimulation of melanocyte proliferation and pigmentation. The treatment methods encompass phototherapy, topical application of corticosteroids and/or calcineurin inhibitors, among others.³¹ However, the effectiveness of these treatments remains unsatisfactory as they do not work for all patients. Additionally, there is a high recurrence rate of 40% within the first year after discontinuing treatment.³² Emerging JAK inhibitors are currently the most promising new drugs for treating vitiligo.^{31,33} However, similar to other immunosuppressive drugs, JAK inhibitors may cause adverse reactions such as opportunistic infections, anemia, and rare malignant tumors.^{31,34} Therefore, further exploration of effective and targeted treatment methods is still needed.

Radix Polygalae is a dried root of either *Polygala tenuifolia* Willd. or *Polygala sibirica* L., and is widely used in traditional Chinese medicine.³⁵ It exhibits various pharmacological activities, including improving cognitive ability,³⁶ combating neurodegenerative diseases,^{37,38} exhibiting anti-viral properties,³⁹ and regulating the biological clock system.⁴⁰ Clinically, Radix Polygalae has also been formulated as an external preparation for vitiligo management, largely based on empirical experience. Chemically, Radix Polygalae is rich in bioactive constituents such as triterpenoid saponins (eg., Tenuifolin), xanthenes (eg., polygalaxanthone III), and oligosaccharide esters.⁴¹ Some related natural products, including triterpenoid saponins derived from *Gynostemma pentaphyllum*, have been reported to modulate melanogenesis and tyrosinase activity, suggesting a possible link between Radix Polygalae and pigmentation-related processes.^{42,43} However, the molecular mechanisms underlying these potential effects of Radix Polygalae in vitiligo remain unclear. Therefore, this study aimed to identify the potential active ingredients and target genes of Radix Polygalae in vitiligo through network pharmacology and to further investigate the mechanism of the key active ingredient using in vitro experiments.

Materials and Methods

Network Pharmacology Analysis

Screening Target Genes Related to Radix Polygalae

By searching the keyword “Radix Polygalae” in ETCM database (<http://www.tcmip.cn/ETCM/index.php/Home/Index/index.html>), TCMIP database (<http://www.tcmip.cn/TCMIP/index.php/Home/Index/index.html>), HERB database (<http://herb.ac.cn/>) and SymMap database (<http://www.symmap.org/>), detailed information regarding the ingredients of Radix Polygalae can be obtained from different websites. The information includes the chemical formula, molecular weight, CAS number, PubChem CID, Ingredient ID, and more. After removing duplicates, the final active ingredients of Radix Polygalae can be determined. The PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>) is a widely used and comprehensive compound database, which provides information such as the standard chemical name, 2D/3D structure, and Canonical SMILES number of each ingredient. The unique structure of the ingredients can be obtained through the Canonical SMILES number. Only certain ingredients in the aforementioned four databases can retrieve the corresponding target gene names on the respective websites, but this coverage is not exhaustive. Therefore, we predict the target genes of each ingredient retrieved on the SwissTargetPrediction website (<http://www.swisstargetprediction.ch/>) using the Canonical SMILES number or 2D structure, and set the research species as “Homo sapiens” to screen potential effective target genes with the “probability” greater than 0. The TCMSP database (<https://old.tcmsp-e.com/tcmsp.php>) is a commonly used Chinese herbal medicine retrieval database. There is no result in the search for “Radix Polygalae” in this database, but some single ingredients can be retrieved. We sequentially searched the ingredients of Radix Polygalae obtained in the above four databases to obtain their Mol ID, oral bioavailability (OB), drug-like properties (DL) and corresponding target protein name, etc., and screened target proteins with OB value $\geq 30\%$ and DL value ≥ 0.18 .^{44,45} Next, we used the UniProt database (<https://www.uniprot.org/>) to convert the target protein names into the corresponding target gene names, and remove the target genes without the corresponding standard gene names. Finally, we summarized all the target genes from the aforementioned sources, removing any duplicate values. This allowed us to obtain the final ingredients and target genes of Radix Polygalae.

Screening of Vitiligo-Related Target Genes

Using “Vitiligo” as the keyword, potential target genes involved in vitiligo were obtained from GeneCards database (<https://www.genecards.org/>), OMIM database (<https://omim.org/>), TTD database (<https://db.idrblab.net/ttd/>) and DisGeNET database (<https://www.disgenet.org/>). In the GeneCards database, target genes were screened according to the Score value $\geq$ the median of the Score column. In the DisGeNET database, target genes were screened based on the Score value ≥ 0 . The higher the Score value of the target gene, the more relevant it is to the occurrence of the disease. Finally, the genes from the four databases were merged, and any duplicates were removed to obtain the final target genes of vitiligo.

Intersection of Target Genes of Active Ingredients and Vitiligo

To elucidate the interaction between the target genes associated with the active ingredients of Radix Polygalae and those of vitiligo, the Venn diagram was drawn through the jvenn online website (<http://jvenn.toulouse.inra.fr/app/example.html>) and the intersection target genes were obtained.

Construction of PPI and Screening of Core Genes/Ingredients

The protein-protein interaction (PPI) network model of the intersection target gene was constructed in the STRING database (<https://string-db.org/>), the species “Homo sapiens” and “medium confidence (0.400)” were set, while the free nodes were hidden and the rest were set by default.⁴⁶ The results were then imported into Cytoscape 3.6.1 software to construct PPI network. In this network, the nodes represent active ingredients or target genes, and the edges represent the interactions between active ingredients and target genes. Through the “NetworkAnalyzer” and “CytoNCA” functions in the software, the degree value, betweenness centrality (BC) value, and Closeness Centrality (CC^a) value of the network can be calculated. When constructing the PPI network diagram, nodes are assigned different sizes based on their degree values. Nodes with larger size or darker color imply a higher likelihood of playing a significant role within the network. After obtaining the PPI network, the first step is to screen out the nodes with degrees greater than 2 times the median degree.⁴⁷ If there are still a substantial number of genes remaining, the next step is to screen out the network nodes that

have degrees greater than the medians of BC and CC^a. Finally, the core target genes of Radix Polygalae for treating vitiligo were identified, along with the corresponding core ingredients.

Bioinformatics Analysis and Construction of “Radix Polygalae Core Ingredients-Core Target Genes-Vitiligo” Network Diagram

The intersection target genes were subjected to Gene Ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. From the results, the top 20 pathways were chosen to generate a visualization graph. The path map of “Radix Polygalae-core ingredients-core target genes-vitiligo” was constructed by Cytoscape 3.6.1 software.

Molecular Docking

Molecular docking was performed to evaluate the interactions between Tenuifolin and the predicted target proteins. The three-dimensional (3D) structures of the target proteins were obtained from the RCSB Protein Data Bank (PDB, <https://www.rcsb.org>) with the criteria of Homo sapiens, X-ray diffraction, and a resolution ≤ 2.5 Å. The 3D structure of Tenuifolin was retrieved from the PubChem database (<https://pubchem.ncbi.nlm.nih.gov>) as a 3D conformer in SDF format. Docking simulations were performed using the CB-Dock2 server (<https://cadd.labshare.cn/cb-dock2/>), and the binding affinity predicted by AutoDock Vina was used to evaluate the binding stability between the ligand and receptor.

Prediction of Toxicity Risk

The potential toxicity risks of the candidate compounds identified from network pharmacology analysis were predicted using the OSIRIS Property Explorer software.⁴⁸ This tool evaluates several toxicity-related parameters, including mutagenicity, tumorigenicity, irritant effects, and reproductive effects. The prediction results were used to preliminarily assess the safety profile of the compound prior to subsequent experimental validation.

Experimental Materials and Methods

Reagents

Fetal bovine serum (FBS) was purchased from ThermoFisher SCIENTIFIC. Penicillin-streptomycin solution was purchased from G-CLONE (Beijing, China). Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco (#C11995500BT). Non-Essential Amino Acids solution was purchased from MeisenCTCC (#CTCC-009-345). Phosphate buffered saline (PBS) was purchased from Gibco (#C11995500BT). Dimethyl sulfoxide (DMSO) was purchased from BioFroxx (Guangzhou, China). Cell Counting Kit-8 (CCK8), paraformaldehyde solution (4%) and 5% Nonfat-Dried Milk were purchased from Biosharp (Beijing, China). TEN was purchased from ChemFaces (Wuhan, China). Masson-Fontana melanin stain kit was purchased from G-CLONE (Beijing, China). The mounting medium was purchased from Wuxi Jiangyuan Industrial Technology and Trade Corporation (Jiangsu, China). Goat anti-rabbit immunoglobulin G (IgG) was purchased from G-CLONE (Signalway Antibody, America). Vitamin C tablets were purchased from HUAZHONG Pharmaceutical Co., Ltd.

Cell Culture

The human immortalized keratinocyte (HaCaT) used in this study was purchased from otwo biotech. The MNT1 cells, a research tool for melanogenesis, was purchased from the ATCC cell bank by MeisenCTCC (Zhejiang, China) Co., Ltd. HaCaT cells were cultured in DMEM high-glucose medium containing 5% or 10% FBS and 1% penicillin-streptomycin solution. The culture medium for MNT1 cells was DMEM high-glucose medium, which contained 20% FBS, 1% of penicillin-streptomycin solution and 1% non-essential amino acids. The cells were cultured at 37°C and 5% CO₂.

Drug Preparation and Cell Treatment

TEN was dissolved in DMSO, resulting in a final concentration of DMSO of less than 0.1%. According to the objective of the experiment, HaCaT and MNT1 cells were treated with different concentrations of TEN. In addition, conditional culture of MNT1 cells was also employed in this study. The treatment methods were as follows: HaCaT cells were seeded in 6-well plates. The NC group and TEN treatment groups with different concentrations of TEN were established. After a certain period of treatment, the old medium was discarded and the cells were washed with PBS 2–3 times. Then, fresh

drug-free culture medium was added and the culturing process was continued for 24 hours. The supernatant of each group was collected and stored after centrifugation. Subsequently, the supernatant was mixed with MNT1 culture medium in a ratio of 7:3 and used for treating MNT1 cells.⁴⁹

Cell Viability Assay

The CCK-8 kit was utilized to assess cell viability. HaCaT cells and MNT1 cells were inoculated in 96-well plates at a density of 5000 cells per well. Subsequently, the cells were treated with TEN at concentrations of 10, 20, 40, 80, and 160 µg/mL for 24 hours. Next, 10 µL of CCK8 reagent was added to each well. The 96-well plates were then transferred to the incubator for a period of 1–4 hours. The incubation process was terminated once the medium attained an orange hue. The absorbance of each well at 450nm was determined using a multifunctional microplate reader (PerkinElmer, America) and subjected to statistical analysis.

Fontana-Masson Melanin Staining of Cells

After treating the MNT1 cells, they were washed with PBS 2–3 times. Following that, 1000µL of a 4% paraformaldehyde solution was added to each well to fix the cells for 30 minutes. After washing with distilled water 5–6 times, 1000µL of an ammonia silver solution was added to each well. The cells were then incubated in a water bath at 56°C for 15–30 minutes. After another round of washing, the melanin was observed under an inverted light microscope.

Fontana-Masson Melanin Staining of Tissue Slices

Human foreskin tissue samples were obtained from three independent healthy adolescent donors undergoing circumcision surgery, with informed consent obtained from their legal guardians. The subcutaneous tissue was removed, and the remaining skin was cut into approximately 0.5×1 cm slices, which were placed in 12-well plates, adhered to the walls, and treated with various concentrations of TEN for 7 days. New drugs were applied daily. Afterward, the tissue was fixed, sliced, dewaxed, and stained with an ammonia silver solution (Fontana-Masson staining). Melanin was then observed under an inverted light microscope.

Determination of Tyrosinase Activity by TYR Probe

The TYR probe used in this study was independently designed by Professor Sheng Yang. This probe has the capability to perform more precise, quantitative, and spatio-temporal analyses of TYR activity in cells.⁵⁰ The specific operation methods are as follows: After MNT1 cells were inoculated into 6-well plates, 1 mL of diluted probes was added to each well and incubated in a cell incubator for 30 minutes. Then, the cells were washed 4–5 times with PBS to remove any excess probe. The fluorescence photos were taken using an inverted fluorescence microscope (CARL ZEISS, Germany).

Determination of pH Value of Acidic Organelles by LysoSensor Green DND-189 Probe

The LysoSensor™ Green DND-189 probe used in this study was purchased from Shanghai Maokang Biotechnology Co., Ltd (Shanghai, China). Currently, there are literature sources discussing the application of this probe for determining the pH levels of intracellular acidic organelles such as lysosomes and melanosomes.²⁰ The preparation and usage protocol for this probe is identical to that of the TYR probe.

Real-Time Fluorescence Quantitative Polymerase Chain Reaction (qRT-PCR)

After HaCaT cells were treated, total RNA was extracted using the RNA fast 200 total RNA rapid extraction kit (Shanghai Feijie Bio-Technology Co., Ltd). The extracted RNA was then reverse transcribed and subjected to qRT-PCR reaction using the Fluorescence quantitative gene amplification instrument (LightCycler480II, Roche, Switzerland). The results were standardized and statistically analyzed. The primer sequences can be found in [Supplementary Table S1](#).

Western Blotting (WB)

After MNT1 cells were treated, the protein was extracted using the RIPA Lysis Buffer (G-CLONE, China) along with a protease inhibitor (#4693159001, Roche) and a phosphatase inhibitor (#4906837001, Roche) system. The protein concentration was determined using the BCA Protein Assay Kit (Biosharp, China). Then, electrophoresis, membrane transfer, and blocking (with 5% Nonfat-Dried Milk) were performed. The membranes were subsequently incubated overnight at 4°C with primary antibodies against TYR (BS1484, Bioworld), MITF (STJ94134, St. John's Laboratory),

and GAPDH (#AP0066, Bioworld). After washing the membrane the next day, it was incubated with goat anti-rabbit immunoglobulin G (IgG) for 1 hour and then developed with ECL Chemiluminescence Kit (Biosharp, China).

Browning Experiment of Apple

The apples purchased from the market were divided into several equal-sized blocks using a cutting mold. Afterward, the corresponding reagents were applied to the surface of each apple block, and they were placed in a 24-well plate. The grouping settings were as follows: blank group, PBS group, TEN group, and vitamin C group. Subsequently, the 24-well plate was placed in a natural environment at room temperature, and the color changes of the apples in each group were recorded.

Statistical Analysis

All experiments were performed independently at least three times ($n = 3$ biological replicates). Biological replicates refer to independent cell culture experiments or tissues obtained from different donors, whereas technical replicates refer to parallel wells or repeated measurements within the same experiment. SPSS 22.0 was used to perform statistical analysis on the experimental results, and GraphPad Prism 9.0 was employed to generate histograms. Differences among multiple groups were analyzed using one-way analysis of variance (ANOVA), followed by appropriate post hoc tests for multiple comparisons. A p value < 0.05 was considered statistically significant. Statistical significance was indicated as follows: *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$. To create relevant graphics, tools such as R 4.1.1, Adobe Illustrator 2021, and online websites were used.

Ethical Approval and Consent to Participate

Human foreskin tissue samples were obtained from healthy adolescent donors following circumcision surgery, with informed consent obtained from their legal guardians. The study was reviewed and approved by the Institutional Review Board (IRB) of Third Xiangya Hospital, Central South University (approval No. 2021-S137). All experiments involving human tissues were conducted in accordance with institutional guidelines and the principles of the Declaration of Helsinki.

Results

Network Pharmacology Analysis Results

Screening the Active Ingredients of Radix Polygalae

Eighty-six active ingredients of Radix Polygalae were obtained from both the ETCM and TCMIP databases, which had the same ingredients. The HERB database revealed 101 active ingredients, while the SymMap database showed 139 active ingredients. However, some of these ingredients could not be found in the PubChem database. After removing these ingredients, a total of 86 active ingredients were included in the network pharmacology analysis ([Supplementary Table S2](#)). Subsequently, the target genes for each active ingredient were obtained and screened in each database, resulting in a final tally of 884 target genes after deduplication.

Screening Vitiligo Disease-Related Target Genes

A total of 1270 target genes of vitiligo were obtained from the GeneCards database. After screening, 885 genes were selected based on a Relevance score greater than the median of the column. Using the DisGeNET database, 395 disease target genes were obtained by further screening the target genes with $\text{Score} > 0$. Additionally, the OMIM database provided 9 vitiligo target genes, while the TTD database contributed 10 ([Figure 1a](#)). Combining the genes from all four databases and removing duplicates resulted in a total of 1070 vitiligo disease target genes.

Intersection of Target Genes of Active Compounds and Vitiligo

After entering the target genes related to each active ingredient of Radix Polygalae and the target genes associated with vitiligo into the jvenn online website, a total of 129 intersecting target genes were obtained. Subsequently, a Venn diagram ([Figure 1b](#)) was created to represent the intersection of these genes.

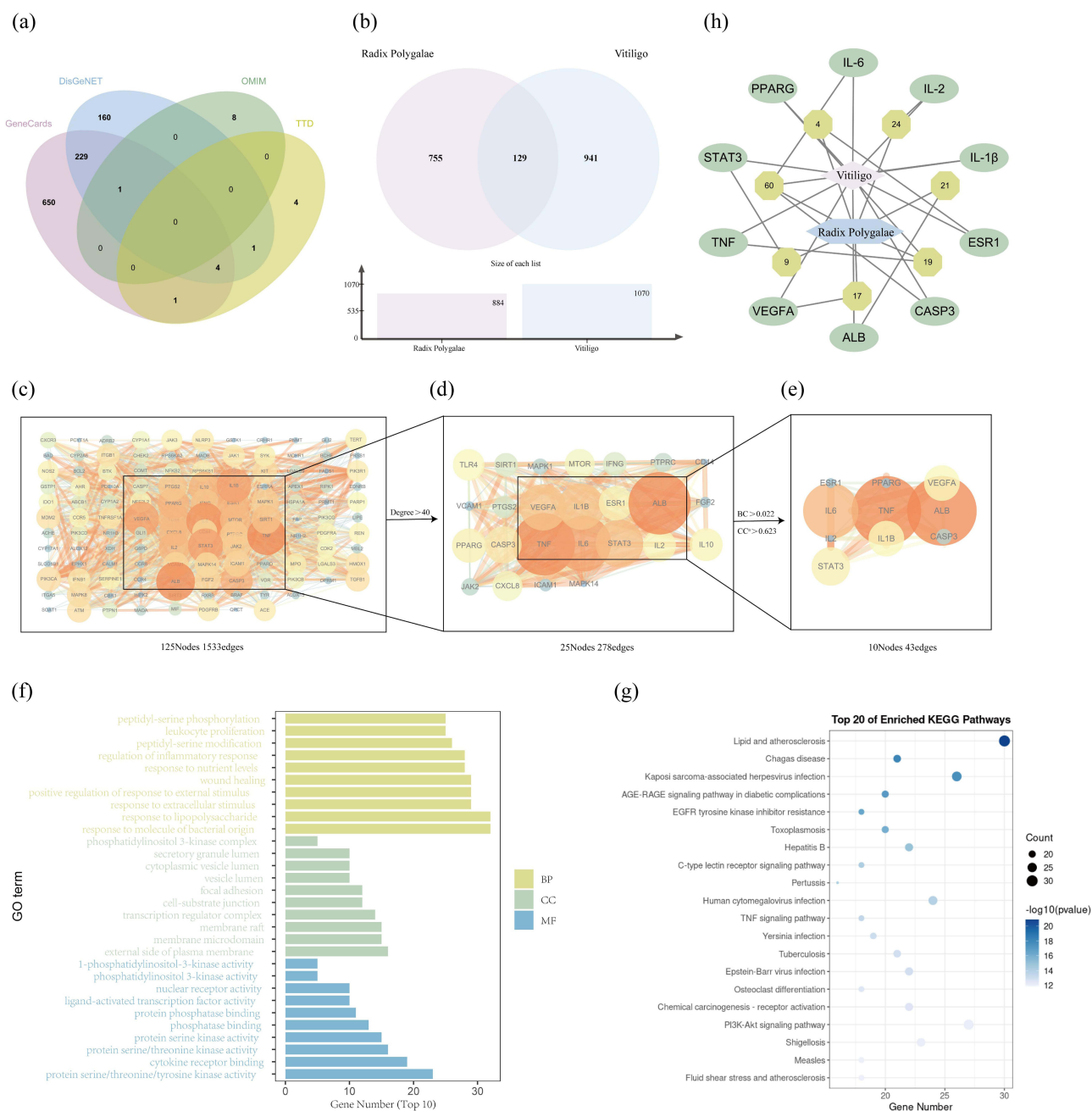


Figure 1 Identification of active ingredients, target genes, and signaling pathways of Radix Polygalae in vitiligo based on network pharmacology analysis. **(a)** Venn diagrams of vitiligo disease target genes in four databases. **(b)** Intersection of target genes of active ingredients and vitiligo. **(c-e)** Intersection target gene PPI network diagram and core target gene screening. **(f)** GO functional enrichment bar graph and network graph of target genes of Radix Polygalae treating vitiligo. **(g)** KEGG enrichment bubble map of target genes of Radix Polygalae. The active ingredients of Radix Polygalae, represented by numbers, are as follows: 4: 1,6-Dihydroxy-3,7-dimethoxyxanthone. 9: 1-Hydroxy-3,7-dimethoxyxanthone. 17: aristolactam a. 19: N-Acetyl-D-Glucosamine. 21: carvacrol. 24: cordarine. 60: Tenuifolin.).

Construction of PPI Network and Screening Core Genes/Ingredients

The 129 intersection target genes were imported into the STRING database to obtain the protein-protein interaction (PPI) network diagram. After importing the results into Cytoscape 3.6.1 software, the degree value was calculated using the NetworkAnalyzer function. The PPI network diagram was then drawn, with node sizes reflecting the degree value (Figure 1c). By screening the network with degree value > degree column median, we obtained 25 nodes and 278 edges (Figure 1d). Further screening for nodes with a degree greater than the median values of BC and CC^a resulted in the

identification of 10 core target genes: TNF, CASP3, IL-1 β , IL-6, ESR1, STAT3, VEGFA, PPARG, ALB, IL-2 (Figure 1c and Supplementary Table S3).

By searching for the active ingredients corresponding to the intersection target genes, we obtained 16 core ingredients that may play a role (Table 1). We then identified the top core ingredients based on the number of core genes they contained. These included Tenuifolin (TEN), N-Acetyl-D-Glucosamine, and a group of five ingredients (1,6-Dihydroxy-3,7-dimethoxyxanthone, 1-Hydroxy-3,7-dimethoxyxanthone, aristolactam a, carvacrol, and cordarine) that tied for third place. Therefore, TEN was selected for further exploration in subsequent in vitro experiments.

Molecular Docking Results

To further evaluate the potential interactions between Tenuifolin and the core targets identified from the PPI network, molecular docking analysis was performed. Binding affinity ≤ -6.0 kcal/mol is generally considered to indicate favorable binding activity. The results showed that Tenuifolin exhibited strong binding affinity with the selected targets, with binding energies of -8.5 kcal/mol for TNF, -8.0 kcal/mol for IL1B, and -8.7 kcal/mol for CASP3 (Supplementary Figure S1). These results suggest that Tenuifolin and its potential targets may represent key mediators through which Radix Polygalae exerts its regulatory effects on melanogenesis.

Toxicity Risk Prediction of TEN

To preliminarily evaluate the safety profile of TEN, toxicity risk prediction was conducted using the OSIRIS Property Explorer. The analysis indicated that TEN showed no predicted risks of mutagenicity, tumorigenicity, irritant effects, or reproductive effects, suggesting a relatively favorable safety profile for further experimental investigation (Supplementary Table S4).

Bioinformatics Analysis Results

The intersection target genes were annotated by GO through R 4.1.1 software, and the top entries were selected for visual display (Figure 1f). In terms of the biological process (BP), it is mainly concentrated in the response to molecule of bacterial origin, to lipopolysaccharide, to extracellular stimulus, to positive regulation of external stimulus, and so on. As for the molecular function (MF), protein serine/threonine/tyrosine kinase activity, cytokine receptor binding, protein serine/threonine kinase activity, protein serine kinase activity were found to be relatively important. The cellular components (CC^b) include external side of plasma membrane, membrane microdomain, membrane raft, transcription regulator complex and so on. A total of 162 pathways were identified by KEGG enrichment analysis, including lipid and

Table 1 The 16 Potential Core Active Ingredients of Radix Polygalae in the Treatment of Vitiligo and the Corresponding Target Genes

Serial Number	Ingredient Name	Included Intersection Target Gene Names
1	1,2,3,6,7-Pentamethoxyxanthone-9-one	MAOA, PTPN1, PIK3CD, PIK3CG, PIK3CA, MTOR, PIK3CB, MAPK8, HIPK2, ATM, CDK2, QPCT, JAK3, JAK1, JAK2, PDE10A, PIK3R1, CASP7, VCAM1
4	1,6-Dihydroxy-3,7-dimethoxyxanthone	ESR1
5	Gentisin	CHEK2, MAOB, SERPINE1, PDGFRA
7	1-Ethoxycarbonyl-beta-carboline	ALOX15, FADS1, NRIH3, NRIH2, TLR4, CCR4, LIPE
9	1-Hydroxy-3,7-dimethoxyxanthone	ABAT, STAT3
11	3,4,5-Trimethoxycinnamic acid	ABCB1, CYP1A1, CYP1A2, NFE2L2, GSTP1, ITGB1, ITGA5, APEX1, CXCL8
12	3,4-Dimethoxycinnamic acid	PTPRC, REN
17	Aristolactam a	COMT, VEGFA, CALM1
19	N-Acetyl-D-Glucosamine	NFKB2, TNF, ABO, ACE, ACHE, CD44, ICAM1, IFNG, MBL2
21	Carvacrol	ALB
24	Cordarine	IL-2, EDNRB
27	Harmine	MDM2, HMOX1, SIRT1
43	Perlolyrine	PTGS2
59	Sucrose	FGF2
60	Tenuifolin	CASP3, IL-1 β , IL-6, VDR
73	Palmitic Acid	BCL2, IL-10, PCYT1A

atherosclerosis, phosphatidylinositol 3 kinase-protein kinase B (PI3K-Akt) signal pathway, and Kaposi sarcoma-associated herpesvirus infection. The bubble chart in [Figure 1g](#) represents the top 20 pathways sorted by gene number.

Construction of Network Diagram of “Radix Polygalae-Core Ingredients-Core Target Genes-Vitiligo”

The four elements, “Radix Polygalae”, “selected core ingredients of Radix Polygalae”, “core target genes”, and “vitiligo”, were imported into Cytoscape3.6.1 to create a network map of “Radix Polygalae-core ingredients-core target genes-vitiligo” ([Figure 1h](#)). The octagon’s sequence number corresponds to the serial number of the potential core active ingredients of Radix Polygalae could be found in [Table 1](#). The network illustrates how Radix Polygalae can be effective in treating vitiligo through its multiple ingredients and targets.

Experimental Verification and Mechanism of Melanin Production Promoted by TEN

TEN Promotes Melanin Production in Foreskin Tissue

We collected foreskin tissue to verify phenotypic characteristics. After treating the foreskin tissue with TEN for 7 days, we tested the melanin content in the tissue section using Masson-Fontana staining. It was observed that as the concentration of TEN increased, there was an increase in melanin granules in the basal layer of the tissue ([Figure 2a](#)).

TEN Promotes the Production of Melanin in Cells

We explored the cytotoxicity of TEN to HaCaT and MNT1 cells by CCK8 assay, as described in [Supplementary Text S1](#) and shown in [Supplementary Figure S2](#). Non-toxic dose of TEN was utilized for subsequent experiments. After the MNT1 cells were treated with different concentrations of TEN for 48 hours, we measured the melanin content using Masson-Fontana staining. Remarkably, TEN significantly enhanced the melanin content ([Figure 2b](#)). Additionally, we developed a conditional culture model of MNT1 cells to investigate whether TEN could influence melanin production through paracrine effects. HaCaT cells were treated with different concentrations of TEN for 24 hours, following which the medium was replaced with fresh medium and HaCaT cells were cultured for an additional 24 hours. The supernatant was then collected to treat MNT1 cells for 48 hours. The results of Masson-Fontana melanin staining indicated that conditioned medium from TEN-treated HaCaT cells also stimulated melanin production in MNT1 cells ([Figure 2c](#)), suggesting that TEN may also promote melanin production through a paracrine pathway.

TEN Can Promote Apple Surface Browning

Since tyrosinase (PPO) is also found in plants, we conducted additional phenotyping experiments on apples. In this experiment, we established the NC group (without applying any liquid), the PBS group as the control, and used vitamin C (VC), a common antioxidant, as the reference. We also dissolved TEN in PBS and used it as the efficacy determination group for TEN. Each group had apples of the same size and thickness, and the corresponding solution was applied to their surfaces. The apples were then placed in a natural environment for 24 hours. The results confirmed that TEN promoted browning on the surface of the apples ([Figure 2d](#)), providing further evidence of its blackening effect.

TEN Does Not Have an Effect on the pH Value of Acidic Organelles

The pH value in melanocytes plays a significant role in regulating tyrosinase function and melanosome maturation. To determine the pH value of acidic organelles in MNT1 cells treated with TEN or conditioned medium from TEN-treated HaCaT cells, we utilized the LysoSensor GreenDND-189 probe. Our findings indicated that TEN and conditioned medium had no impact on the pH value of acidic organelles in MNT1 cells, either directly or through the paracrine pathway ([Figure 2e and f](#)).

TEN Can Increase the Tyrosinase Activity

We further determined the impact of TEN on the expression of MITF and several melanogenesis-related genes regulated by MITF. After treating MNT1 cells with different concentrations of TEN for 48 hours, qRT-PCR was performed to detect the RNA levels of MITF, TYR, TYRP1 and DCT. The results indicated that TEN had no effect on the RNA levels of these genes ([Figure 3a](#)). To further assess the influence of TEN on the protein levels of MITF and TYR, Western Blotting was performed on MNT1 cells treated with TEN or conditioned medium obtained from TEN-treated HaCaT cells. It was observed that TEN did not impact their expression levels ([Figure 3b and c](#)). Additionally, TYR activity in

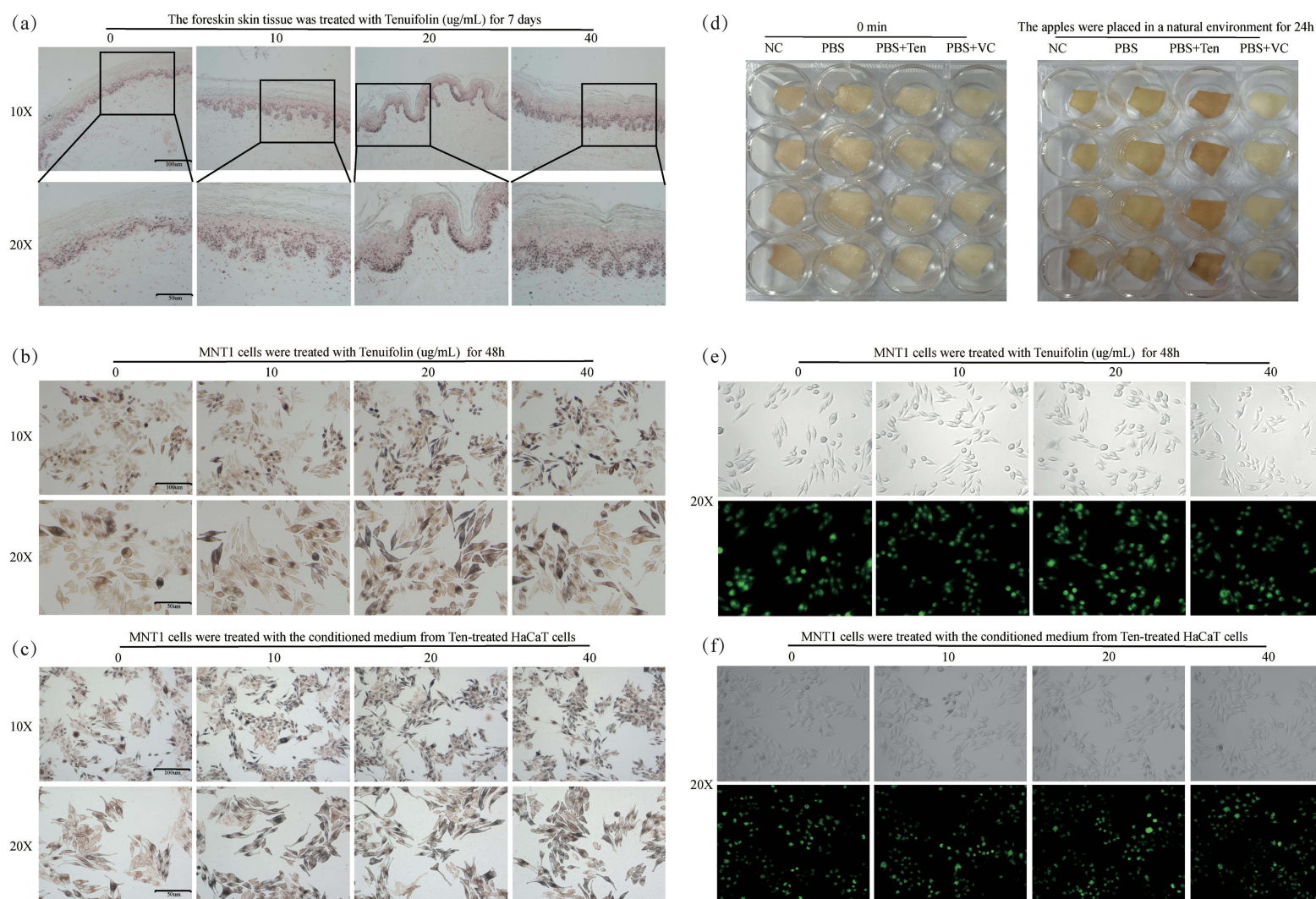


Figure 2 Tenuifolin promotes melanin production in skin tissue and melanocytes without altering the pH of acidic organelles. **(a)** After treating the foreskin tissue (n=3) with TEN (0 µg/mL, cell culture medium; 10 µg/mL; 20 µg/mL; 40 µg/mL) for 7 days, the melanin content in the tissue sections was assessed using Masson-Fontana staining. **(b)** MNT1 cells were treated with TEN (0 µg/mL, cell culture medium; 10 µg/mL; 20 µg/mL; 40 µg/mL) for 48 h, and melanin content was detected using Masson-Fontana staining. **(c)** HaCaT cells were treated with TEN (0, 10, 20, 40 µg/mL) for 24 h, and the supernatant was collected to treat MNT1 cells for 48h. Melanin content in MNT1 cells was detected by Masson-Fontana staining. **(d)** Apple slices were treated with different groups (NC group: no treatment; PBS group; PBS + TEN group; PBS + VC group) and placed in a natural environment for 24 h. Surface browning was observed thereafter. **(e)** MNT1 cells were treated with TEN (0, 10, 20, 40 µg/mL) for 48h, and the pH value of acidic organelles was detected using the LysoSensor GreenDND-189 probe. **(f)** HaCaT cells were treated with TEN (0, 10, 20, 40 µg/mL) for 48h, and the supernatant was collected to treat MNT1 cells for 72h. The pH value of acidic organelles was detected using the LysoSensor GreenDND-189 probe.

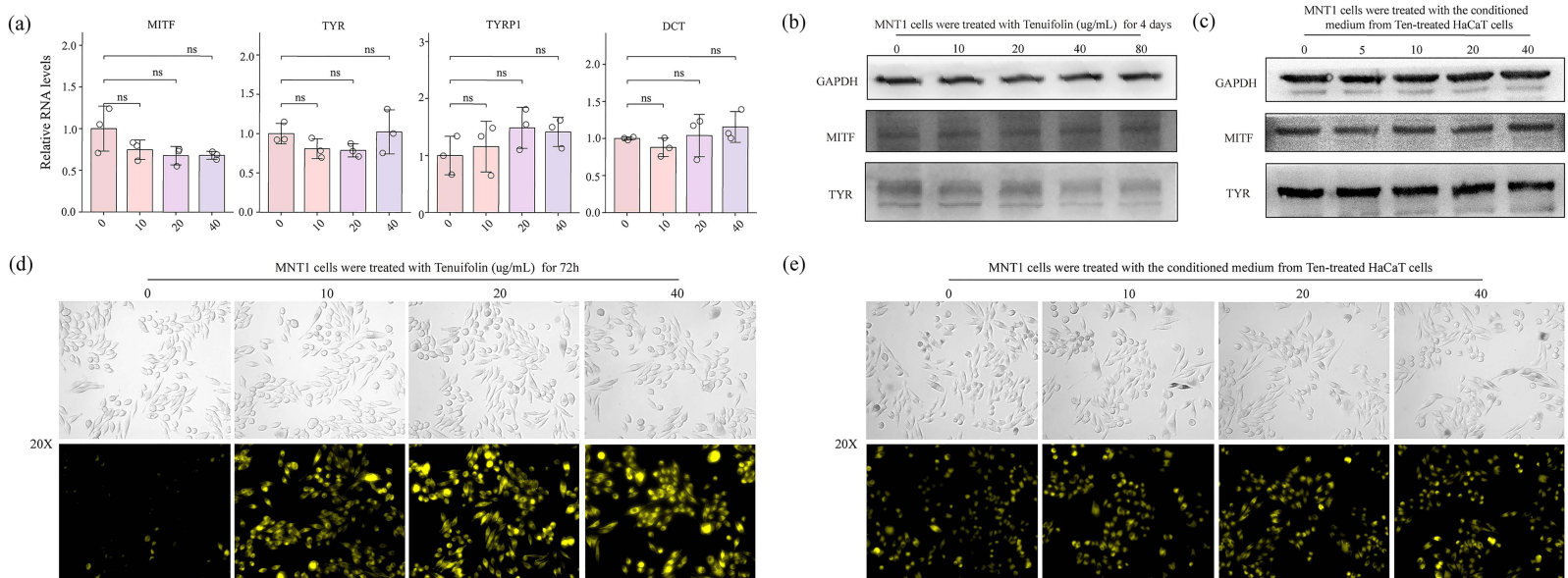


Figure 3 Tenuifolin can increase the tyrosinase activity. **(a)** MNT1 cells were treated with TEN (0 µg/mL, cell culture medium; 10 µg/mL; 20 µg/mL; 40 µg/mL) for 48 h, and the RNA levels of melanogenesis-related genes were detected using qRT-PCR. **(b)** MNT1 cells were treated with TEN (0, 10, 20, 40 µg/mL) for 4 days, and the protein levels of melanogenesis-related genes were detected through WB. **(c)** HaCaT cells were treated with TEN (0, 10, 20, 40 µg/mL) for 48h, and the supernatant was collected to treat MNT1 cells for 72h. The protein levels of melanogenesis-related genes were detected by WB. **(d)** MNT1 cells were treated with TEN (0, 10, 20, 40 µg/mL) for 72h, and TYR activity was detected using a TYR probe. **(e)** HaCaT cells were treated with TEN (0, 10, 20, 40 µg/mL) for 48h, and the supernatant was collected to treat MNT1 cells for 72 h. TYR activity in MNT1 cells was detected using a TYR activity probe. ns, not significant.

MNT1 cells was measured using a TYR probe. It was discovered that TEN could enhance TYR activity in MNT1 cells treated with TEN or conditioned medium from TEN-treated HaCaT cells (Figure 3d and e). Consequently, we speculate that TEN may enhance TYR activity through non-MITF pathways.

Effect of TEN on RNA Level of Cytokines in HaCaT Cells

Through preliminary network pharmacology analysis, we found that the core target genes of TEN are inflammatory factors like IL-1 β and IL-6. Therefore, we used qRT-PCR to measure the RNA levels of IL-1 β and IL-6 in HaCaT cells treated with TEN. However, we did not observe any changes in the expression of IL-1 β and IL-6. Additionally, we also assessed the RNA level of the key chemokine CXCL10, which is involved in the pathogenesis of vitiligo. Interestingly, as the concentration of TEN increased, the expression level of CXCL10 decreased. This finding suggests that TEN may prevent the occurrence and development of vitiligo by reducing the level of CXCL10 (Figure 4a).

TEN Has No Significant Effect on the RNA Levels of Paracrine Factors Such as ET-1 in HaCaT Cells

Since TEN can promote the production of melanin in MNT1 cells through a paracrine pathway, we conducted an experiment to measure the RNA levels of the main paracrine factors (ET-1, VEGF) in HaCaT cells after TEN treatment. However, TEN did not affect the expression of ET-1 and VEGF (Figure 4b). The results suggested that TEN may activate the paracrine function of keratinocytes through other paracrine factors, which remains to be further explored.

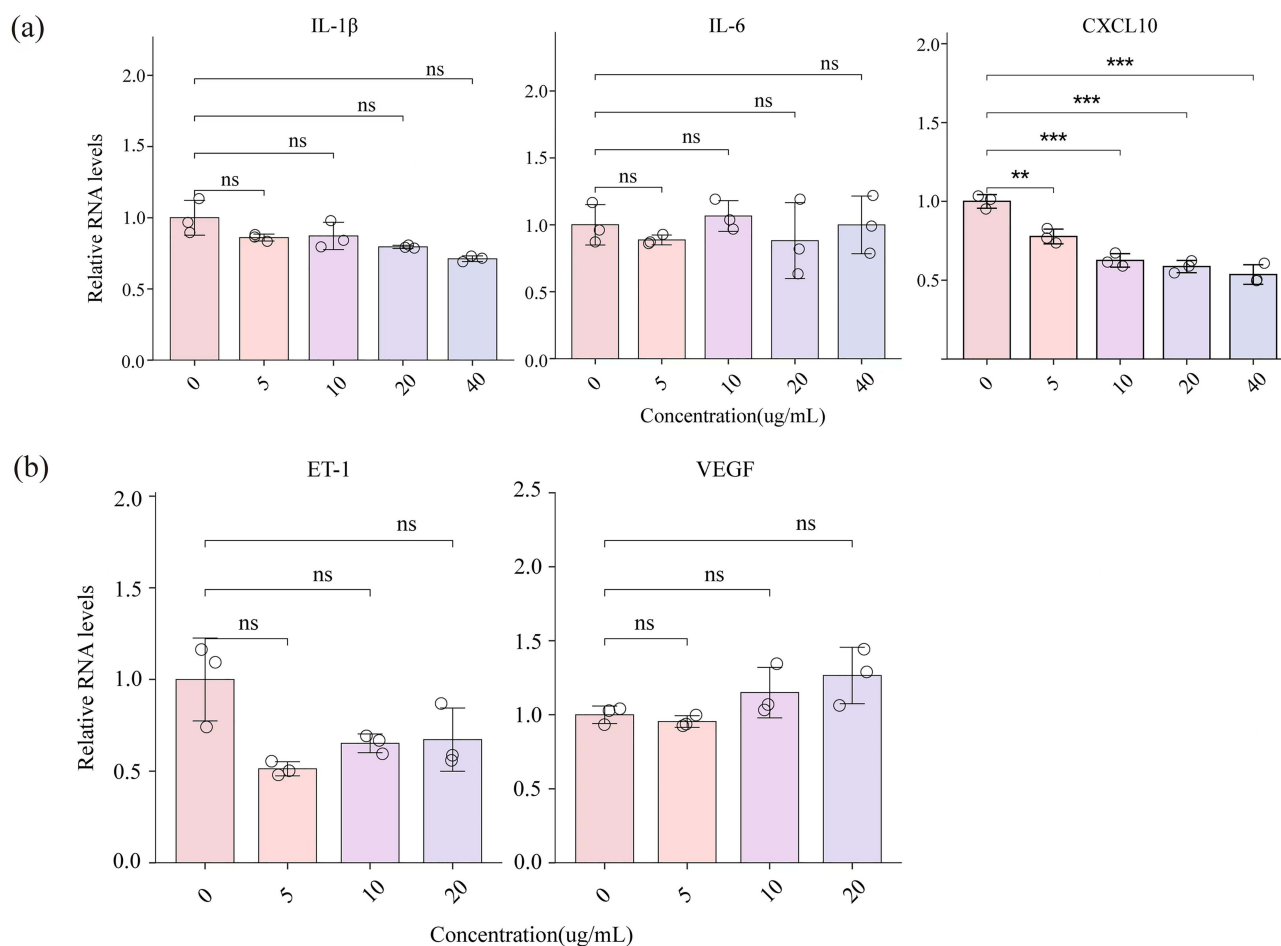


Figure 4 Effect of TEN on RNA level of cytokines and paracrine factors. (a) HaCaT cells were treated with TEN (0 µg/mL, cell culture medium; 5 µg/mL; 10 µg/mL; 20 µg/mL; 40 µg/mL) for 24 h, then the RNA levels of cytokines were detected by qRT-PCR. (b) HaCaT cells were treated with TEN (0, 5, 10, 20 µg/mL) for 48h, then the RNA levels of major paracrine factors were detected by qRT-PCR. ** $p < 0.01$; *** $p < 0.001$; ns, not significant.

Discussion

Network pharmacology has emerged as an effective approach for exploring the multi-component, multi-target, and multi-pathway characteristics of traditional Chinese medicines. Previous studies have demonstrated that this strategy can be used to predict active compounds, potential target genes, and relevant signaling pathways involved in disease regulation.^{45,51,52} In the present study, network pharmacology analysis was employed as the initial step to systematically investigate the potential therapeutic mechanisms of Radix Polygalae in vitiligo. A total of 86 candidate active ingredients of Radix Polygalae were identified, among which seven core ingredients were predicted to be closely associated with vitiligo-related targets. These core ingredients include Tenuifolin (TEN), N-acetyl-D-glucosamine, 1,6-dihydroxy-3,7-dimethoxyxanthone, 1-hydroxy-3,7-dimethoxyxanthone, aristolactam A, carvacrol, and cordarine. Among these compounds, Tenuifolin (TEN) showed the highest degree of connectivity with the core target genes in the compound–target network, suggesting that it may play an important role in the pharmacological effects of Radix Polygalae. Furthermore, the PPI network analysis identified several key target genes, including TNF, CASP3, IL-1 β , IL-6, and STAT3, which are closely related to inflammatory responses and melanocyte regulation. These findings provided a theoretical basis for subsequent experimental validation and guided the selection of TEN as the representative compound for further in vitro investigation. Although network pharmacology provides a useful strategy for predicting potential compounds, targets, and pathways, several limitations should be acknowledged. Network pharmacology is primarily a prediction-based approach, and the identified compound–target interactions may include indirect targets or potential biases due to database and algorithm limitations. In addition, ADME-based screening criteria such as oral bioavailability and drug-likeness may introduce bias in compound selection.^{53,54} Therefore, experimental validation is necessary to confirm the predicted pharmacological effects.

Based on the network pharmacology results, we further performed in vitro experiments to validate the predicted pharmacological activity. Regarding TEN, current research mainly focuses on exploring its effects on the nervous system, such as reducing the secretion of Amyloid- β (A β) and reducing cellular inflammatory response.^{55,56} Our study verified its melanogenesis-stimulating effect for the first time. As there are no prior studies investigating TEN in skin-derived cells, the working concentrations were determined based on preliminary dose-ranging experiments combined with cell viability and morphological assessments. The results of this study suggest that TEN can promote melanin production in pigment cells and skin. We further found that TEN can increase the activity of tyrosinase in MNT1 cells, indicating that TEN may promote melanogenesis by enhancing TYR activity. Similar phenomena have been reported for other natural compounds. For example, *Eclipta herba* has been shown to increase the activity of intracellular tyrosinase, which aligns with the increase of melanin content.⁵⁷ Apigenin-7-butylene glucoside has also been found to enhance the melanin level and tyrosinase activity in B16-F10 cells.⁵⁸ However, in the present study, TEN did not significantly alter the expression of melanogenesis-related genes. Previous studies have suggested that tyrosinase activity can also be regulated at the post-transcriptional or post-translational level. For example, vitamin E has been reported to inhibit tyrosinase activity by acting as a secondary molecule at the post-translational level.^{59,60} Therefore, the pro-melanogenic effect of TEN observed in this study may involve post-transcriptional regulation of TYR activity rather than transcriptional regulation of melanogenesis-related genes. In our study, we have also verified the blackening effect of TEN on apples. Yu et al discovered that *Rosa roxburghii* can effectively inhibit the activity of PPO and browning of apple juice,⁶¹ Rai et al also conducted research on the anti-browning properties of banana and apple juice.⁶² In both of these studies, apple juice was used as the experimental raw material. These findings provide methodological references for further optimization in future studies.

The study further found that TEN can reduce the RNA level of CXCL10, which plays a key role in the progression and maintenance of vitiligo. Utilizing CXCL10 as a therapeutic target not only has the potential to prevent disease development but also, surprisingly, to induce disease reversal through repigmentation in mice with established vitiligo.²⁸ However, it should be noted that the present experiments were performed under in vitro conditions without inflammatory stimulation. Previous studies have shown that CXCL10 expression in vitiligo is largely induced by inflammatory signaling, particularly through the IFN- γ –JAK/STAT pathway. In this process, keratinocytes can contribute to anti-melanocyte immune responses by presenting melanocyte-related antigens and promoting IFN- γ production from activated T cells, which subsequently induces the release of CXCL chemokines such as CXCL9–11 that recruit cytotoxic T cells to lesional skin.⁶³ Therefore, the reduction of CXCL10 observed in this study should be interpreted with caution. Nevertheless, these findings suggest that TEN may have the potential

to influence CXCL10-related inflammatory signaling in vitiligo, which warrants further investigation under inflammatory stimulation conditions.

In addition, our network pharmacology analysis revealed that the core target genes of *Radix Polygalae* in the treatment of vitiligo include IL-1 β , IL-6 and other inflammatory factors, all of which can inhibit melanogenesis and inhibit TYR activity.⁶⁴ However, in this study, TEN did not cause any changes in the RNA levels of these genes. First, it should be noted that the RNA expression levels of predicted targets do not necessarily reflect their functional activity. Therefore, the lack of significant changes in IL-1 β and IL-6 mRNA levels does not exclude their potential involvement in the regulatory network. Second, inflammatory cytokines are not the only regulators of melanogenesis, and the pro-melanogenic effect of TEN may involve alternative regulatory pathways rather than direct modulation of IL-1 β or IL-6 expression.⁶⁵ Previous studies have shown that melanogenesis is regulated through multiple signaling mechanisms, including oxidative stress responses, inflammatory signaling, and melanogenesis-related pathways. Inflammatory mediators can influence melanocyte function and pigmentation through downstream signaling pathways rather than solely through transcriptional regulation of cytokines. Natural compounds have also been reported to protect melanocytes from oxidative stress through pathways such as Nrf2/HO-1, or to promote melanin synthesis by activating key melanogenic signaling pathways including MAPK, cAMP/PKA, and Wnt/ β -catenin. In addition, melanogenesis can be influenced by immune regulation and the inhibition of cytotoxic T-cell-mediated melanocyte destruction.^{65,66} Therefore, the pro-melanogenic effect of TEN observed in this study may involve multiple regulatory pathways beyond the inflammatory mediators predicted by network pharmacology. In particular, keratinocyte-derived paracrine signaling has been reported to play an important role in the regulation of melanogenesis.⁶⁵ Proopiomelanocortin (POMC) and α -melanocyte stimulating hormone (α -MSH) can stimulate melanin.⁶⁷ Endothelins (EDNs), such as endothelin 1 (ET1), can stimulate MC proliferation and promote pigmentation by binding to the EDN receptor of melanocytes.⁶⁸ Activated VEGF can promote the expression of melanogenesis-related proteins in melanocytes.⁶⁹ In our study, we found that TEN promotes melanin production in conditioned MNT1 cells through the paracrine pathway. However, when we measured the RNA levels of the paracrine factors ET-1 and VEGF in HaCaT cells after TEN treatment, no statistically significant differences were observed under the current experimental conditions. It is important to note that this study only focused on a few factors. Considering the extensive variety of inflammatory and paracrine factors within the family, we cannot conclude that TEN has no effect on the RNA expression levels of inflammatory and paracrine factors. Further studies are needed to clarify these mechanisms.

In summary, network pharmacology analysis identified seven potential core active compounds of *Radix Polygalae* for the treatment of vitiligo, including Tenuifolin (TEN), N-acetyl-D-glucosamine, 1,6-dihydroxy-3,7-dimethoxyxanthone, 1-hydroxy-3,7-dimethoxyxanthone, aristolactam A, carvacrol, and cordarine. Among these compounds, experimental validation suggested that Tenuifolin promotes melanogenesis, possibly through enhancing TYR activity. Nevertheless, further studies are required to clarify the molecular mechanisms underlying TYR activation by TEN and to investigate the potential roles of other active components. In addition, in vivo and clinical studies are needed to further evaluate their therapeutic potential.

Data Sharing Statement

All data generated or analyzed during this study are included in this published article and its [Supplementary Information Files](#).

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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