

Analysis on the Active Components and Mechanisms of Action of Ecliptae Herba Intervention on Melanin Synthesis Applying UHPLC-Q-Orbitrap HRMS and Network Pharmacology in Combination with Molecular Docking Screening

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ABSTRACT

Background: White hair, a prevalent condition associated with melanin metabolism, has become increasingly common among the modern young population. Ecliptae Herba has been used since ancient time for white hair treatment, but its active components and underlying mechanisms of action remain to be explored. **Materials and Methods:** The chemical compositions of Ecliptae Herb were analyzed using liquid chromatography-mass spectrometry and UHPLC-Q-Orbitrap HRMS. To evaluate its effects on melanin synthesis, a zebrafish model was employed and treated by different concentrations of Ecliptae Herba extract. The TCMSP and PharmMapper platform were utilized to identify the chemical compounds of Ecliptae Herba and its potential targets. Additionally, melanin metabolism-related targets were screened using the GeneCards and OMIM databases and integrated to construct a drug-target-disease network. Finally, the structural data of the target proteins were obtained from Uniprot and PDB databases and pre-processed by PyMOL 2.6.0 software for molecular docking using Autodock Tool. **Results:** UHPLC-Q-Orbitrap HRMS screened a total of 34 components of Ecliptae Herb. Network pharmacology and molecular docking analysis showed that linarin, pratensin, demethylwedelolactone, echinocystic acid, Ecliptasaponin A, and quercetin were the active compounds. These components affected the drug metabolism, cytochrome P450, EGFR tyrosine kinase inhibitor activity, PPAR signaling pathway, and other signaling pathways by regulating the targets, such as GSTP1, PPARA, IGF1, PAH, NR1I2, and MAPK1, suggesting a potential mechanism underlying the effect of Ecliptae Herba on melanin metabolism. **Conclusion:** This study elucidated the multi-component, multi-target, and multi-pathway mechanisms by which Ecliptae Herba regulated melanin metabolism, contributing to future research on developing novel products for growing black hair.

Keywords: Ecliptae Herba, Melanin, Molecular Docking, Network Pharmacology, Zebrafish.

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INTRODUCTION

The occurrence of white hair, characterized by the abnormal whitening of partial or complete hair strands, is closely related to human health.¹ Melanin is a high molecular weight polymer with an uncertain molecular structure. Synthesis of melanin is realized through a series of reactions in the melanosome of melanocytes. Firstly, tyrosine is catalyzed by Tyrosinase (TYR) to produce

L-DOPA, which is further oxidized to obtain DOPAquinone.² Eumelanin and zeaxanthin are two types of melanin generated by dopaquinone through different pathways, and the colors of these two types of melanin are also different. Tyrosinase-Related Protein-1 (TRP1) and TRP2 play important role in the formation of melanin.³⁻⁵ In recent years, the increasing incidence of white hair among younger population has emerged as a significant dermatological problem,⁶ especially among young and middle-age individuals. According to research, the effects of traditional Chinese and Western medical products for black hair on the market are not satisfactory.^{7, 8} Current therapeutic products for white hair, exemplified by minoxidil in Western medicine, are limited by its low efficacy and inconvenience in daily usage. Though hair dyeing has become a solution for white hair, this approach may potentially compromise hair quality and scalp



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health.⁹ Therefore, it is urgent to discover and produce safe drugs that control white hair problems through oral or external use.

According to traditional Chinese Medicine (TCM), the pathogenesis of melanin deficiency is attributed to the factors such as qi and blood blockage, liver qi stagnation, kidney yin deficiency, and spleen dysfunction due to overexertion. TCM with blood nourishing, blood activating, and qi-regulating effects is used to restore the formation of melanin by nourishing the blood, promoting blood circulation, and relieving liver qi stagnation.^{10,11} Ecliptae Herba, the dry aerial part of the Asteraceae plant *Eclipta prostrata* L., is mainly used to treat liver and kidney yin deficiency, loose teeth, premature graying of the beard, dizziness and tinnitus, soreness and weakness of the waist and knees, yin deficiency with blood heat, vomiting of blood, bleeding, hematuria, blood dysentery, collapse and bleeding, and external bleeding.¹² Modern pharmacological studies have shown that Ecliptae Herba has liver protective, anti-inflammatory, melanin-promoting, antioxidant and some other effects. Clinical studies also validated that Ecliptae Herba can be used to treat symptoms such as bleeding, white hair, vitiligo, etc.¹³⁻¹⁵ Previous study investigated the effects of ethanol extract from Ecliptae Herba on seven cancer cell lines, and discovered that the growth and movement of breast cancer cells were significantly restricted.¹⁶ Nevertheless, the specific mechanism by which Ecliptae Herba influences human melanin metabolism remains unclear, necessitating further investigation into the mechanism underlying the effects of Ecliptae Herba on the prevention and treatment of white hair.

To elucidate the material basis and molecular mechanism underlying the effect of Ecliptae Herba on enhancing melanin production, this study used UHPLC-Q-Orbitrap HRMS to identify the components of Ecliptae Herba and employed a zebrafish juvenile model for verification. The underlying mechanism was explored by applying network pharmacology and molecular docking methods. As the first study to investigate the effects of Ecliptae Herba on melanin metabolism, the current research provided valuable insights into the therapeutic potential of Ecliptae Herba for treating white hair, laying a solid foundation for further investigation on Ecliptae Herba.

MATERIALS AND METHODS

Experimental reagents and instruments

Ethanol was provided by Xilong Science Co., Ltd., (Shantou, China, lot No. 24030201). Acetonitrile was provided by Anaqua Anke Chemical (Cleveland, America, lot No. Y24AC0110K). Formic acid was provided by Tianjin Kemio Chemical Reagent Co., Ltd., (Tianjin, China, lot No. 20220710). Tricaine (lot No. C12590064) and 1-phenyl-2-thiourea (PTU, lot No. N816213) were provided by Shanghai McLean Biochemical Technology Co., Ltd., (Shanghai, China). Dimethyl Sulfoxide (DMSO) was provided by Tianjin Zhiyuan Chemical Reagent Co., Ltd., (Tianjin, China, lot No. 20190111). Melanocyte Stimulating Hormone

(α -MSH) was provided by GloBio Technology (Shanghai, China, lot No. GC14374). UHPLC-Q-Orbitrap HRMS was purchased from Thermo Scientific (Waltham, MA, USA).

Medicinal plant materials

The dried aerial part of *Eclipta prostrata* L. was purchased from Anhui Tonghuatang Traditional Chinese Medicine Decoction Technology Co., Ltd., in 2023, with batch number No. 20230901.

Preparation of Ink Lotus Samples

Sample 1: Approximately 1 g of the powder of Ecliptae Herba (sieved through the third sieve) was accurately weighted, and placed in a conical flask with a stopper. After adding 50 mL of 70% ethanol, the total volume was weighted. The mixture was subjected to heat reflux for 1 hr and allowed to cool down. After weighing, weight loss was compensated with 70% ethanol. Next, the mixture was well shaken, filtered, and the filtrate was collected as the final product.

Sample 2: Precisely 5 g of Ecliptae Herba powder (sieved through a 65-mesh sieve) was soaked in 50 mL of cold pure water for 60 min, then boiled for 60 min, then cooled down and filtered. The medicine residue was added again with 50 mL of pure water and boiled for 30 min. The filtrate was combined, heated and concentrated, and then diluted to 100 mL using a 100 mL volumetric flask. The solution was to be sociated for 30 min to prepare a mother liquor at a concentration of 50g/L,¹⁷ followed by sterilization in a high-temperature sterilization pot and storage in a refrigerator for later use. For the experiments, the mother liquor diluted with 0.1% DMSO embryo culture water to the desired concentration was used as the treatment group of drought lotus, while the control group contained an equal volume of 0.1% DMSO embryo culture water solution.

Testing conditions

Chromatographic conditions: Using a Waters ACQUITY UPLC BEH C18 column (2.1 mm×100 mm, 1.7 μ m) with a flow rate of 0.3 mL/min and a column temperature of 30°C, gradient elution was performed using acetonitrile (A) -0.1% formic acid (B) as the mobile phase, with an injection volume of 1 μ L. The specific elution gradient program was as follows: 0-7 min, 5% A -13% A; 7-25 min, 13% A-30% A; 25-30 min, 30% A-45% A; 30-40 min, 45% A-95% A; 40-45 min, 95% A-95% A; 45-50 min, 95% A-5% A.

Mass spectrometry conditions: Analysis was conducted using a Heated Electrospray Ionization (H-ESI) in both positive and negative ion modes. The parameters of the instrument were as follows: nitrogen as the carrier gas is, with a sheath gas pressure and auxiliary air pressure at of 3.5 MPa and 1.0 MPa, respectively; spray voltage set at 3.80 kV(+), 2.50 kV(-); capillary temperature and auxiliary heating temperature set at 350°C and 350°C, respectively; resolution set at 120000 and Normalized Collision

Energy (NCE) set at 20, 40, 60 eV. The scanning range was between m/z 100 and 1500.

Drug administration and sample collection

Four days post-fertilization (dpf), zebrafish fries were obtained through normal fertilization and exposed to 0.03 g/L PTU solution for 24 hr. The fries were divided into six groups: control group, model group (PTU group), positive control group (α -MSH group), and three treatment groups (treated by 0.5 g/L, 1 g/L, and 2 g/L PTU solution). Each group of 20 juvenile fishes were placed in a 6-well plate, with three treatment groups administered with PTU solution at the concentrations of 0.5 g/L, 1 g/L, and 2 g/L. In addition, a positive control group was processed with 0.01 g/L α -MSH (positive drug), according to literature. The model group was treated by PTU after 24 hr and continuously processed until the end of the experiment,¹⁸ which was set at 7dpf (zebrafish fry can no longer absorb drugs through the skin and gills starting from 7dpf). At the end of the experiment, 5 fries from each group were anesthetized with 0.01% lidocaine solution and observed using a stereomicroscope. The images were then processed using ImageJ-win64 software.

Target collection

The chemical constituents of Ecliptae Herba were searched in The Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP) using the keyword "Ecliptae Herba" under the initial screening criteria of Oral Bioavailability (OB) $\geq 30\%$ and Drug-Like index (DL) ≥ 0.18 . Potential molecular targets of the identified chemical constituents were then predicted using PharmMapper (<http://www.lilab-ecust.cn/pharmmapper/>) database. Subsequently, the resulting targets were processed by UniProt database (<https://www.uniprot.org/>) to standardize the gene names and establish a database of monomeric active ingredients and target genes.

Targets related to melanin metabolism, including melanin, melanogenesis, melanin biosynthesis, melanin metabolism, and melanin pigmentation were collected from the GeneCards (<http://www.genecards.org/>) and OMIM (<https://omim.org/>) databases. After removing duplicate data, a dataset of targets linked to melanin metabolism was combined for subsequent analysis.

Protein-Protein Interaction (PPI) network

The active component targets and disease targets were processed using Venny (<https://bioinfogp.cnb.csic.es/tools/venny/index.html>) tool to obtain the intersection targets. The common targets were imported into STRING (<http://string-db.org>) platform for PPI network construction, under the species of *Homo sapiens*. Cytoscape 3.9.1 software was employed to develop a network of the target genes in the active components of Ecliptae Herba.^{19,20}

Enrichment analysis

Using the Metascape tool (<https://metascape.org/gp/index.html#/main/step1>), Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were conducted on the 48 common targets between the chemical components of the herb and disease-related targets. Enrichment entries for the three GO terms were obtained under a significance threshold of $p < 0.05$ as the screening criterion.^{21,22}

Molecular docking

Based on the degree value, the top-ranked key targets and key components were selected from both the PPI network and the "Ecliptae Herba Single Ingredient-Active Ingredient-Action Target" network diagram for molecular docking. The mol2 format structure of the active ingredients were downloaded from the TCMSP database, and the pdb format structure of the target protein was obtained from the Uniprot and PDB database. Using PyMOL2.6.0 and Autodock Tool-v1.5.7 software,²³ dehydration, hydrogenation, ligand removal, and charge removal on the target protein and target molecules were performed. Finally, Autodock Tool-v1.5.7 was employed to dock the processed active molecules with the target protein. Binding energy is a parameter that reflects the interaction strength between pharmacological molecules and their target proteins. Specifically, binding energy < -4.25 kcal/mol indicates that the ligand may bind to the receptor, binding energy < -5.0 kcal/mol indicates a relatively high binding activity, and binding energy < -7.0 kcal/mol indicates an excellent binding activity.^{24,25}

Statistical analysis

The images were processed using Image J-win64 software, and the data were analyzed using GraphPad Prism 8.0.1 and Excel 2019 software. The experimental data were expressed as $\pm s$, and one-way analysis of variance was used to compare differences between groups. $p < 0.05$ indicated a significant difference.

RESULTS

Identification of the components of Ecliptae Herba

Analysis of the main molecular chromatogram of Eclipta Herba in positive and negative ion modes identified 34 compounds (Figure 1). In positive ion mode, 6 compounds, namely, wedelolactone, Demethylwedelolactone, chlorogenic acid, isoquercetin, D,L-glutamine, L-glutamate, were identified (Table S1). In negative ion mode, 32 compounds were identified, such as wedelolactone, echinocystic acid, quercetin, luteolin, Ecliptasaponin A/D, Demethylwedelolactone, pratensein, chlorogenic acid, isoquercetin, apigenin, quinic acid, caffeic acid, Glucose, Shikimic acid, etc. (Table S2).

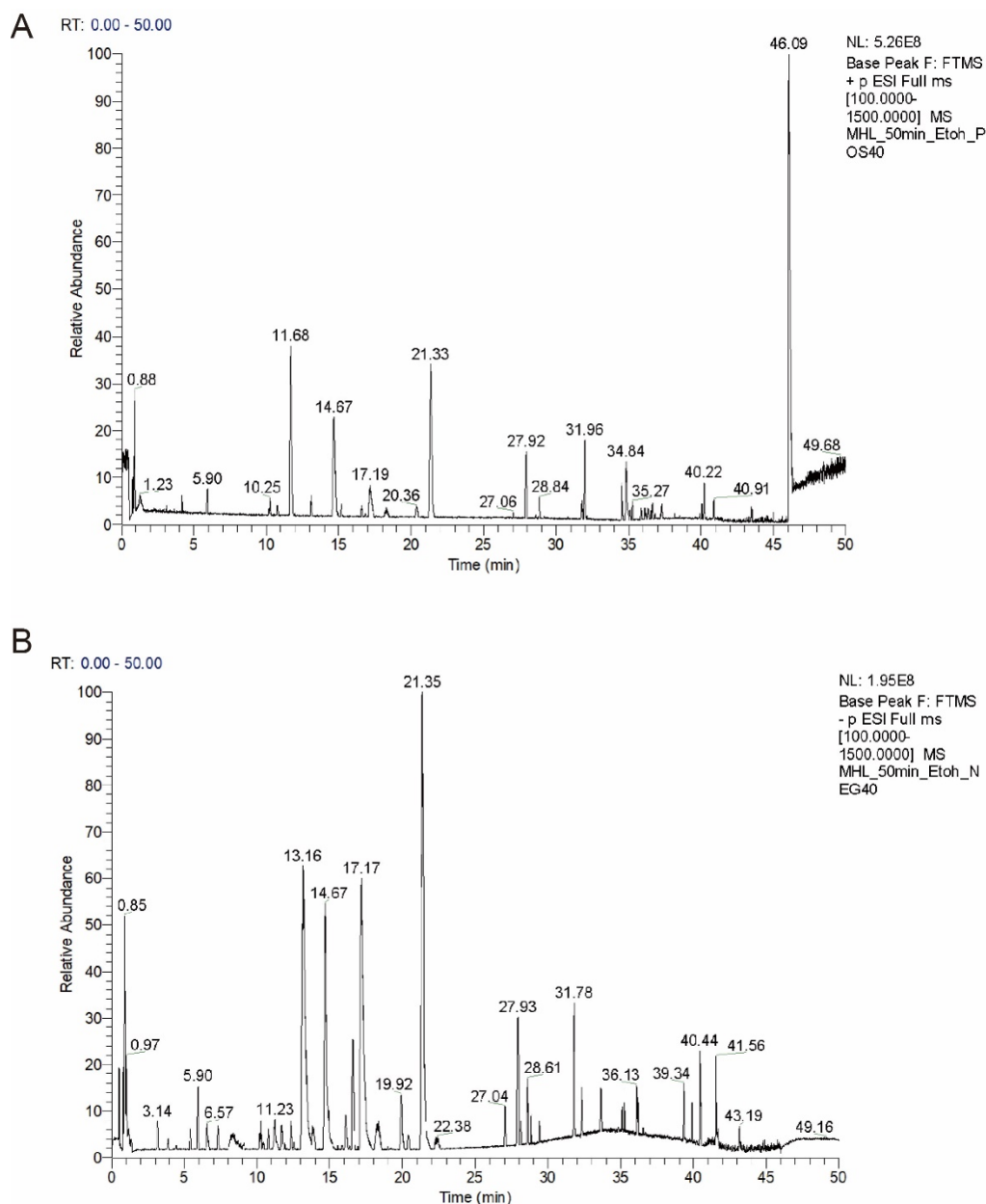


Figure 1: Ion diagram of Ecliptae Herba using UHPLC-Q-Orbitrap HRMS. (A) Total ion current diagram of Ecliptae Herba in positive ion mode. (B) Total ion current diagram of Ecliptae Herba in negative ion mode.

Potential effects of Ecliptae Herba on melanin metabolism

We used zebrafish fry to further explore the potential effects of Ecliptae Herba on melanin metabolism. As shown in Figures 2A-2D, 0.5 g/L of Ecliptae Herba significantly reduced gray values of eye and eye end to neck when compared to the treatment with α -MSH. These data revealed that Ecliptae Herba may possess a potential capacity to promote melanin formation and modulate pigmentation in specific body regions.

Construction of component-target network diagram and PPI network diagram

Using Venny 2.1.0 software, a total of 51 common targets in the intersection between 437 chemical targets of Ecliptae Herba and 652 targets related to melanin metabolism were obtained (Figure 3A). These 51 common targets were imported into Cytoscape 3.9.1 software to generate a component-target network diagram for Ecliptae Herba and melanin metabolism (Figure 3B). The network contained 63 nodes and 487 edges. According to the degree value, the top five chemical components in the network diagram were Pratensein, Wedelolactone, butin, acacetin, and Linarin, and the top five targets were GSTP1, PAH, NR1I2, MAPK1, and ADH1B. After loading the 51 common targets into

the STRING platform, the interaction relationships between the 51 potential targets were obtained based on the condition of confidence>0.4 (Figure 3C).

Enrichment analysis

GO enrichment analysis showed that the therapeutic targets of Ecliptae Herba on melanin metabolism mainly exerted their effects through Biological Process (BP) such as hormone response, hormone level regulation, and MARK cascade regulation. The Cellular Component (CC) were mainly related to acrosome vesicles, receptor complexes, and mitochondrial matrix. The activity of nuclear receptors, monooxygenase activity, insulin-like growth factor receptor binding, protein serine/threonine/tyrosine kinase activity, etc. were the main Molecular Function (MF) involved. These results were shown in Figure 4A and Table S3. KEGG pathway enrichment analysis showed that the signaling pathways significantly affected by Ecliptae Herba

on melanin metabolism were insulin resistance pathway, EGFR tyrosine kinase inhibitor activity, etc. (Figure 4B, Table S4).

Molecular docking

The heatmap of the docking result was shown in Figure 4C. Specifically, Linarin showed a high binding affinity with GSTP1, PPARG, and IGF1; pratensin showed a high binding affinity with PPARG; Demethylwedelolactone showed a high binding affinity with GSTP1; Echinocystic acid showed a high binding affinity with GSTP1, PAH, NR1I2, MAPK1, IGF1; Ecliptaaponin A showed a high binding affinity with PPARG and IGF1; quercetin showed a high binding affinity with GSTP1.

DISCUSSION

The Chinese Pharmacopoeia currently considers pyrethroid as the indicator component of Ecliptae Herba, but a single indicator cannot fully represent the quality of traditional Chinese

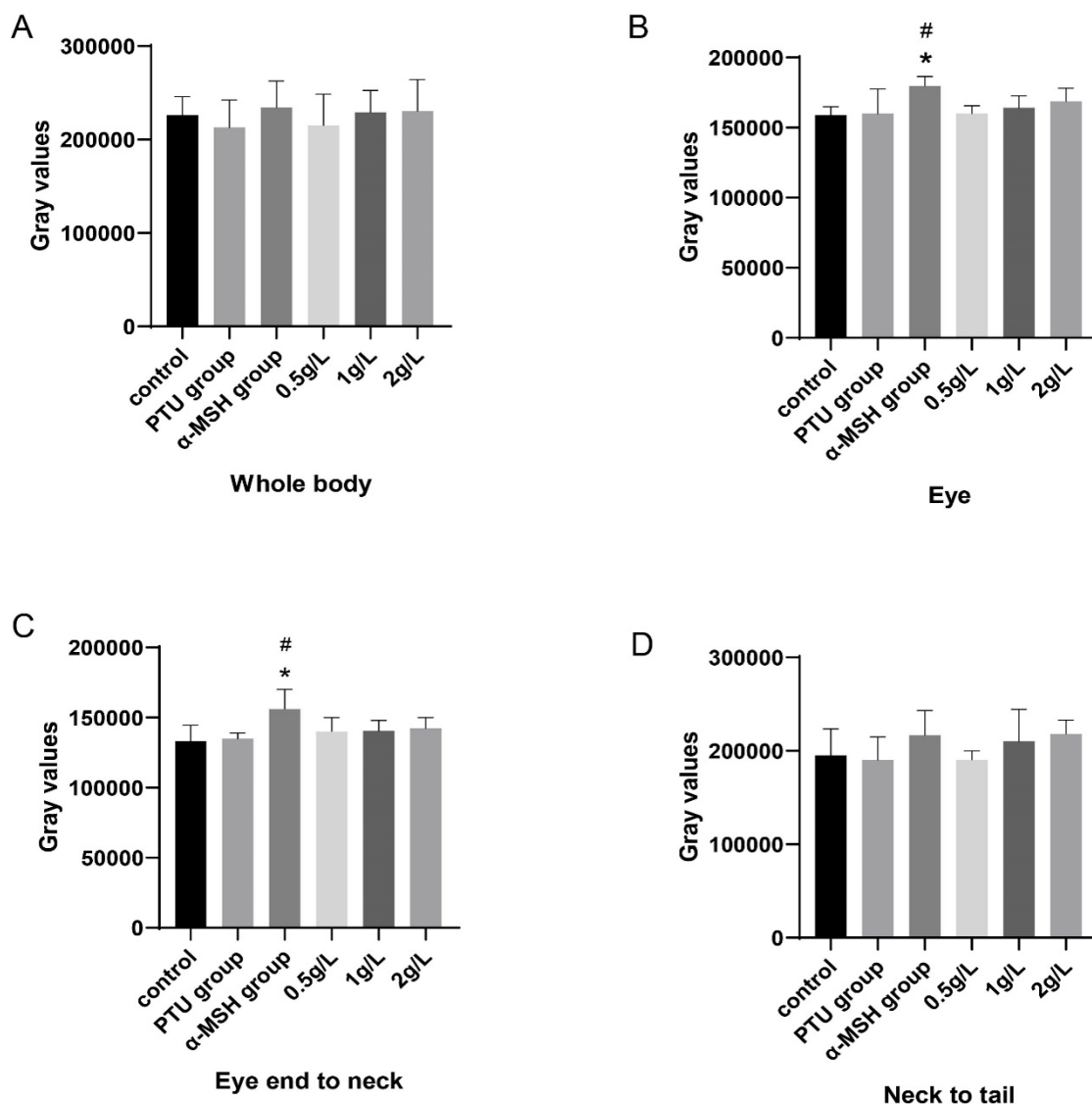


Figure 2: Effects of Ecliptae Herba on different parts of the body of zebrafish fry, including whole body (A), eyes (B), eye end to neck (C), and neck to tail (D).

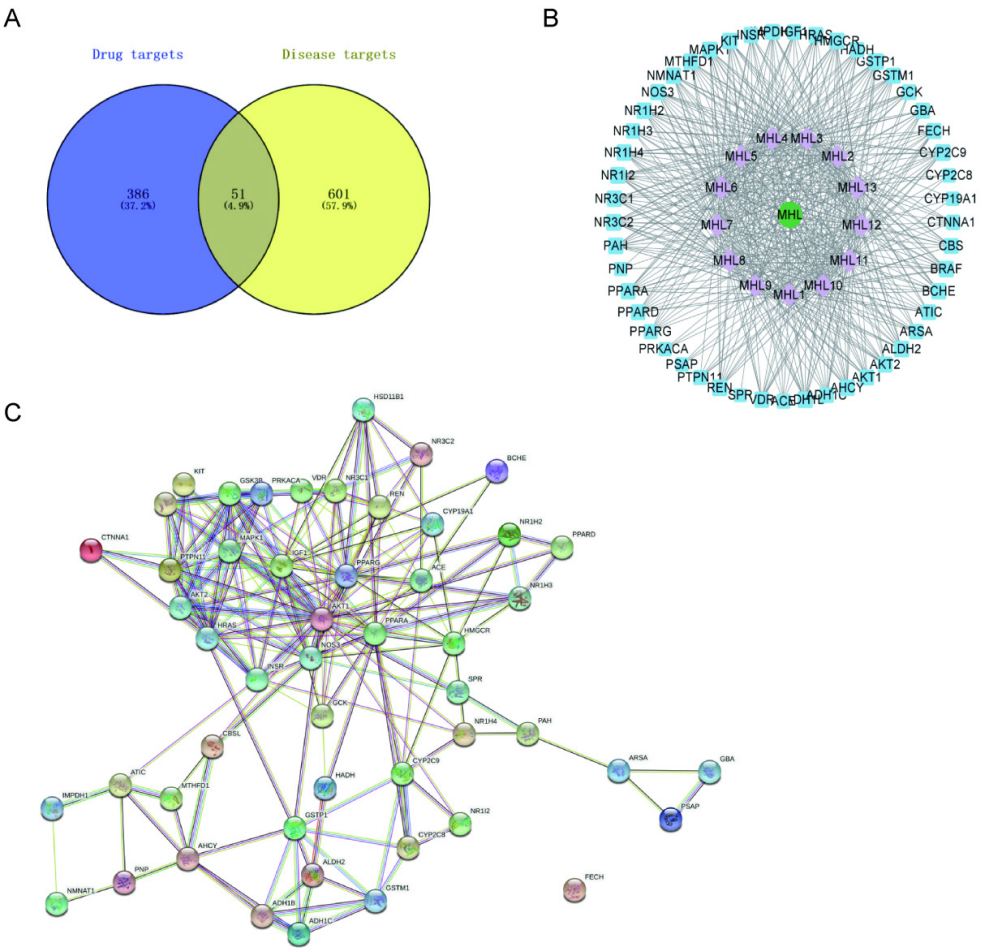


Figure 3: Multidimensional network analysis diagram of Ecliptae Herba based on the association of targets and components. (A) Venn diagram showing the screening of 51 target genes from Ecliptae Herba drug targets and disease targets. (B) Network diagram of single herb-active ingredient-target of Ecliptae Herba. Blue represents the target, purple represents the active ingredient of Ecliptae Herba, and green represents the single herb of Ecliptae Herba. (C) PPI network diagram of active component target genes in Ecliptae Herba.

Table S1: Identification of Chemical Components of Ecliptae Herba under LC-MS Positive Ion Mode.

Sl. No.	tR/min	Ionic mode	Molecular formula	Relative molecular weight	error	chemical compound	Secondary mass spectrometry fragments(m/z)
1.	21.33	+	C16H10O7	315.0496	-0.6	wedelolactone	297.03961,287.05524, 271.06055,269.04453, 243.06551
2.	14.68	+	C15H8O7	301.0341	-0.5	Demethylwedelolactone	283.02371,273.03918
3.	5.90	+	C16H18O9	355.1022	-0.5	chlorogenic acid	163.03903,135.04422
4.	10.74	+	C21H20O12	465.1027	-0.0	isoquercetin	303.04990
5.	0.84	+	C5H10O3N2	147.07649	0.5	D,L-glutamine	130.02884,102.09171, 84.04498
6.	0.84	+	C5H9O4N	148.06049	0.4	L-glutamate	130.02884,102.09171, 84.04498

medicine. This study investigated the effects of Ecliptae Herba on melanin synthesis using UHPLC-Q-Orbitrap HRMS, network pharmacology, and molecular docking. Employing a zebrafish model, we identified key components such as linarin, pratensein,

and quercetin, which targeted pathways such as drug metabolism. The present results indicated that Ecliptae Herba enhanced melanin production through multiple components and pathways,

Table S2: Identification of Chemical Components of Ecliptae Herba by LC-MS Negative Ion Mode.

Sl. No.	tR/min	Ionic mode	Molecular formula	Relative molecular weight	error	chemical compound	Secondary mass spectrometry fragments(m/z)
1.	21.35	-	C ₁₆ H ₁₀ O ₇	313.0356	4.0	wedelolactone	298.01205,270.01691,242.02057
2.	36.13	-	C ₃₀ H ₄₈ O ₄	471.3484	3.1	echinocystic acid	471.34824,407.33243,453.33719
3.	20.36	-	C ₁₅ H ₁₀ O ₇	301.0357	4.7	quercetin	273.04175,151.00270,121.02834,107.01261
4.	20.37	-	C ₁₅ H ₁₀ O ₆	285.0407	4.6	Luteolin	175.03954,151.00276,133.02829
5.	31.78	-	C ₃₆ H ₅₈ O ₉	633.4014	2.6	Ecliptasaponin A/D	587.39362,161.04463,113.02319
6.	9.08	-	C ₁₅ H ₈ O ₇	299.0200	4.6	Demethylwedolactone	299.01965,271.02518,227.03479,199.04007,183.04451
7.	24.29	-	C ₁₆ H ₁₂ O ₆	299.0564	4.7	pratensein	284.03293
8.	5.90	-	C ₁₆ H ₁₈ O ₉	353.0879	3.2	chlorogenic acid	191.05544,173.04439,161.02347
9.	10.75	-	C ₂₁ H ₂₀ O ₁₂	463.0889	3.8	isoquercetin	463.08469,301.03522,300.02789,255.02884
10.	19.92	-	C ₁₅ H ₁₀ O ₅	269.0454	3.5	apigenin	269.04572,159.04472,151.00327
11.	0.88	-	C ₇ H ₁₂ O ₆	191.0553	1.6	Quinic acid	191.05537,173.04453,127.03889
12.	6.65	-	C ₉ H ₈ O ₄	179.0341	1.1	caffeic acid	179.03427,135.04401
13.	0.83	-	C ₆ H ₁₂ O ₆	179.0553	1.3	Glucose	161.04459,117.01807,89.02309,71.01247
14.	4.87	-	C ₇ H ₁₀ O ₅	173.0447	1.2	Shikimic acid	155.03409,136.86159,129.31128,111.04359
15.	0.97	-	C ₄ H ₆ O ₅	133.0131	-0.7	Malic acid	115.00243,71.01249
16.	1.01	-	C ₆ H ₈ O ₇	191.0189	1.6	Citric acid	173.00734,154.99715,129.01807,103.03882
17.	1.62	-	C ₇ H ₆ O ₅	169.0133	1.0	Gallic acid	125.02319,107.01245,97.02811,69.03322
18.	3.41	-	C ₁₃ H ₁₈ O ₈	301.0931	4.4	Tachioside	139.00240,123.86333
19.	11.82	-	C ₁₃ H ₁₆ O ₁₀	331.0658	-0.6	Gallic acid 3-O-β-D-glucopyranoside	169.01337,125.02319
20.	3.14	-	C ₇ H ₆ O ₄	153.0183	0.1	Protocatechuic acid	109.02820,91.01732,81.03316
21.	6.32	-	C ₉ H ₈ O ₃	163.0391	0.7	P-Coumaric acid	119.04897
22.	4.65	-	C ₁₅ H ₂₀ O ₁₀	359.0988	4.1	Glucosyringic acid	197.04495,153.05473,121.02814
23.	15.48	-	C ₂₁ H ₂₀ O ₁₀	431.0988	3.6	Cosmosiin	269.04413,151.00262
24.	9.41	-	C ₁₄ H ₁₈ O ₉	329.0883	4.8	Phloracetophene 4'-O-glucoside	167.03410,123.04431

Sl. No.	tR/min	Ionic mode	Molecular formula	Relative molecular weight	error	chemical compound	Secondary mass spectrometry fragments(m/z)
25.	24.29	-	C ₁₆ H ₁₂ O ₆	299.0564	4.7	3'-Hydroxybiochanin A	284.03293,256.03690, 255.02956,239.03397, 227.03429
26.	37.53	-	C ₃₀ H ₄₆ O ₄	469.3326	2.9	3-Oxo-16 α -hydroxyolean-12-en-28-oic acid	423.33286,407.33228
27.	10.27	-	C ₂₁ H ₂₀ O ₁₃	479.0834	3.3	Quercetagitin-7-O-glucoside	317.03040,271.02435
28.	19.50	-	C ₁₅ H ₁₂ O ₆	287.0565	5.3	Isookain	151.00266,135.04401
29.	12.52	-	C ₂₁ H ₂₀ O ₁₂	463.0890	4.0	Quercetin-7-O- β -D glucopyranoside	301.03552,151.00272
30.	16.59	-	C ₂₅ H ₂₄ O ₁₂	515.11987	2.9	3,5-O-dicaffeoylquinic acid	191.05542,173.04477, 161.02350,135.04402
31.	13.11	-	C ₂₁ H ₂₀ O ₁₁	447.0937	3.3	Astragalin	285.04044,284.03296
32.	1.27	-	C ₁₀ H ₁₃ O ₅ N ₅	282.0845	4.4	Guanosine	150.04547,133.06430

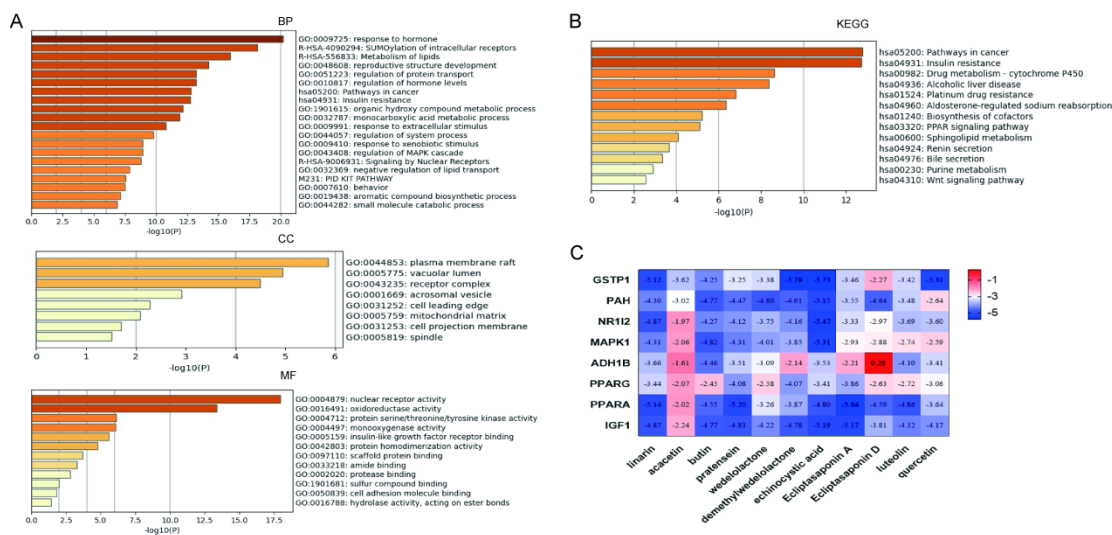


Figure 4: Multifaceted exploration of Ecliptae Herba's regulatory mechanisms on melanin-related targets. (A) GO enrichment analysis (including BP, CC, and MF terms) of common targets between the active components of Ecliptae Herba and melanin-related genes. (B) KEGG enrichment analysis of common targets between the active components of Ecliptae Herba and melanin-related genes. (C) Molecular docking results of key pharmacological components in Ecliptae Herba with target proteins.

providing a foundation for developing novel treatments for gray hair.

GSTP1, PAH, NR1I2, MAPK1, and ADH1B were identified as the key targets that played significant roles in melanin formation. GSTP1, a glutathione S-transferase, is involved in detoxification and oxidative stress regulation, which are critical for melanocyte function and melanin synthesis.²⁶ PAH is essential for the conversion of phenylalanine to tyrosine, a precursor for melanin production, highlighting its importance in melanogenesis.²⁷ Nuclear receptor subfamily 1 group I member 2 (NR1I2), also known as PXR, regulates drug metabolism and detoxification pathways and may indirectly influence melanin synthesis.⁵ Mitogen-Activated Protein Kinase 1 (MAPK1) is a key gene in the MAPK signaling pathway, which regulates melanocyte

proliferation and differentiation.³ Alcohol Dehydrogenase 1B (ADH1B) is implicated in retinol metabolism and is linked to melanocyte function and skin pigmentation.⁶

According to the existing research, *Scutellaria baicalensis* lactone has been identified as an effective tyrosinase inhibitor that can inhibit the enzyme in a reversible and competitive manner.²⁸ Red clover extract inhibits the pharmacological activity of melanin and also has anti-inflammatory and antioxidant effects.²⁹ Prickling acid can not only inhibit the expression of pro-inflammatory cytokines IL-1 β and TNF- α , but can also suppress the activation of NF- κ B and MAPKs, thereby improving inflammation.³⁰ Butin has a therapeutic effect on vitiligo. Studies have shown that butin treats vitiligo by elevating the expressions of TYR and TRP-1 proteins, reducing the activity

Table S3: GO enrichment analysis results.

Sl. No.	Category	Name	Gene target
1.	BP	response to hormone	AKT1 AKT2 BCHE CTNNA1 ACE GBA GCK NR3C1 GSK3B HADH INSR KIT NR3C2 NOS3 PPARA PPARD PPARG PTPN11 REN NR1H3
2.	BP	regulation of protein transport	AKT2 GCK GSK3B HADH HMGCR HRAS IGF1 PPARD PPARG PRKACA PTPN11 NR1H2 NR1H4 NR1H3
3.	BP	reproductive structure development	AKT1 CTNNA1 CYP19A1 ACE INSR KIT NOS3 PPARD PPARG MAPK1 PTPN11 REN VDR
4.	BP	regulation of hormone levels	ADH1C BCHE CYP2C8 CYP2C9 CYP19A1 ACE GCK HADH PPARD PPARG PTPN11 REN NR1H4
5.	BP	monocarboxylic acid metabolic process	ADH1B ADH1C CYP2C8 CYP2C9 GCK GSTM1 GSTP1 HADH IGF1 PPARA PPARD PPARG NR1H4
6.	BP	organic hydroxy compound metabolic process	ADH1C ALDH2 CYP2C8 CYP2C9 CYP19A1 GBA HMGCR IGF1 PAH PPARD SPR NR1H4
7.	BP	regulation of system process	ACE GBA IGF1 KIT NOS3 PPARA PPARG PRKACA PTPN11 REN NR1H2 NR1H3
8.	BP	regulation of MAPK cascade	BRAF GBA GSTP1 HMGCR HRAS IGF1 INSR KIT PPARG MAPK1 PTPN11 REN
9.	BP	Behavior	AKT1 BCHE ACE GBA HMGCR INSR KIT PPARA MAPK1 REN
10.	BP	response to xenobiotic stimulus	BCHE CYP2C8 CYP2C9 ACE GSTM1 GSTP1 HADH PNP REN NR1I2
11.	BP	aromatic compound biosynthetic process	ATIC FECH IMPDH1 MTHFD1 PNP PAH PPARG SPR VDR NR1H3 NMNAT1
12.	BP	negative regulation of lipid transport	AKT1 AKT2 PTPN11 NR1H2 NR1H3
13.	BP	small molecule catabolic process	ALDH2 CBS GCK HADH NOS3 PNP PAH PPARD
14.	CC	plasma membrane raft	CTNNA1 INSR NOS3 PRKACA MAPK1
15.	CC	vacuolar lumen	ARSA GBA IMPDH1 MAPK1 PSAP
16.	CC	acrosomal vesicle	CTNNA1 KIT PRKACA
17.	CC	receptor complex	INSR KIT NR3C2 PPARG VDR NR1H4 NR1H3
18.	CC	cell leading edge	AKT1 AKT2 CTNNA1 INSR
19.	CC	mitochondrial matrix	ALDH2 FECH NR3C1 HADH
20.	CC	cell projection membrane	AKT2 ACE INSR
21.	CC	spindle	AKT1 NR3C1 MAPK1
22.	MF	nuclear receptor activity	NR3C1 NR3C2 PPARA PPARD PPARG NR1H2 VDR NR1I2 NR1H4 NR1H3
23.	MF	oxidoreductase activity	ADH1C ALDH2 CBS CYP2C8 CYP2C9 CYP19A1 GSTP1 HADH HMGCR HSD11B1 IMPDH1 MTHFD1 NOS3 PAH SPR
24.	MF	monooxygenase activity	CYP2C8 CYP2C9 CYP19A1 NOS3 PAH
25.	MF	insulin-like growth factor receptor binding	IGF1 INSR REN
26.	MF	protein serine/threonine/tyrosine kinase activity	AKT1 AKT2 BRAF GSK3B INSR KIT PRKACA MAPK1

Sl. No.	Category	Name	Gene target
27.	MF	protein homodimerization activity	AKT1 ATIC CBS FECH GSTM1 HSD11B1 KIT
28.	MF	scaffold protein binding	BRAF NOS3 PSAP
29.	MF	amide binding	BCHE GSTM1 INSR PPARG PSAP
30.	MF	cell adhesion molecule binding	ATIC CTNNA1 IGF1 PTPN11
31.	MF	hydrolase activity, acting on ester bonds	ALDH2 ARSA BCHE PTPN11
32.	MF	protease binding	GSK3B KIT PSAP
33.	MF	sulfur compound binding	CBS GSTM1 GSTP1

Table S4: KEGG enrichment analysis results.

Sl. No.	KEGG ID	KEGG Pathway	Gene target
1.	hsa04931	Insulin resistance	AKT1 AKT2 GSK3B INSR NOS3 PPARA PTPN11 NR1H2 NR1H3
2.	hsa05200	Pathways in cancer	AKT1 AKT2 BRAF CTNNA1 GSK3B GSTM1 GSTP1 HRAS IGF1 KIT PPARD PPARG MAPK1
3.	hsa00982	Drug metabolism - cytochrome P450	ADH1B ADH1C CYP2C8 CYP2C9 GSTM1 GSTP1
4.	hsa01524	Platinum drug resistance	AKT1 AKT2 GSTM1 GSTP1 MAPK1
5.	hsa04960	Aldosterone-regulated sodium reabsorption	IGF1 INSR NR3C2 MAPK1
6.	hsa01240	Biosynthesis of cofactors	ALDH2 FECH MTHFD1 SPR NMNAT1
7.	hsa03320	PPAR signaling pathway	PPARA PPARD PPARG NR1H3
8.	hsa04936	Alcoholic liver disease	ADH1B ADH1C AKT1 AKT2 ALDH2 GSK3B PPARA
9.	hsa00230	Purine metabolism	ATIC IMPDH1 PNP
10.	hsa00600	Sphingolipid metabolism	ARSA GBA PSAP
11.	hsa04924	Renin secretion	ACE PRKACA REN
12.	hsa04976	Bile secretion	HMGCR PRKACA NR1H4
13.	hsa04310	Wnt signaling pathway	GSK3B PPARD PRKACA
14.	hsa01521	EGFR tyrosine kinase inhibitor resistance	AKT1 AKT2 BRAF GSK3B HRAS IGF1 MAPK1

of malondialdehyde and cholinesterase.³¹ Meanwhile, coumarin, isodemethylated coumarin, luteolin, and apigenin have been found to have strong anti-hepatotoxic activity.³² Linarin could inhibit changes in the inflammatory system by reducing NF- κ B p65/ Inhibitor of Nuclear Factor Kappa-B Kinase Subunit Beta (IKK β), IL-6, and TNF- α in animal bones, confirming that Linarin can suppress oxidative stress and inflammation and regulate the RANK/RANKL/OPG pathway.³³ In recent years, studies reported that inflammatory response plays an important role in the regulation of melanin production.³⁴ In addition, the active components of Ecliptae Herba regulate melanogenesis by binding to key proteins involved in melanogenesis and oxidative stress. Molecular docking results showed that Linarin, Pratensein, Demethylwedelolactone, Echinocystic acid, Ecliptasaponin A,

and Quercetin had a high affinity with multiple targets, suggesting that they may act synergistically through multiple targets. For instance, Leis *et al.* found that the extracts of French maritime pine bark not only downregulates tyrosinase, but also reduces pigmentation-related mediators (including PPAR α), which in turn inhibits melanin production *in vitro* and the pathways associated with hyperpigmentation of the skin.³⁵ Moreover, Hu *et al.* showed that administering IGF1 at a concentration of 10 ng/mL significantly influences melanogenesis in alpaca melanocytes via the cAMP pathway.³⁶ The binding of the active components of Ecliptae Herba to these targets indicates that they may enhance melanin formation through multifaceted interactions, highlighting the potential of Ecliptae Herba as a therapeutic agent for treating melanin-related diseases.

However, there were also some limitations in our study. Firstly, although the zebrafishes were an effective model for pigmentation studies, its melanogenesis mechanism cannot fully mimic the melanin physiological processes in mammals and humans. Future studies should include mammalian systems such as mouse pigmentation model or human melanocyte culture to enhance the clinical applicability of the current findings. Secondly, due to the synergistic action of multiple components in Ecliptae Herba, analysis on the specific function of a single component may be less reliable. Therefore, these components should be isolated and purified, and their individual effects are required to be verified by melanocyte models or enzyme activity assay systems. Finally, although three dose gradients were analyzed in this study, a systematic and complete dose-effect relationship has not been established, making it difficult to clarify the optimal effective dose and safety range of Ecliptae Herba. Therefore, further studies are encouraged to analyze the concentration of the gradients to clarify the effective dose range for the development of medicinal or functional products of Ecliptae Herba for white hair treatment.

CONCLUSION

In this study, UHPLC-Q-Orbitrap HRMS identified 34 active components of Ecliptae Herba, and network pharmacology combined with molecular docking revealed key targets and pathways involved in melanogenesis. Functional enrichment analysis showed that Ecliptae Herba regulated melanogenesis through oxidative stress modulation, hormone response, and PPAR signaling pathways. Importantly, the experiments using the zebrafish model demonstrated that Ecliptae Herba water extract significantly reduced the gray values in specific body regions of the model, indicating a potential role of Ecliptae Herba in promoting melanin formation. In summary, this study offered valuable insights into the mechanisms by which Ecliptae Herba promoted melanin production, establishing a solid foundation for future investigations in this field.

ABBREVIATIONS

UHPLC-Q-Orbitrap HRMS: Ultra-High Performance Liquid Chromatography-Quadrupole-Orbitrap High-Resolution Mass Spectrometry; **TCMSP:** The Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform; **OMIM:** Online Mendelian Inheritance in Man; **PDB:** the Protein Data Bank; **TRP1:** Tyrosinase-related protein-1; **NP:** network pharmacology; **DMSO:** Dimethyl Sulfoxide; **OB:** oral bioavailability; **DL:** drug-like index; **PPI:** Protein-protein interaction; **GO:** Gene Ontology; **KEGG:** Kyoto Encyclopedia of Genes and Genomes.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

All authors contributed to this present work: MYS and SYG designed the experiment, conducted the experiment, and analyzed the data, MYS and SYG designed the study, MYS and SYG acquired the data. MYS and SYG drafted the manuscript, MYS and SYG revised the manuscript. All authors have been involved in revising it critically for important intellectual content. All authors read and approved the final manuscript.

SUMMARY

This study explored the active components and mechanisms of Ecliptae Herba in regulating melanin synthesis. UHPLC-Q-Orbitrap HRMS identified 34 compounds, while network pharmacology and molecular docking revealed key targets such as GSTP1, PPARA, and IGF1 involved in oxidative stress and PPAR signaling pathways. Zebrafish experiments showed that the Ecliptae Herba extract can reduced gray values in specific regions. These results suggest that Ecliptae Herba exerts multi-component, multi-target effects on melanin regulation, offering a scientific basis for its development in treating pigmentary disorders.

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