The effective components and mechanism of the kernel of Prunus mira Koehne to promote hair growth

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Research Article

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Abstract

Objective

The aim of the present study is to reveal the effective components and their mechanism of hair growth promotion in the kernel of *Prunus mira* Koehne, and to improve its quality control level.

Methods

Network pharmacology was used to predict the mechanism of action and active components in the treatment of the kernel of *P. mira*. The contents of oleic acid, linoleic acid and amygdalin in 12 batches of the kernel of *P. mira* were determined by HPLC. An animal model of the depilation of KM mice induced by sodium sulfide was created, and then 5 effective components that promoted hair growth were initially screened. An animal model of C57BL/6 mice depilation model induced by sodium sulfide was created, and the 5 effective components selected above were used for re-evaluating, so as to clarify the 3 effective components that promote hair growth. RT-PCR and immunohistochemistry were used to evaluate the influence of the expression of indicators in the skin Wnt/β-catenin signaling pathway, including *β*-catenin, GSK-3β, mRNA and protein expression of Cyclin D 1 and LEF 1.

Results

In the study of network pharmacology, it was found that there were 149 targets for the chemical composition and alopecia in the kernel of *Prunus mira* Koehne, and the "protein-protein network interaction diagram" and the network diagrams of "components in the kernel of *Prunus mira* Koehne - chemical -targets-hair loss" were constructed. KEGG enrichment resulted in 12 alopecia related pathways, involving 25 targets and 13 components. The methodological investigation of oleic acid, linoleic acid and amygdalin in the kernel of *Prunus mira* Koehne were carried out by HPLC. Furthermore, The average content of oleic acid, linoleic acid and amygdalin in 12 batches from different producing areas is 428.91mg/mL, 99.89mg/mL, 17.27 mg/mL. Vitamin E (3.125 mg/cm^2/d), β-sitosterol (0.061 mg/cm^2/d), linoleic acid (0.156 mg/cm^2/d) had the effect of promoting hair growth in mice, and could up-regulate Wnt/β-catenin signaling pathway, the mRNA expression of LEF 1, GSK-3β, and β-catenin also showed an upward trend, but they had no significant effect on the protein expression of the four indicators.

Conclusions

The determination method of oleic acid, linoleic acid and amygdalin in the kernel of *Prunus mira* Koehne was established by HPLC, which improved the quality standard. Vitamin E (3.125 mg/cm^2/d), β-sitosterol (0.061 mg/cm^2/d) and linoleic acid (0.156 mg/cm^2/d) in the kernel of *Prunus mira* Koehne can promote hair growth in mice, and its mechanism of action may be related to the Wnt/β-catenin pathway.

1. Introduction

Prunus mira Koehne, a plant of the genus *Prunus* in the *Rosaceae* family, is named *P. mira* in Latin and □□□□□ in Tibetan (Kangbu, Holdkan [1]). It is the direct origin of peach in Yunnan Province and the southwestern of Sichuan Province [2], and it is a rare "living fossil group" of peach genetic resources in the world. It has the characteristics of fast growth, long life, drought and cold tolerance. According to *Commonly used Chinese medicinal materials quality sorting and quality research* (1997)[3], *P. mira* is distributed in the border areas of Sichuan Province, Yunnan Province, and Tibet Autonomous Region, with large reserves, as shown in Fig. 1. The main distinguishing points of *P. mira* and other species such as *Amygdalus kansuensis* (Rehd.) Skeels and peach are that the surface of the pit is smooth and has no holes and has shallow and wide longitudinal grooves. Both *Jingzhu Materia Medica* (□□□□□□□□□□□□□□□□□□□□) (the classic of Tibetan medicine, compiled by the renowned Tibetan pharmacist Diamer Danzeng Pengcuo over roughly a period of 20 years, was finished in 1743 [4] and *Dictionary of Chinese Ethnic Medicine* record that the kernel oil of *P. mira* can darken and grow hair, it can treat alopecia. In addition, the *Dictionary of Chinese Ethnic Medicine* also records that the burnt ash of fruits of *P. mira* can be used to treat trauma and constipation, and grasserie [5]. According to *Sichuan Province Standard for Chinese Medicinal Materials* (2010)[6], its efficacy is consistent with that of *Amygdalus persica* L., which is used in traditional Chinese medicine. At present, in addition to the kernel of *A. persica*. and *Amygdalus davidiana* (Carrière) de Vos ex Henry, there are still some the kernel of *P. mira* in the market for sale [7].

The characteristics of fast growth, strong adaptability, cold tolerance, longevity and other characteristics of *P. mira* have laid a good foundation for its development and utilization. It could provide good peach varieties for high altitude areas such as Tibet Autonomous Region, the southwestern of Sichuan Province etc, which is easy to cultivate and plant, and save manpower cost, and less manual intervention makes the fruit less polluted, so it is a natural green organic food. At the same time, due to the special geography and climate of Tibetan areas, the fruit has low sugar content [8] and is rich in mineral elements such as Zn, Ca, and K. Compared with the fruits of *Amygdalus persica* L., the content of Zn is 2–3 times higher, and the content of Vc is more than 10 times [9]. At present, herdsmen in Tibetan areas will pick up the ripe fruit of *P. mira* as feed for yaks, Tibetan pigs and other livestock, saving feeding costs.The lifespan of *P. mira* is longer than that *Amygdalus persica* L., and the tree age can reach a hundred years, and it is cold-resistant. It is a good original material for cultivating cold-resistant and long-lived peach varieties. The existence of plant cold-resistant genes can also provide huge genetic and hybrid value [10]. At present, the GC-MS method is used to identify the fat-soluble components of light walnut, and a total of 28 chemical components have been found [11]. The content of vitamin E, squalene, *β*-sitosterol, and *α*-tocopherol was determined by HPLC [12]. However, the composition of the kernel of *P. mira* is complex, and the current control indicators are difficult to control its quality.

Hair is an important accessory structure of the skin, which has the function of protecting the body. For example, hair can reduce the heat loss of the head [13]. The hair growth cycle can be divided into growth period, regression period, and resting period. In the growth period, the cells at the bottom of the hair follicles receive proliferation signals and extend and differentiate into the dermis to form hair follicles. During the growth period, the central column layer of the hair follicle first extends to the subcutaneous tissue, and then grows back to form the hair shaft after reaching a certain depth. Growing period can be divided into six sub-periods. There is little difference in the growth status of the hair follicles in the first to five sub-phases, the last growth period of time determines the length of the hair shaft. During the regression period, the growth of the hair shaft stops. The growth and differentiation of hair follicle cells stop and gradually undergo apoptosis, and the hair papilla separates from the hair bulb. In the resting period, hair follicle is located in the upper layer of the dermis during the resting period, and a layer of fibroblasts is formed at the bottom, that is, the secondary hair embryo is the basis for the next hair follicle growth. There is no DNA transcription synthesis activity in the secondary hair embryo [14]. In some species, the hair follicles at the same location may have their own growth rhythms (such as humans, guinea pigs), and in some rodents (mice), the hairs at the same location are almost in the same hair cycle. At present, it is considered that the factors affecting the hair follicle cycle include hair follicle damage, abnormal growth cycle, surrounding hair follicle growth cycle, endocrine and so on.[15]

Alopecia is a skin disease characterized by hair loss [16], which is divided into 7 levels according to the area of hair loss. Studies have found that its pathogenesis is related to heredity [17], endocrine [18], mental state [19], etc. In recent years, China Association for health promotion and education, two major e-commerce platforms of China have released relevant statistics on the current situation of hair loss in China. It is pointed out that about 1/6 people in China have hair loss, among which 64% are men, and hair loss tends to be younger. Alopecia can cause a psychological burden on young people and even lead to a decline in the quality of life [20], so the treatment of alopecia has become the focus of attention in the field of dermatology and the public. At present, the drugs commonly used to treat hair loss are minoxidil and finasteride. Minoxidil can make hair grow and thicken, with an effective rate of about 30–50%, and requires long-term medication, and it is easy to relapse after stopping the drug [21]. Finasteride inhibits the conversion of testosterone to dihydrotestosterone, thereby improving alopecia caused by strong androgen secretion, but it is easy to cause problems in sexual function [22]. The commonly used drugs for hair loss treatment in traditional Chinese medicine include *Fallopia multiflora* (Thunb.) Harald., *Ligustrum lucidum* Ait., *Platycladus orientalis* (L.) Franco., *Polygonum multiflorum* Thunb, etc. The commonly used traditional Chinese medicine compounds include traditional Chinese medicine hair growth liquid, Sangbai hair growth recipe, hair growth mixture of *Ginkgo biloba* L., hair growth tincture, etc [23]. In addition to drug treatment, plum blossom needle tapping, silver needle acupuncture, point injection, moxibustion and other methods are commonly used methods for the treatment of alopecia. The literature reports that vitamin E combined with plum blossom needle tapping is effective in treating alopecia areata [24]. At present, in addition to modern medicine treatment and Chinese medicine treatment, hair transplantation is also a common treatment at present. Hair transplantation is achieved by transplanting autologous hair follicles and surrounding tissues to the treatment area to achieve the purpose of local hair distribution density. The advantage of hair transplant is that it is suitable for patients with large or severe hair loss, and usually the effect after surgery is ideal. However, hair transplants are expensive and may leave scars after surgery, and may even cause scalp bleeding, swelling, inflammation, and infection.

Network pharmacology is a technology that combines multiple disciplines such as systems biology, pharmacology and computer science to mine and analyze drug targets. It is mainly by searching databases and high-throughput omics analysis, systematically interpreting the interaction between drugs and the body, visualizing the relationship between drugs, targets, multi-pathway regulatory signal pathways, and diseases, so as to predict the effective components, targets and mechanism of action [25]. Traditional Chinese medicine monomer and compound are the main application methods of modern Chinese medicine in the prevention and treatment of diseases. However, due to the complex composition, numerous targets and difficult research of traditional Chinese medicine, the modernization and internationalization of traditional Chinese medicine progress slowly. The systemic nature of network pharmacology is consistent with the overall view of traditional Chinese medicine. Therefore, the application of network pharmacology methods to study the relationship between the components of traditional Chinese medicine and the complex system of biological organisms is more conducive to the discovery of the main compounds in traditional Chinese medicine that exert efficacy, find the molecular mechanism of drug prevention and treatment, and then explain the mechanism of action of traditional Chinese medicine from a molecular perspective [26]. Network pharmacology is currently used to explain mainly the active components and mechanism of action of preparations or medicinal materials [27]. The traditional method of researching active components in Chinese pharmacy, which include the physical and chemical separation and identification with multilevel pharmacodynamic evaluation of animal, tissue, and cell models and study of mechanism of action, has laid a good foundation for the development of network pharmacology, but this method has a large workload, time-consuming, low efficiency, and network pharmacology through the existing research results to simulate the interaction between the drug and the body can reduce the workload and save manpower and material resources [28]. Wu guosong [29] applied network pharmacology and found that the compounds such as geranidin, dihydrochelerythrine and sesamin contained in *Zanthoxylum nitidum* (Roxb.) DC. showed potential anti-inflammatory activities, and COX-2 and MAPK14 exerted anti-inflammatory effects. TNF signaling pathway is a potential signaling pathway for *Zanthoxylum nitidum* (Roxb.) DC. to achieve anti-inflammatory effects. Tao et al. [30] used the method of network pharmacology to study the relationship between the prescriptions of *Curcuma aromatica* Salisb. recipe and found that this prescription can maintain the maximum effective concentration, and also found that *C. aromatica* recipe may have the effect of improving nutritional and metabolic diseases. The databases commonly used in network pharmacology can be divided into chemical information-related databases and biological information-related databases. Commonly used chemical information databases are: Traditional Chinese Medicine System Pharmacology Database and Analysis Ping (TCMSP), Taiwan Traditional Chinese Medicine Database (TCM Datebase@Taiwan), and Traditional Chinese Medicine Comprehensive Database (TCMID). TCMSP is currently the most commonly used database, containing 499 Chinese medicines, 29384 chemical and related metabolic components, 3311 targets, and 837 diseases. TCMID is currently the world's largest computer-based drug screening TCM database, containing about 47000 TCM prescriptions, 8159 medicinal materials, 25210 compounds, 6828

drugs, 3791 diseases, and 17521 targets. TCM Database@ Taiwan is the earliest and second largest traditional Chinese medicine database, containing more than 2000 compounds discovered from 453 Chinese medicines, and provides the cdx (2D) and Tripos mol2 (3D) structure diagrams of each compound [31]. Commonly used biological information databases can be roughly divided into three categories: First, it is used to find information about biological targets related to drugs or chemical components, such as Uniprot, Parmmaper, Drug Bank, etc. Second, databases used to find disease-related targets, such as OMIM, Genecard. Third, a database of protein interaction relationships was established, such as Sring, Mint, IntAct, etc. [32]. UniProt database is currently the most commonly used database with the most abundant protein information. Its information source is the protein sequence obtained by genome sequencing and the protein function reported in the literature. The Sring database is a commonly used protein interaction relationship database, which records 9 643 763 proteins and 1 380 838 440 interaction information. There are other databases for other purposes, such as KEGG, Matescape and other biological information GO analysis and pathway analysis databases.

Previous studies have found that the fat-soluble components of the kernel oil of *P. mira* have a certain effect on hair growth within the range of 15.06 ~ 60.26 mg /cm^2/d, but the effective components is still unclear [33]. The acute toxicity of the kernel oil of *P. mira* to mice, rats and rabbits was studied, and it was found that the maximum dose of the kernel oil of *P. mira* administered orally to rats and mice was 144.612 g/kg/d and 289.224 g /kg/d, the maximum dose of the kernel oil of *P. mira* administered to rabbits through the skin is 482.28 mg/cm^2/d, which is safe [34]. The kernel of *P. mira* is safe for clinical use. The half lethal dose of its aqueous extract to mice is 238 times higher than that of the commonly used dose (0.18g/kg)[3]. It's fat-soluble components had no obvious long-term toxicity on rats, and had no obvious irritation on skin. But the application of fat-soluble components on the scalp of the patient will cause discomfort and cannot achieve the effect of clinical application. By looking for the main components or parts of the kernel of *P. mira* that exerts the medicinal effect, and the mechanism to promote hair growth can be further clarified, and the form of a drug will be improved in the later stage to make it more suitable for clinical use. This article first studied the effective components of the kernel oil of *P. mira* to promote hair growth. According to the previous research results and network pharmacology technology, 8 kinds of components including β-sitosterol and linoleic acid, were selected to evaluate the pharmacodynamics of promoting hair growth in KM mice and C57BL/6 mice, and passed the pharmacodynamic evaluation screening. The components that have obvious effects on promoting hair growth, namely β-sitosterol, linoleic acid and vitamin E, have also been initially explored to provide reference for clinical promotion and use.

2. Materials And Methods

2.1 Network pharmacology predicts the mechanism and active components of the kernel of P. mira in the treatment of hair loss

Network pharmacology data collection first collected and sorted out the chemical components of the kernel of *P. mira* through literature search, collected the components and the corresponding targets of the disease with the help of the database, and then matches the two targets to predict the mechanism of the kernel of *P. mira* treatment for alopecia, for subsequent research and treatment and the mechanism of alopecia provides a reference. The database and analysis software used in network pharmacology were shown in Table 1. Since TCMSP, TCMD@Taiwan, PubChem and other databases did not contain information about the chemical composition of the kernel of *P. mira*, the chemical composition of the kernel of *P. mira* was comprehensively collected through modern biomedical literature databases such as CNKI, VIP database, Pubmed, and Wanfang database. Drugs and disease-related targets were analyzed and screened. The chemical structure of its chemical components was searched in Pubchem database, 2D and 3D SDF files were downloaded, and then the Palmmapper database is used to collect target proteins of the components, according to the synthesis of the Palmmapper database Score, genes with Score > 0.6 were selected. GeneCards was used to search the keywords "hair loss" and "alopecia" to search disease targets. The component-related targets of the kernel of *P. mira* and the alopecia disease-related targets were taken to intersect, so as to obtain a common potential target and draw a Venn diagram. The relationship files between drugs, components, targets, and diseases were sorted out, and Cytoscape software was used to draw a network diagram of drugs-components-targets-disease. Potential targets were imported into String, and Score > 0.4 was selected to predict the interaction relationship between protein and protein. The degree of topological parameter was used to evaluate the importance of the target, and the top 30 Hub genes and PPI files were obtained. The PPI relationship files obtained by String were imported into Cytoscape software to draw PPI diagrams. The Metascape database was used for Gene oncology (GO) enrichment analysis to find the biological processes, molecular functions and cell composition of potential targets involved in the body. The Metascape database was used for KEGG enrichment analysis.

Table 1
databases and analysis software related to Network pharmacology

Tools	Function	Website
Pubchem	Compound structure search	https://pubchem.ncbi.nlm.nih.gov
Pharmmapper	Compound target prediction	http://www.lilab-ecust.cn/Pharmmapper
UniProt	Protein name correction	https://www.Uniprot.org
Genecards	Disease target prediction	http://www.genecards.org
String	Construct protein interaction map (PPI)	https://String—db.org
Venny 2.1	Drawing	https://bioinfogp.cnb.csic.es/tools/Venny/
Metascape	Analyze the database	https://metascape.org/gp/index.html
Cytospase3.7.1	Drawing software	——

2.2 The contents of oleic acid, linoleic acid and amygdalin in the kernel of P. mira were determined by HPLC

2.2.1 Materials

Twelve batches of the kernel of *P. mira* were collected from Sichuan Province, Yunnan Province and other places in China. The specimens collected from each place were identified as *P. mira* by Minru Jia, who was the professor of Chinese medicine identification at Chengdu University of Traditional Chinese Medicine. The voucher specimen was preserved in the college of ethnomedicine, Chengdu University of Traditional Chinese Medicine. HPLC analyses were carried out using Shimadzu LC-2030 high performance liquid chromatograph (Shimadzu, Japan), and the purity by HPLC detection was greater than 97%. The CPA2250 electronic balance was purchased from Sartorius, Germany. The reference substances of oleic acid (batch number PS20190843), linoleic acid (batch number PS20190832), and amygdalin (batch number PS20190922) were purchased from Chengdu Pusi Biotechnology Co., Ltd. The purity by HPLC detection was greater than 97%. Acetonitrile (chromatographically pure) was purchased from Anhui Tiandi High Purity Solvent Co., Ltd., and methanol (analytical pure) was purchased from Sigma-Aldrich Trading Co., Ltd., and water was ultra-pure water, and other reagents were analytical pure.

2.2.2 HPLC conditions

Using Shimadzu LC-2030 HPLC (Shimadzu, Japan), the content of oleic acid, linoleic acid and amygdalin were determined, and the processing of chromatogram and the calculation of peak area was automatically integrated by the computer using LabSolution software. By investigating the chromatographic conditions of different wavelengths, different mobile phases, different column temperatures, and different flow rates, the best chromatographic conditions are obtained. The chromatographic column is Hypersil ODS2 (250×4.6 mm, 5 μm). When determining oleic acid and linoleic acid, the column temperature was 30℃, and the flow rate was 1.0 mL/min, and the injection volume was 10μL, and the detection wavelength was 203nm, and the mobile phase was acetonitrile-0.1% phosphoric acid water (88:12). When determining amygdalin, the column temperature was 30℃, and the flow rate was 0.8 mL/min, and the injection volume was 10μL, and the detection wavelength was 203nm, and the mobile phase was acetonitrile-water (80:20).

2.2.3 Preparation and calibration of standard solutions

Linoleic acid, oleic acid and amygdalin were accurately weighed 4.0607mg, 25.8510mg, and 6.4001 mg respectively, and the volume was fixed into 5 mL volumetric flasks. The concentrations of the reference substance stock solution were respectively 812.14, 5170.2, and 1280.00 μg/mL. The stock solutions of the reference substance of oleic acid, linoleic acid, and amygdalin were diluted 2 times in sequence, and the concentration of linoleic acid standard solution were 812.14, 406.07, 203.035, 101.5175, 50.75875 μg/mL; the concentration of oleic acid were 5170.2, 2585.1, 1292.55, 646.275, and 323.1375 μg/mL; the concentration of amygdalin were 1280.00, 640.00, 320.00, 160.00, 80.00 μg/mL. 10μL of sample was taken to determine the peak area, and draw the standard curve equation of each component.

2.2.4 Sample preparation

Desheng brand oil press was used to squeeze the seeds at 40°C, and the best extraction conditions were obtained through experiments on different temperatures, different solvents, different extraction times, different volumes, and different extraction methods. Based on the above research results, the preparation method of the test solution of oleic acid and linoleic acid was to weigh 25 g of the kernel of *P. mira*, and extract the fat-soluble components of the kernel of *P. mira* at 40°C. The calculated oil yield was 38.08%. The method was to accurately weigh 0.5 g of the obtained components, place it in a round bottom flask, add 20 mL of 0.5 mol/L potassium hydroxide ethanol solution, weigh, reflux in a water bath for 30 min, cool, and add 50 μL of phenolphthalein, 1 mol/L hydrochloric acid solution until the red color just fades away, transfer the solution to a 50 mL

volumetric flask, and dilute to volume with ethanol. 1 mL was accurately measured and placed in a 5 mL measuring flask, the volume of ethanol was constant, and it was passed through a 0.45 μm microporous membrane.

The preparation method of the test sample of amygdalin was to accurately weigh 0.5 g of the kernel of *P. mira*, add 25 mL of petroleum ether, ultrasonic (200W, 40 KHz) for 30 minutes, and filter out the petroleum ether, add 25 mL of methanol solution, ultrasonically extract for 30 min, filter, transfer the filtrate to a 25 mL volumetric flask, make constant volume of methanol, and pass through a 0.45 μm microporous membrane.

2.3 Screening of the effective components in the treatment of two depilatory model animals with chemical components in P. mira

2.3.1 Animals and test sites

430 KM mice (SPF grade, 20 ± 2 g, male) and 170 C57BL/6 mice (SPF grade6 weeks old, 20 ± 2 g, male and female) were provided by Chengdu Dashuo Experimental Animal Co., Ltd. The experimental animal production license number were SCXK (Sichuan Province) 2015-030 and SCXK (Sichuan Province) 2015-030, and the numbers of experimental animal quality certificate were 51203500006767 and 51203500009764. All animals were raised in the Science and Technology Building of Chengdu University of Traditional Chinese Medicine. Experiments were carried out in the Ethnomedicine Resource Evaluation Laboratory of Chengdu University of Traditional Chinese Medicine (the third-level scientific research laboratory of the State Administration of Traditional Chinese Medicine, №TCM-2009-320).The above-mentioned experimental animals had been reviewed and approved by the Experimental Animal Ethics Committee of Chengdu University of Traditional Chinese Medicine.

2.3.2 Preparation of experimental samples

Vitamin E soft capsules (batch number 10891003) were purchased from Xingsha Pharmaceutical (Xiamen) Co., Ltd, with National Medicine Standard H35020242. β-sitosterol (batch number AF8102901), trans-squalene (batch number AF8091427), oleic acid (batch number AF8062708), campesterol (batch number AF8112891), fucosterol (batch number AF8112891), and amygdalin (batch number AF8051847) were purchased from Chengdu Alpha Biotechnology Co., Ltd.. Linoleic acid (batch number Y-096-170426) was purchased from Chengdu Pusi Biotechnology Co., Ltd., Minoxidil liniment (batch number 1805 – 932) was purchased from Sichuan Meidakang Huakang Pharmaceutical Co., Ltd., with National Medicine Standard H20052642, as a positive control drug [35].

Clinically, the concentration of minoxidil liniment is 20 mg/mL, once a day, 0.1 mL each time, the area of hair removal is 4 cm^2, and the dosage is 0.5 $mg/cm^2/d$. The dose setting method of the test substance is the same as above. The method is through consulting literature [33] and previous experiments. Peanut oil is used as a solvent for drugs with less polarity, and physiological saline is used for drugs with greater polarity. Pre-experiments have been conducted on peanut oil and physiological saline. Peanut oil and physiological saline as solvents do not affect the efficacy. Through reviewing literature [36] and previous experiments, peanut oil is used as a solvent for drugs with less polarity, and physiological saline is used for drugs with greater polarity, and preliminary experiments had been conducted on peanut oil and saline, as solvents do not affect the efficacy. The corresponding medicine w accurately weighed and placed in a bottle, and the solvent is added and shaken with a vortex instrument until it is completely dissolved or evenly dispersed in the solvent. The prepared medicine is stored in a refrigerator at 4°C.

2.3.3 Preliminary screening of the dose-effect relationship of 8 components in the kernel of P. mira in the treatment of KM mice after depilation induced by sodium sulfide sodium

430 KM mice were divided into 43 groups randomly after stratification by body weight, including blank control group, model control group, minoxidil control group and 8 different test drug groups; each test drug was set 5 doses. There were 10 animals in each group, reared in 2 cages. Since 430 KM mice are needed for 8 components, and the number of animals was large, it was carried out in batches. Each batch of experiments involved each drug and subgroup of animals. The mice in the blank control group only shaved their back hair of about 2×2 cm, the other groups first shaved the back hair of the same area as the mice in the blank control group, and then evenly spreaded 6% Na_2S on the area, and washed off after about 2 minutes, and selected mice with smooth and undamaged skin for follow-up experiments [37]. 24 hours after making the model, the drug solution was evenly applied to the depilatory area, and was allowed to absorb. The mice were observed to prevent them from licking their backs. After the drug solution dries, they were returned to the cage. Once a day, 0.1 mL each time, for 7 consecutive days, before each administration, the back area was washed with physiological saline and didn't receive any drug before it dried up [38]. On the second, fourth, and sixth days, the depilated parts of the mice were graded according to the standard [39], as shown in Table 2. 24 hours after the last administration, 5 hairs were plucked to measure the hair length of the mice in the middle of the administration area, and the average hair length was measured (cm) [40]. After the animal was sacrificed, the same position was used by a 7 mm punch to take a piece of skin, and all the hairs on the skin were scraped off and weighed[41].

Table 2
Rating standard table of KM mice and C57BL/6 mice newborn hair condition

Rating	Standard(KM mice)	Standard(C57BL/6 mice)
	Hairless growth	The skin of the administration area is pink
	Shallow hair overgrown depilated area	The skin in the depilatory area is gray
	The length and density of new hair is about one-half of the unhaired area	The skin in the epilation area is black
	No difference between newborn hair growth and unhaired areas	Hair grows in the depilatory area

2.3.4 To verify the effect of five active components in the kernel of P. mira on C57BL/6 mice after depilation induced by sodium

170 C57BL/6 mice were randomly divided into 17 groups after stratified by body weight. There were 10 animals in each group, reared in 2 cages. According to the literature [42] and preliminary experiments, it was found that C57BL/6 mice needed external stimulation such as plucking and sodium sulfide to enter the growth phase from the resting phase to the hair follicles. Therefore, all experimental mice needed to be treated with Na_2S. There was not established blank control group in this experiment. Because not all 170 mice were in the resting phase (the skin is pink), only mice in the resting phase could be modeled and administered. Considering the operability of the experiment, the mice were tested in batches. Each batch of experiment involved each drug and subgroup of animals. The time when the skin color changed from pink to black was recorded, and rated and photographed according to the standard on the 7, 14 and 21 days. The grading standards were shown in Table 2. Since the C57BL/6 mice gradually developed hair growth after 7 days of administration, the hair length was measured on the 14th and 21st days. The method was the same as the above, and the new hair weight was measured as above. 0.5cm^2C57BL/6 mice back skin tissues of each group were taken for subsequent HE, immunohistochemistry and RT-PCR detection.

2.4 To study the mechanism of the three active components in the kernel of P. mira in the treatment of C57BL/6 mice depilation model induced by sodium sulfide

2.4.1 Grouping and general indicators

Through comprehensive analysis of the results of skin color change time, hair growth status, hair length and weight of C57BL/6 mice, *β*-sitosterol group 2(0.061 mg/cm^2/d) and the second group of linoleic acid group 2(0.156 mg/cm^2/d), vitamin E group 2 (3.125 mg/cm^2/d) had a better effect on promoting hair growth, so one dose group for each of the 3 components was selected for the mechanism of action.

2.4.2 Skin histological observation

On the 22nd day, the skin of the administration area of C57BL/6 mice was fixed, and the inspection and image collection were performed according to the pathological examination SOP procedure. The number of primary hair follicles, the number of secondary hair follicles, the total number of hair follicles and the thickness of the dermis were observed and counted, and the average value was calculated [43].

2.4.3 RT-PCR detection of mRNA expression of four targets in the skin Wnt/β-catenin pathway

On the 22nd day, the skin of the depilated area on the back of the C57BL/6 mice was taken, and total RNA was extracted from the dorsal skin tissue using the RNA extraction kit (RE-03014, Forgene Biotechnology Co., Ltd.). Total RNA was reverse-transcribed using the PrimeScript RT reagent kit (RR047A; Bio-Biology, Dalian, China) according to the manufacturer's recommendations. The reagents were added to the kit in sequence at a temperature of 42°C for 2 min, the genomic DNA removal reaction was shown in Table 3, and the reverse transcription reaction was shown in Table 4 on the PCR machine. The full sequence of the gene was searched in the NCBI database. The Primer Premier primer design software was designed to screen specific primers for each gene and purify them by ULTRAPAGE, as shown in Table 5. The real-time fluorescence quantitative PCR reaction was shown in Table 6–7. The *β*-actin of the sample skin tissue and the dissolution curves of the four indicators were all single peaks, which indicating that the amplified product was the target product of the corresponding gene, and there were no primer dimers and non-specific products, as shown in Fig. 2a. The amplification curves of the tested genes were smooth and all could enter the plateau phase, which indicating that the PCR reaction system and reaction program were set reasonably, as shown in Fig. 2b. The CT value of each test sample in the PCR process was analyzed, and the relative mRNA expression of X was calculated by $2^{-\triangle\triangle CT}$.

Table 3
Genomic DNA removal reaction system

Reagent	Volume (μL)
5×gDNA Eraser Buffer	2
gDNA Eraser	1
Total RNA	2
RNase Free dH_2O	5
Total	10
Note: After adding each reagent in sequence, 42°C, 2 min.	

Table 4
Reverse transcription reaction system

Reagent	Volume(μL)
5×PrimeScript Buffer 2	4
PrimeScript RT Enzyme Mix I	1
RT Primer Mix	1
RNA	10
RNase Free dH_2O	4
Total	20
Note: Add the reagents one by one and put them on the PCR machine for reaction.	

Table 5
Primers and base sequences used in detection

Primer name	Upstream	Downstream	Citation length, bp
β-actin	gaagatcaagatcattgctcc	tactcctgcttgctgatcca	111
Cyclin D 1	ccagaggcggatgagaacaagcagac	tgtgcggtagcaggagaggaagttgt	183
GSK3β	acagtggtgtggatcagttggtggaa	ccagaggcggatgagaacaagcagac	153
LEF 1	caacgggcatgaggtggtcagacaag	agtgctcgtcgctgtaggtgatgagg	293
β-actin	gaagatcaagatcattgctcc	tactcctgcttgctgatcca	296

Table 6
PCR reaction system

Reagent	Volume(μL)
2×Real PCR EasyTM Mix-SYBR	10.0
Forward Primer(10μM)	0.8
Reverse Primer(10μM)	0.8
Template(DNA)	2.0
ddH_2O	6.4
Total	20.0

Table 7
PCR reaction program

Reaction temperature	Time	Remarks
95°C	30s	Predenaturation
95°C	5s	transsexual
55°C	30s	annealing
72°C	30s	Fully extend to collect fluorescence
Note: 45 cycles.		

2.4.4 Immunohistochemical method to detect the protein expression of four targets in the skin Wnt/β-catenin pathway

On the 22nd day, the skin of the depilated area on the back of the mice was fixed. After pretreatment, the blocking solution was added dropwise at room temperature for 20 minutes; then the mice GSK-3β antibody (1:200), rabbit β-catenin antibody (1:300), rabbit Cyclin D 1 antibody (1:50), and rabbit LEF 1 antibody (1:100) were added in it, rinsed, overnight at 4°C; The treated skin was then dripped with biotinylated goat anti-rabbit/mouse antibody, incubated at 37°C for 30 min; washed 3 times with PBS; developed at room temperature, counterstained, mounted, and finally checked and calculated the optical density.

2.5 Statistical methods

The measurement data were compared between groups by "independent sample T test" in SPSS 17.0 software, which was expressed by "mean ± standard deviation ()". The data was analyzed by the Mann-Whitney test in SPSS 17.0 software for comparison between groups. A single asterisk ($*$, $p \leq 0.05$) indicates that there is a statistical difference between the averages, and a double asterisk ($**$, $p \leq 0.01$) indicates that there is a more significant difference between the averages.

2.6 Principal component analysis method

SIMCA-P + software (Version 14.1) was used to perform principal component analysis. A total of 16 measurement indicators of the hair loss model (total number of hair follicles, number of primary hair follicles, number of secondary hair follicles, dermal thickness, Cyclin D 1, β-catenin, GSK-3β, LEF 1 mRNA and protein expression) were included, involving 5 groups, which are model control group, minoxidil control group, β-sitosterol group 2, vitamin E group 2, and linoleic acid group 2.

3. Results

3.1 Network Pharmacology

According to the literature, 17 chemical constituents of the kernel of *P. mira* were obtained, that included Amygdalin, Beta-sitosterol, Cyclododecene, Glyceryl monooleate, Methyl oleate, Lauric acid, Oleic acid, Stearic acid, Vitamin E, Arachidic acid, Campesterol, Fucosterol, Methyl linoleate, Myristic acid, Linoleic acid, Squalene, Trans-Squalene. The relevant targets of the components were searched through the Pharmmapper database. After the duplicate was deleted, the final corresponding targets totaled 5,085, and the targets with a score greater than 0.6 were selected. After the duplicate was deleted, 216 targets were obtained. The GeneCards library was searched to obtain 9640 disease targets. After matching the two related targets, a total of 149 potential targets for the treatment of alopecia diseases were obtained and the Venn diagram was drawn, as shown in Fig. 3 and Table 8. The network diagram of "the kernel of *P. mira*-components-Target-Disease" established by Cytoscape software was shown in Fig. 4. The network included 168 nodes and 842 edges. The relationship between the kernel of *P. mira*, chemical composition, potential target and hair loss was demonstrated by different colors and shapes. Then the "protein-protein" network topology was analyzed, and the PPI network was constructed using String website and Cytoscape, as shown in Fig. 5. If the circle in the figure is the greater, the gene is more important. The degree of freedom was used as the selection condition for the Hub gene. If the degree of freedom is the greater, the greater possibility is that the kernel of *P. mira* will function through the target gene. The top 30 degrees of freedom are regarded as the Hub gene, as shown in Fig. 6. The biological process and molecular function analysis of targets of the kernel of *P. mira* are carried out, and the first 20 processes with the least significance are selected, as shown in Fig. 7a. Through GO analysis, the following reasons may be related to alopecia, such as heme binding, tetrapyrrole binding, nuclear receptor activity, transcription factor activity, steroid hormone receptor, S100 protein binding, damaged DNA binding, hydrolase activity, vitamins Binding, fatty acid derivative binding, antioxidant activity, etc. KEGG analysis was performed on 149 potential targets and a total of 89 signal pathways were obtained, of which 12 pathways were related to alopecia, as shown in Fig. 7b. Among them, thyroid hormone pathway (hsa04919) and HIF-1 pathway (hsa04066) were related to angiogenesis. Rap1 pathway (hsa04015) and AGE-RAGE pathway (hsa04933) were related to inflammation. The p53 pathway (hsa04115) and FoxO pathway (hsa04068) were related to death. PI3K-Akt pathway (hsa04151), Wnt pathway (hsa04310) and TGF-beta pathway (hsa04350) were related to cell proliferation and differentiation. Estrogen pathway (hsa04915), neurotrophin pathway (hsa04772); cAMP pathway (hsa04024) was related to lipid metabolism. From Fig. 8, it was found that 25 of the 149 potential targets were related to these signal pathways. Among them, CREBBP, EIF4E, SOD2,

CYCS, etc., had greater degrees of freedom, and the 25 targets were mainly derived from 13 components. According to the results of previous research and literature review, it was found that stimulating the Wnt signaling pathway could regulate the growth of hair follicles [44]. In this paper, the Wnt signaling pathway was selected as the main research object, and five related targets were found, namely CREBBP 1, CCND1, and CCND1. RAC1, CUL1 and EP300, meanwhile 13 chemical components related to these 5 targets, such as transsqualene, VE, fucosterol, linoleic acid, oleic acid, campesterol, etc. It was suggested that it may be a potential effective ingredient of *P. mira* to promote hair growth, but the results of network pharmacology still need further experimental verification.

Table 8
Potential targets of the kernel of *P. mira* in the treatment of alopecia

Gene Name	Gene Name	Gene Name	Gene Name	Gene Name	Gene Name
ABO	ACADVL	ACHE	ALAD	ALOX12	APIP
AR	ARHGAP11	ARHGEF12	ARL2	AZGP1	B2M
CALmL3	CAMK4	CAPN2	CAPN9	CCND1	CCNE1
CD1B	CD2	CD8A	CDCA8	CDKN1B	CEL
CES1	CETN2	CMAS	CREBBP	CUL1	CYB5A
CYB5B	CYB5R3	CYCS	CYP2E1	DDC	DGCR8
DHPS	DLG1	DRAP1	E2F1	ECHS1	EDC3
EEF1A1	EGF	EIF2AK2	EIF4A1	EIF4E	EP300
ESR2	ETFB	EZR	F2	FLOT2	FUT8
GAD1	GC	GCDH	GLB1	GLYCTK	GOT2
GSS	GSTA1	GYG1	HBA2	HBB	HEXA
HMOX1	HSD17B11	HSD17B4	HSP90AB1	KCNIP3	LAP3
LDHC	LIG1	LIG4	LTF	MAGEA4	MAGI2
MAN2A1	MAN2B1	MB	MDM2	MEX3D	MMP2
MOG	MRI1	MTAP	MUC1	NGLY1	NPL
NR1I3	NUCB1	OGG1	P4HB	PAPOLA	PEBP1
PFN2	PHGDH	PMM1	POLRMT	POT1	PPP1R8
PPP2R1A	PRPS1	PTGS1	PTPRJ	PTPRN	RAB5C
RAB7A	RAC1	RANBP2	RARG	RBP4	RGS18
RGS6	RHO	RPS3	RRM1	RUNX1T1	RXRB
RXRG	S100A11	S100A6	S100A8	S100B	SCO2
SDHA	SF1	SIN3A	SMS	SOD2	SRP54
SUB1	SUOX	TCEA3	THBS1	THRB	TJP1
TOX	TRPV6	TUFM	TXNRD1	UNG	USF1
USH1C	USP14	USP19	USP8	VCP	—

3.2 HPLC analysis

3.2.1 Optimization of chromatographic separation conditions

The content of oleic acid, linoleic acid and amygdalin were determined by HPLC, and different detection wavelengths (203, 210, 220, 230, 242 nm), mobile phase (oleic acid and linoleic acid were Acetonitrile-pure water, 0.1% phosphoric acid water, 0.2% phosphoric acid water; amygdalin is acetonitrile: water = 30:70, 20:80, 10:90), column temperature (25℃, 30℃ and 35℃) and flow rate (0.8, 1.0, 1.2 mL/min), according to the peak resolution, peak shape, peak area, etc., the best chromatographic conditions were determined. In the preparation of the test product, the following conditions were considered to ensure the best test conditions, such as the extraction method (cold soaking for 12 h, heating and refluxing for 30 minutes, and ultrasonic for 30 minutes), extraction solvent (oleic acid and linoleic acid were 0.5, 1.0, 1.5 mol/L potassium hydroxide ethanol solution;

amygdalin were 50% methanol, 70% methanol, methanol solution), extraction volume (oleic acid and linoleic acid were 10, 20, 30 mL; amygdalin were 5, 15, 25 mL), extraction time (15, 30, 60 min).

3.2.2 Quantitative analysis of oleic acid, linoleic acid and amygdalin in the kernel of *P. mira*

The linear relationship of oleic acid, linoleic acid and amygdalin is good. The measured oleic acid was323.14 ~ 5170.20 μg/mL, linoleic acid was 50.76 ~ 812.14 μg/mL, and amygdalin was 80.00 ~ 1280.00 μg/mL. The methodological investigation RSD% was less than 3%, see Fig. 9 and Table 9. The content of oleic acid and linoleic acid in the kernel of *P. mira* was relatively high, and the content of oleic acid was close to 4 times the content of linoleic acid. In 12 batches, kernels of *P. mira* in Xingduo Village, Nixi Township, Shangri-La City, The content of linoleic acid was significantly lower than that of other producing areas. The content of amygdalin in Xinlong County, Jiulong County and Kangding City was significantly higher than that of other producing areas. The contents of oleic acid, linoleic acid and amygdalin in the kernel of *P. mira* from other producing areas had little difference (see Table 10, Fig. 10 and Fig. 11)

Table 9
Linear regression equation of 3 chemical components in the kernel of *P. mira*

chemical component	Linear regression equation	Correlation coefficient (r)	Linear range(μg/mL)	Precision (RSD)	Repeatability (RSD)	Stability (RSD)	Average value of sample recovery	Sample recovery rate(RSD)
Oleic acid	y = 968.03x + 122110	0.9991	323.14-5170.20	0.10%	2.78%	0.14%	96.95%	2.22%
Linoleic acid	y = 10472x + 183930	0.9993	50.7587–812.14	0.10%	2.57%	0.09%	96.83%	2.86%
Amygdalin	y = 10749x + 1018982	0.9993	80.00-1280.00	2.60%	2.40%	2.8%	95.04%	1.60%

Table 10
Contents of 3 chemical components in the kernels of *P. mira* (n = 3)

Origin	Altitude (m)	longitude	latitude	Oil yield (%)	Content(mg/g)		
					Oleic acid	Linoleic acid	Amygdalin
Xulong Township, Derong County	2935	99°13′7005″	28°74′1808″	38.08	442.30	107.58	14.81
Zhongza Town, Batang County	2929	99°19′1089″	29°21′2186″	37.89	423.16	130.52	11.00
Bajiaolou Township, Yajiang County	2719	101°06′1872″	30°06′0709″	37.94	449.63	91.58	15.14
Chitu Township, Daocheng County	3164	100°16′1899″	28°37′3726″	38.13	537.88	103.67	16.18
Malkang Forestry Bureau	2630	102°13′4203″	31°55′2159″	38.11	482.09	90.54	13.86
Xinlong County	3066	100°31′1368″	30°93′9169″	38.04	390.13	92.72	25.85
Zhengdou Township, Xiangcheng County	2750	99°31′2148″	29°05′4042″	37.53	420.97	112.24	11.09
Wachang Town, Muli County	2577	100°50′2772″	28°09′4138″	38.23	419.74	84.35	15.09
Pusharong Township, Kangding City	2922	101°19′1446″	29°32′1447″	38.05	481.71	102.82	19.61
Jiaer Town, Jiulong County	2823	101°30′3892″	28°59′1512″	38.04	434.97	115.48	21.19
Nixi Township, Shangri-La City*	3135	99°50′6456″	28°04′7398″	37.87	187.54	47.70	12.14
Benzilan Town, Deqin County*	2220	99°16′4288″	28°14′2346″	37.94	476.76	119.48	13.55

Note: Those marked with * are areas under the jurisdiction of Yunnan Province, China, and the others are areas under the jurisdiction of Sichuan Province, China.

3.3 The dose-effect relationship of 8 chemical components in the kernel of P. mira to promote hair growth

KM mice which depilation induced by sodium sulfide was used to evaluate the efficacy of eight components (*β*-sitosterol, linoleic acid, vitamin E, oleic acid, trans-squalene, campesterol, fucosterol, and amygdalin) of the kernel of *P. mira*. It was showed that *β*-sitosterol groups 2, 3, 4, and 5, vitamin E groups 1, 2, linoleic acid group 2, fucosterol group 3 and amygdalin group 1, 3 could increase the grade of newborn hair condition of mice on the 4th day. Amygdalin group 1 and fucosterol group 3 could increase the hair length of mice. Vitamin E groups 1, 2, 3 and *β*-sitosterol group 2 could significantly increase the weight of new hair, and see Table 11 and Fig. 12 for details. After considering the above results comprehensively, it could be considered that vitamin E, *β*-sitosterol, linoleic acid, amygdalin, and fucosterol have obvious promoting effects. Therefore, three doses of these five components were selected for subsequent sodium sulfide-inducedhair loss model in C57BL/6 mice for re-evaluation of the efficacy, namely *β*-sitosterol groups 2, 3, and 4 (0.061, 0.031, 0.016 mg/cm^2/d), linoleic acid groups 1, 2, and 3 (0.313, 0.156, 0.078 mg/cm^2/d), vitamin E groups 1, 2, and 3 (6.125, 3.125, 1.563 mg/cm^2/d), amygdalin groups 2, 3, and 4 (0.061, 0.031, 0.016 mg/ cm^2/d), fucosterol groups 2, 3 and 4 (0.061, 0.031, 0.016 mg/cm^2/d).

Table 11
Newborn hair growth rating, the newborn hair length and hair weight in KM mice (n = 10)

Group	Dose (mg/cm2/d)	Newborn hair growth rating															Newborn hair length(mm)	Newborn hair weight(mg)
		Day 2					Day 4					Day 6						
		I	II	III	IV	P	I	II	III	IV	P	I	II	III	IV	P		
Blank group	—	5	5	0	0	0.660	0	3	7	0	0.024*	0	0	3	7	0.129*	6.72 ± 0.53**	2.82 ± 1.26**
Model control group	—	7	3	0	0	—	0	8	2	0	—	0	2	4	4	—	3.73 ± 1.7	1.33 ± 0.59
Minoxidi control group	0.500	7	3	0	0	1.000	0	2	5	3	0.003**	0	0	3	7	0.129	4.63 ± 0.85	2.03 ± 0.51*
β - sitosterol group 1	0.123	8	2	0	0	0.016*	3	4	3	0	0.113	1	3	5	1	0.129	3.64 ± 1.70	1.86 ± 0.74
β - sitosterol group 2	0.061	9	1	0	0	0.001*	0	4	6	0	0.044*	0	4	4	2	0.227	3.91 ± 1.95	2.38 ± 1.27*
β - sitosterol group 3	0.031	8	2	0	0	0.016*	0	7	3	0	0.628*	0	2	6	2	0.262	3.51 ± 0.55	1.95 ± 0.98
β - sitosterol group 4	0.016	7	3	0	0	1.000	0	7	3	0	0.628*	0	3	6	1	0.227	3.23 ± 1.42	1.49 ± 0.75
β - sitosterol group 5	0.008	7	3	0	0	1.000	1	2	6	1	0.063*	1	1	3	5	0.815	4.02 ± 1.83	2.20 ± 1.36
linoleic acid group 1	0.313	2	8	0	0	0.064	1	6	3	0	0.928	2	3	3	2	0.137	3.35 ± 2.69	1.64 ± 1.42
linoleic acid group 2	0.156	0	7	3	0	0.021*	0	2	8	0	0.005**	0	2	2	6	0.525	5.33 ± 1.69	1.82 ± 1.42
linoleic acid group 3	0.078	1	7	2	0	0.003**	1	3	6	0	0.177	1	1	4	4	0.380	4.28 ± 2.11	1.64 ± 1.11
linoleic acid group 4	0.039	3	7	0	0	0.081	1	4	5	0	0.350	1	2	3	4	0.758	3.97 ± 12.33	1.26 ± 0.71
linoleic acid group 5	0.020	2	5	3	0	0.181	1	2	7	0	0.060	1	2	0	7	0.137	4.36 ± 2.41	1.38 ± 0.81
vitamin E group 1	6.250	7	3	0	0	1.000	0	3	7	0	0.024*	0	2	5	3	0.754	4.33 ± 2.03	2.50 ± 1.21*
vitamin E group 2	3.125	7	3	0	0	1.000	0	4	6	0	0.044*	0	2	4	4	1.000	4.28 ± 1.66	2.56 ± 0.97**
vitamin E group 3	1.563	6	4	0	0	0.673	0	7	3	0	0.628	0	3	4	3	0.596	3.62 ± 1.55	2.59 ± 1.03**
vitamin E group 4	0.781	6	4	0	0	0.673	0	7	3	0	0.628	0	4	3	3	0.475	2.92 ± 2.38	1.66 ± 1.02
vitamin E group 5	0.391	8	2	0	0	0.660	2	4	3	1	0.805	1	4	1	4	0.439	3.52 ± 2.12	1.90 ± 1.33
oleic acid gruop 1	0.00055	3	7	0	0	0.081	2	1	5	2	0.132	2	0	0	8	0.240	4.65 ± 2.34	2.05 ± 1.08
oleic acid gruop 2	0.00028	1	9	0	0	0.058	1	1	5	3	0.051	1	1	5	3	0.686	4.70 ± 2.39	2.14 ± 1.32
oleic acid gruop 3	0.00015	3	7	0	0	0.081	4	3	3	0	0.375	2	4	0	4	0.279	3.90 ± 2.20	1.91 ± 1.35
oleic acid gruop 4	0.00008	3	7	0	0	0.081	1	2	4	3	0.054	3	1	1	5	0.700	3.67 ± 2.25	2.30 ± 1.32
oleic acid gruop 5	0.00004	8	2	0	0	0.897	7	1	2	0	0.022*	5	3	2	0	0.001**	2.58 ± 2.36	1.66 ± 1.55

Note: Compared with the model control group, *$P \leq 0.05$, **$P \leq 0.01$.

Group	Dose (mg/cm2/d)	Newborn hair growth rating															Newborn hair length(mm)	Newborn hair weight(mg)
		Day 2					Day 4					Day 6						
		I	II	III	IV	P	I	II	III	IV	P	I	II	III	IV	P		
Trans squalene group 1	0.123	7	3	0	0	1.000	6	3	1	0	0.013*	6	2	2	0	0.002**	1.52 ± 1.73**	0.90 ± 1.10*
Trans squalene group 2	0.061	2	8	0	0	0.064	1	5	2	2	0.493	0	2	4	4	1.000	4.03 ± 2.06	1.72 ± 0.77
Trans squalene group 3	0.031	3	7	0	0	0.081	2	2	4	2	0.267	2	0	3	5	0.876	4.97 ± 2.34	2.27 ± 1.26
Trans squalene group 4	0.016	1	6	3	0	0.840	1	2	4	3	0.054	1	2	1	6	0.753	4.88 ± 1.44	3.03 ± 1.85
Trans squalene group 5	0.008	2	8	0	0	0.064	0	4	3	3	0.050	0	2	4	4	1.000	4.47 ± 1.38	2.29 ± 1.54
Campesterol gruop 1	0.060	3	7	0	0	0.081	2	2	6	0	0.330	2	2	1	5	0.758	3.40 ± 2.75	1.68 ± 1.59
Campesterol gruop 2	0.030	4	6	0	0	0.196	3	2	5	0	0.812	3	0	4	3	0.438	2.96 ± 2.48	1.86 ± 0.77
Campesterol gruop 3	0.015	1	6	3	0	0.840	1	5	3	1	0.546	1	2	3	4	0.758	3.72 ± 2.45	1.42 ± 0.86
Campesterol gruop 4	0.008	3	6	1	0	0.840	2	4	4	0	0.868	2	2	1	5	0.758	3.68 ± 2.89	1.15 ± 0.97
Campesterol gruop 5	0.004	1	7	2	0	0.053	1	3	6	0	0.177	1	2	2	5	1.000	4.14 ± 2.04	1.97 ± 0.97
Fucosterol group 1	0.123	2	5	3	0	0.053	2	2	3	3	0.237	1	1	3	5	0.815	4.32 ± 2.20	2.88 ± 1.86
Fucosterol group 2	0.061	7	2	1	0	0.892	2	4	4	0	0.868	2	0	2	6	0.049*	3.53 ± 2.38	2.29 ± 1.53
Fucosterol group 3	0.031	1	4	5	0	0.011	0	2	4	4	0.002**	0	0	4	6	0.037*	5.14 ± 1.21*	2.83 ± 0.99
Fucosterol group 4	0.016	5	5	0	0	0.412	3	2	5	0	0.812	1	3	3	3	0.395	4.43 ± 1.56	2.59 ± 1.80
Fucosterol group 5	0.008	2	5	3	0	0.053	1	3	4	2	0.138	0	3	1	6	0.693	4.58 ± 1.70	2.64 ± 1.21
Amygdalin group 1	0.123	0	6	4	0	0.055	0	2	4	4	0.014*	0	2	1	7	0.034*	5.58 ± 1.63*	2.29 ± 0.74
Amygdalin group 2	0.061	7	1	2	0	0.384	1	2	4	3	0.054	1	1	3	5	0.815	5.05 ± 2.00	3.46 ± 1.80
Amygdalin group 3	0.031	2	6	2	0	0.376	0	3	3	4	0.031*	0	0	3	7	0.029*	5.04 ± 1.41	2.11 ± 0.97
Amygdalin group 4	0.016	0	8	2	0	0.064	0	5	5	0	0.178	0	2	3	5	0.754	4.43 ± 1.56	2.24 ± 1.28
Amygdalin group 5	0.008	4	4	2	0	0.129	2	2	5	1	0.298	2	1	4	3	0.486	3.78 ± 2.16	1.67 ± 1.23

Note: Compared with the model control group, *$P \leq 0.05$, **$P \leq 0.01$.

3.4 Re-evaluating of the effect of 5 active components in the kernel of P. mira in promoting hair growth

C57BL/6 mice were used to re-evaluate the effectiveness of the 5 active components in the kernels of *P. mira*. *β*-sitosterol groups 2 and 4, linoleic acid group 2 and vitamin E group 2 could significantly shorten the time for the skin to darken, increased the status of new hair, hair length and weight. In addition, *β*-sitosterol group 3, linoleic acid group 1, vitamin E group 3, amygdalin group 2, and fucosterol group 2 could significantly increase the hair length and hair weight of mice on day 14. See Table 12, Table 13. The use of heat maps could show the differences between the data more intuitively. The 17 groups (model control group, positive control group, *β*-sitosterol group 2, 3, 4, linoleic acid group 1, 2, 3, Vitamin E Group 1, 2, 3, fucosterol Group 2, 3, 4, amygdalin Group 2, 3, 4) of 7 efficacy index data (skin darkening time, 7th, 14th, 21st days newborn hair growth rating, 14th, 21st day hair length, hair weight) were normalized to draw a heat map. As shown in Fig. 13, *β*-sitosterol, linoleic acid and vitamin E had a significant promoting effect on mice hair loss induced by sodium sulfide. According to the statistical results of the number of days of skin darkening, the status of new hair, the length of new hair, the weight of new hair, and the results of heat map analysis, it was shown that the three dose groups of *β*-sitosterol, vitamin E, and linoleic acid could make the hair follicles grow in advance, indicating that it had a certain promoting effect on hair growth. Therefore, *β*-sitosterol group 2 (0.061 mg/cm^2/d), linoleic acid group 2 (0.156 mg/cm^2/d) and vitamin E group 2 (3.125 mg/cm^2/d) were selected for observing the effect of drugs on the number of hair follicles, dermal thickness and Wnt/β-catenin pathway.

Table 12
Effects of 5 effective components in the kernel of *P. mira* on the newborn hair growth rating of C57BL/6 mice depilation model induced by sodium sulfide

Gruop	Dose (mg/cm^2/d)	Skin blackening time(day)	Newborn hair growth rating														
			Day 7					Day 14					Day 21				
			I	II	III	IV	P	I	II	III	IV	P	I	II	III	IV	P
Model control group	—	11.70 ± 3.64	8	2	0	0	—	1	1	4	4	—	0	1	2	7	—
Minoxidi control group	0.500	8.70 ± 1.70*	0	9	1	0	P ≤ 0.01	0	0	0	10	*P ≤ 0.05*	0	0	0	10	P ≥ 0.05
β - sitosterol group 2	0.061	8.80 ± 2.04*	3	5	2	0	P ≤ 0.01	0	0	1	9	*P ≤ 0.05*	0	0	0	10	P ≥ 0.05
β - sitosterol group 3	0.031	9.10 ± 1.73	2	8	0	0	P ≤ 0.01	0	0	2	8	P ≥ 0.05	0	0	0	10	P ≥ 0.05
β - sitosterol group 4	0.016	8.80 ± 1.03*	1	8	1	0	P ≤ 0.01	0	0	1	9	*P ≤ 0.05*	0	0	0	10	P ≥ 0.05
linoleic acid group 1	0.313	9.10 ± 1.52	3	6	1	0	P ≤ 0.01	0	0	2	8	P ≥ 0.05	0	0	0	10	P ≥ 0.05
linoleic acid group 2	0.156	8.70 ± 0.82*	2	8	0	0	P ≤ 0.01	0	0	1	9	*P ≤ 0.05*	0	0	0	10	P ≥ 0.05
linoleic acid group 3	0.078	9.10 ± 1.29	2	7	1	0	P ≤ 0.01	0	1	3	6	P ≥ 0.05	0	0	0	10	P ≥ 0.05
vitamin E group 1	0.313	10.20 ± 2.62	3	7	0	0	P ≤ 0.01	0	2	1	7	P ≥ 0.05	0	0	0	10	P ≥ 0.05
vitamin E group 2	0.156	8.50 ± 1.18*	2	7	1	0	P ≤ 0.01	0	0	1	9	*P ≤ 0.05*	0	0	0	10	P ≥ 0.05
vitamin E group 3	0.078	9.10 ± 1.79	3	7	0	0	P ≤ 0.01	0	1	1	8	P ≥ 0.05	0	0	0	10	P ≥ 0.05
Fucosterol group 2	0.061	9.10 ± 1.10*	1	9	0	0	P ≤ 0.01	0	1	3	6	P ≥ 0.05	0	0	0	10	P ≥ 0.05
Fucosterol group 3	0.031	10.20 ± 2.62	5	5	0	0	P ≥ 0.05	1	0	4	5	P ≥ 0.05	0	0	0	10	P ≥ 0.05
Fucosterol group 4	0.016	9.20 ± 2.49	2	7	1	0	P ≤ 0.01	0	1	2	7	P ≥ 0.05	0	0	0	10	P ≥ 0.05
Amygdalin group 2	0.061	9.70 ± 1.83	0	10	0	0	P ≤ 0.01	0	0	2	8	P ≥ 0.05	0	0	0	10	P ≥ 0.05
Amygdalin group 3	0.031	9.40 ± 1.43	3	7	0	0	P ≤ 0.01	0	1	2	7	P ≥ 0.05	0	0	1	9	P ≥ 0.05
Amygdalin group 4	0.016	9.20 ± 0.92	2	7	1	0	P ≤ 0.01	0	1	4	5	P ≥ 0.05	0	0	0	10	P ≥ 0.05

Note: Compared with the model control group, *$P \leq 0.05$, **$P \leq 0.01$.

Table 13
Effects of 5 active ingredients in the kernel of *P. mira* on the newborn hair length and weight of C57BL/6 mice depilation model induced by sodium sulfide

Group	Dose ($mg/cm^2/d$)	Number of animals(number)	Newborn hair length (Day 7, cm)	Newborn hair length (Day14, cm)	Newborn hair weight (g)
Model control group	—	10	0.138 ± 0.151	0.625 ± 0.186	0.00154 ± 0.00071
Minoxidi control group	0.500	10	0.420 ± 0.133**	0.816 ± 0.161**	0.00260 ± 0.00035**
β - sitosterol group 2	0.061	10	0.363 ± 0.203**	0.798 ± 0.116**	0.00284 ± 0.00044**
β - sitosterol group 3	0.031	10	0.336 ± 0.153**	0.740 ± 0.107	0.00258 ± 0.00037**
β - sitosterol group 4	0.016	10	0.373 ± 0.113**	0.786 ± 0.120**	0.00299 ± 0.00051**
linoleic acid group 1	0.313	10	0.296 ± 0.124**	0.693 ± 0.071	0.00277 ± 0.00037**
linoleic acid group 2	0.156	10	0.297 ± 0.152**	0.739 ± 0.152	0.00230 ± 0.00050**
linoleic acid group 3	0.078	10	0.238 ± 0.188	0.787 ± 0.138**	0.00243 ± 0.00053**
vitamin E group 1	0.313	10	0.238 ± 0.173	0.693 ± 0.156	0.00249 ± 0.00029**
vitamin E group 2	0.156	10	0.316 ± 0.167**	0.716 ± 0.131	0.00242 ± 0.00028**
vitamin E group 3	0.078	10	0.314 ± 0.064**	0.755 ± 0.065	0.00261 ± 0.00063**
Fucosterol group 2	0.061	10	0.296 ± 0.145**	0.762 ± 0.131	0.00260 ± 0.00050**
Fucosterol group 3	0.031	10	0.186 ± 0.173	0.735 ± 0.129	0.00272 ± 0.00056**
Fucosterol group 4	0.016	10	0.252 ± 0.155	0.747 ± 0.087	0.00257 ± 0.00041**
Amygdalin group 2	0.061	10	0.362 ± 0.101**	0.717 ± 0.093	0.00216 ± 0.00041**
Amygdalin group 3	0.031	10	0.201 ± 0.146	0.619 ± 0.182	0.00208 ± 0.00056**
Amygdalin group 4	0.016	10	0.208 ± 0.121	0.693 ± 0.100	0.00250 ± 0.00058
Note: Compared with the model control group, *$P \leq 0.05$, **$P \leq 0.01$.					

3.5 The mechanism of 3 active components in the kernel of P. mira to promote hair growth

Through comprehensive analysis of the results of skin color change time, newborn hair growth rating, newborn hair length and weight of C57BL/6 mice, *β*-sitosterol group 2(0.061 $mg/cm^2/d$) and linoleic acid group 2 (0.156 $mg/cm^2/d$), vitamin E group 2 (3.125 $mg/cm^2/d$) had a better effect on promoting hair growth (See Fig. 14), so one dose group for each of the 3 components was selected for the mechanism of action. The number of hair follicles and dermal thickness of the skin of the experimental area of the three groups of mice were tested in the three groups, and the effect of the three groups on the Wnt/β-catenin signaling pathway was studied, please see Table 14 and Fig. 15. There was no significant effect on the thickness of the dermis and the number of hair follicles, but the three groups can up-regulate the mRNA expression of LEF 1 and GSK-3β in the Wnt/β-catenin signaling pathway, and also have a significant up-regulation trend on the mRNA expression of β-catenin, seen as Table 15. All three groups had no significant effect on the protein expression of the four indicators, see Table 16 and Fig. 16.

In order to analysis of the control intensity of the mechanism of action, SIMCA-P (Version 14.1) software was used to perform principal component analysis (PCA-X) on a total of 16 indicators in the 5 groups, and a total of 5 principal components were extracted with R2X = 80.5%, which was the top 5 principal component could reflect 80.5% of the original data. The first two components were extracted to obtain Fig. 17a. From the Fig. 17a, it could be seen that the model control group and the positive control group were at both ends of the Y axis. The samples of the three administration groups are closer to the positive control group, indicating that the hair loss of the mice in the administration group has a tendency to improve. There was a trend of improvement. In addition, the groups in descending order of the area of the circle were β-sitosterol group 4, linoleic acid group 2, and vitamin E group 2.

The strength of the hair growth-promoting effect was also inversely proportional to the area of the circle. Principal component analysis found that the three groups have a certain improvement effect on the alopecia of mice caused by sodium sulfide, as shown in Fig. 17a and Fig. 17b.

Table 14
Effects of three effective components of the kernel of *P. mira* on the number of hair follicles and dermal thickness in the experiment of C57BL/6 mice depilation model induced by sodium sulfide

Group	Dose(mg/cm^2/d)	Number of animals(number)	Dermis thickness (nm)	Dermis thickness(number)	Secondary hair follicle (number)	Total number of hair follicles (number)
Model control group	—	10	360.15 ± 150.44	22.17 ± 12.25	36.33 ± 21.02	59.7 ± 29.26
Minoxidi control group	0.50	10	344.63 ± 137.09	20.10 ± 12.98	43.37 ± 31.69	63.47 ± 44.28
β - sitosterol group 2	0.061	10	345.24 ± 107.93	19.30 ± 11.15	41.17 ± 27.59	60.47 ± 38.15
linoleic acid group 2	0.156	10	381.06 ± 156.68	14.27 ± 10.18	24.83 ± 21.15	39.10 ± 32.96
vitamin E group 2	0.156	10	350.47 ± 150.87	20.63 ± 11.85	37.73 ± 22.60	58.37 ± 34.36

Table 15
Effects of three effective components of the kernel of *P. mira* on the mRNA expression of four targets in the Wnt/β-catenin signaling pathway in the skin tissue of C57BL/6 mice ($\bar{x}\pm s$)

Group	Dose (mg/cm^2/d)	Number of animals(number)	β-catenin	GSK3β	Cyclin D 1	LEF 1
Model control group	—	10	0.23 ± 0.11	0.36 ± 0.16	1.61 ± 0.68	0.43 ± 0.17
Minoxidi control group	0.50	10	0.71 ± 0.27**	0.79 ± 0.27**	1.07 ± 0.21*	0.81 ± 0.20**
β - sitosterol group 2	0.061	10	0.27 ± 0.26	0.57 ± 0.18**	1.31 ± 0.28	0.61 ± 0.18*
linoleic acid group 2	0.156	10	0.39 ± 0.26	0.72 ± 0.26**	1.41 ± 0.26	0.70 ± 0.32*
vitamin E group 2	0.156	10	0.50 ± 0.50	0.73 ± 0.19**	1.21 ± 0.43	0.91 ± 0.49**
Note: Compared with the model control group, *$P \leq 0.05$, **$P \leq 0.01$.						

Table 16
Effects of three effective components of the kernel of *P. mira* on the protein expression of four targets in the Wnt/β-catenin signaling pathway in the skin tissue of C57BL/6 mice ($\bar{x}\pm s$)

Group	Dose (mg/cm^2/d)	Number of animals(number)	β-catenin	GSK3β	Cyclin D 1	LEF 1
Model control group	—	10	0.222 ± 0.008	0.290 ± 0.018	0.195 ± 0.025	0.242 ± 0.010
Minoxidi control group	0.50	10	0.220 ± 0.010	0.278 ± 0.011	0.188 ± 0.028	0.241 ± 0.012
β - sitosterol group 2	0.061	10	0.218 ± 0.006	0.284 ± 0.022	0.183 ± 0.031	0.231 ± 0.015
linoleic acid group 2	0.156	10	0.217 ± 0.008	0.278 ± 0.009	0.171 ± 0.021	0.229 ± 0.016
vitamin E group 2	0.156	10	0.216 ± 0.006	0.279 ± 0.012	0.192 ± 0.030	0.232 ± 0.017

4. Discussion

Alopecia is a common disease in dermatology. It has a greater impact on people's appearance [45] and easily causes psychological burden [46, 47]. Therefore, people pay more and more attention to the research and prevention of hair loss. The drugs and methods used in clinical treatment of hair loss in modern medicine mainly include minoxidil, finasteride, dutasteride, ketoconazole, prostaglandin drugs, laser therapy, hair transplantation, etc.

[48, 49], although they have certain effect, it is easy to relapse. Recent studies have shown that there are 322 kinds of Chinese medicine preparations for the treatment of alopecia, including 135 kinds of preparations prepared by medical institutions, 108 kinds of Chinese patent medicines prepared by pharmaceutical factories, 60 kinds of preparations produced by doctors and 19 kinds of ethnic drugs. The forms of drugs included decoctions, pills, capsules, tablets, granules, tinctures, liniments, and powders [50].

The Convention on Biological Diversity (CBD) was adopted at the United Nations Conference on Environment and Development in 1992 and formally entered into force in December 1993. It is one of the most important multilateral environmental conventions in the world. The CBD establishes "the conservation of biological diversity, the sustainable use of its components and the equitable sharing of benefits arising from the use of genetic resources(ABS)[51]". Article 8 (j) of the CBD introduces the concept of "indigenous and local communities". The Nagoya protocol on access and benefit sharing many of the core terms involves "indigenous and local communities". It requires users to obtain the "prior informed consent" of "indigenous and Local communities" when accessing genetic resources and related traditional knowledge, and to share benefits equally with "indigenous and Local communities[52]". Researchers should have ethnic and folk medicine knowledge from the people to serve the people's idea [53]. Specimens of *P. mira* were collected in Sichuan province, Yunnan province, etc. The research group had good communication with local residents. As researchers, we abide by the above conventions when conducting research of *P. mira*.

A total of 21 chemical components related to the kernel of *P. mira* and corresponding 216 drug targets, 9640 disease target proteins, and 149 drug and disease targets are collected by using network pharmacology technology The Hub gene and protein network interaction map and “the kernel of *P. mira*-chemical composition-target-disease” network map were obtained by using the String website and Cytoscape software. GO analysis find that heme binding, steroid hormone receptor, vitamin binding, antioxidant activity, fatty acid derivative binding, nuclear receptor binding, and so on, may be related to alopecia. KEGG enrichment analysis find that 12 signaling pathways related to alopecia include PI3K/Akt, Wnt, etc. The 12 signaling pathways involve 21 targets and 17 chemical components, of which the Wnt signaling pathway mainly involves 4 targets. The target points are mainly derived from the target prediction of 13 chemical components. The pathway predicted by network pharmacology is related to angiogenesis, inflammation, cell proliferation and apoptosis, and it is consistent with the factors currently considered to affect hair loss, such as inflammation, hair follicle cell cycle, and hair follicle blood circulation. Through literature review, it is found that the mechanism of action for the treatment of alopecia is mostly concentrated in the Wnt and EGFR signaling pathways, which is similar to the predicted results of network pharmacology. Current research on the Wnt pathway mainly involves regulating the proliferation of breast cancer, colon cancer and other cancer cells [54], regulating osteoarthritis [55] and osteoblast differentiation [56], participating in metabolism [57], and regulating hair follicles, and hair regeneration [58]. The PI3K/Akt pathway is also one of the signaling pathways predicted by network pharmacology. It is one of the classic pathways of the EGFR signaling pathway [59]. It can induce or inhibit cell apoptosis [60]. The current research is mainly in tumors [61], Anti-inflammatory[62], osteoblast proliferation and differentiation [63], and growth factor-mediated neuronal activity [64], but there are also reports in the documents that the PI3K/Akt pathway is related to hair growth [65]. There may be a cross link between the Wnt signaling pathway and the EGFR signaling pathway [66]. In the breast epithelial cell line HC11, Wnt can affect the EGFR signaling pathway through Wnt5a, Wnt1 and other cell membrane ligands [67]. Both Wnt and EGFR pathways are involved in Drosophila Polarity of eye cells is formed [68]. From the perspective of research direction and function, they are also similar. There is also the possibility of cross-linking between Wnt signaling pathway and EGFR signaling pathway in hair follicle regulation. Teng et al. found that the EGFR and EGF signals in the hair follicles of mice with mutations in the key genes for hair loss were weakened, and the continued high expression of EGF in the mice could make the hair cycle stay in the growth phase [15]. Yi et al. found that β-catenin can regulate the proliferation of hair follicle stem cells by regulating the PI3K/Akt pathway [69], indicating that the PI3K/Akt signaling pathway is related to the Wnt signaling pathway.

In the study of effectiveness and mechanism of action, firstly, KM mice were used to evaluate the effectiveness of eight chemical components in the kernel of *P. mira*. KM mice after depilation induced by 6% sodium sulfide were used to evaluate the efficacy of the 8 components(β-sitosterol, linoleic acid, vitamin E, oleic acid, campesterol, trans-squalene, amygdalin, fucosterol) by measuring new hair status, hair length and hair weight. The results of the experiment found that the five components (β-sitosterol, linoleic acid, vitamin E, amygdalin, and fucosterol) have obvious promoting effects. Therefore, three doses of the above five components were selected to re-evaluate the pharmacodynamics of the C57BL/6 mice depilation model induced by sodium sulfide, which were vitamin E groups 1, 2, and 3 (6.125, 3.125, 1.563 mg/cm^2/d), β-sitosterol groups 2, 3, and 4 (0.061, 0.031, 0.016 mg/cm^2/d), linoleic acid groups 1, 2, and 3 (0.313, 0.156, 0.078 mg/cm^2/d), amygdalin groups 2, 3, and 4 (0.061, 0.031, 0.016 mg/cm^2/d), fucosterol groups 2, 3, and 4 (0.061, 0.031, 0.016 mg/cm^2/d). Secondly, C57BL/6 mice were used to re-evaluate the effectiveness of the five active components in the kernel of *P. mira*. By observing its skin color change time, new hair status rating, hair length, hair weight and other indicators, it was found that β-sitosterol group 2 (0.061 mg/cm^2/d), linoleic acid group 2(0.156 mg/cm^2/d), vitamin E group 2 (3.125 mg/cm^2/d) could reduce the time of skin darkening in C57BL/ 6 mice, the increase of growth status rating, the increase of hair length and weight. So it indicated that the three dose groups of β-sitosterol, vitamin E, and linoleic acid could make the hair follicles enter the growth phase in advance, which has a certain effect on hair growth. Finally, using C57BL/6 mice depilation model induced by sodium sulfide, the experimental area skin of mice in β-sitosterol group 2 (0.061 mg/cm^2/d), linoleic acid group 2 (0.156 mg/cm^2/d), and vitamin E group 2(3.125 mg/cm^2/d) were tested for the number of hair follicles and dermal thickness, and the effect of the three groups on the Wnt/β-catenin signaling pathway was studied. The results show that the three components can up-regulate the mRNA expression of LEF 1, GSK-3β and β-catenin mRNA expression of the Wnt/β-catenin signaling pathway, and had no significant effect on the mRNA and protein expression of Cyclin D1. Lipids can participate in the development and regulation of hair and skin [70]. Linoleic acid, β-sitosterol and vitamin E are all fat-soluble components. Truong et al. [71] found that oil of red ginseng (*Talinum paniculatum* (Jacq.) Gaertn.) and its main components, linoleic acid and β-sitosterol, can promote hair growth by regulating the Wnt/β-catenin signaling pathway. Upadhyay [72] found that β-sitosterol gel and β-sitosterol-phospholipid complex improved androgenic alopecia by inhibiting 5α-reductase. Linoleic acid derivatives can maintain the stability of the

hair follicles by maintaining the lipid metabolism within the hair and hair follicles [73], so that the hair is in a normal growth state [74]. Vitamin E injection subcutaneously in patients with alopecia areata or plum blossom needle tapping combined with vitamin E rubbing can significantly improve the hair growth of patients with alopecia areata [75].

The Wnt/β-catenin signaling pathway is one of the important pathways related to hair loss. It is a multi-channel signal transduction pathway, which participates in the process of embryonic cell development, division and differentiation and tissue regeneration. It is highly conserved in genetics, and the Wnt pathway among different species is very similar. β-catenin protein is a key factor in activating the pathway [76]. It can be phosphorylated by GSK3β and then degraded [77]. But when it accumulates to a certain amount, it is transferred from the cytoplasm to the nucleus and interacts with TCF/LEF family transcription factors (For example, LEF 1) binds to initiate transcription and translation of target genes, including C-myc, Cyclin D 1, etc. [78] (Fig. 18), so select β-Catenin, GSK3β, LEF 1, Cyclin in the downstream of the Wnt/β-catenin pathway D 1 is four important factors to be studied.

Studies find that β-sitosterol, linoleic acid and vitamin E can up-regulate LEF 1, GSK-3β mRNA expression, β-catenin mRNA expression, and Cyclin D 1 mRNA expression in the Wnt/β-catenin signaling pathway. The expression of mRNA and protein of Cyclin D 1 has no obvious influence. The increase of LEF 1 mRNA expression and the rising trend of β-catenin mRNA expression indicate that the Wnt/β-catenin pathway is in an activated state, but the mRNA expression of GSK-3β in this article increases. This result is consistent with the previous study on the effect of light walnut oil on GSK. The effect of -3β is consistent. It is speculated that the expression of GSK-3β may be regulated by other signal pathways, such as GSK-3β-MCL-1 [44], but further experimental verification is needed. In the results of immunohistochemistry, vitamin E, β-sitosterol and linoleic acid had no obvious effect on the protein expression of GSK-3β, β-catenin, Cyclin D 1 and LEF 1, which may be due to the fact that the skin of the mice in the administration group has changed from black to gray and pink to enter the resting phase after the third week. Although the Wnt/β-catenin pathway is activated, its activity has been reduced and even some mice have stopped hair grows [79], so there is no statistical difference in protein expression overall. It is suggested that mice skins at different administration periods should be selected for testing in subsequent experiments. In this article, the expression of Cyclin D1 mRNA and protein did not change significantly. According to literature reports [80], it was found that the hair of mice knocked out of Cyclin D 1 gene grew normally, and that Cyclin D 1 gene had no obvious effect on the proliferation of hair follicle cells. However, other literatures indicate that Cyclin D 1 is related to hair follicle stem cells [81], and the relationship between Cyclin D 1 gene and related signal pathways and hair follicles should be studied more deeply. In addition, there may be interactions between Wnt/β-catenin pathway and EGFR signaling pathway in the regulation of hair growth. The signal pathways do not exist independently, but are connected to each other to form a complex network. However, there is still little research on the relationship between the signal pathways, which suggests that more attention should be paid to the study of the Wnt/β-catenin pathway and the EGFR signal pathway in subsequent experiments. The connection and interaction of the hair loss treatment, the mechanism network of the treatment of hair loss and the mechanism of its function are more clearly revealed.

The more commonly used hair loss agents in the current literature include hair removal cream, cyclophosphamide, rosin and paraffin, testosterone propionate, and sodium sulfide. The first three reagents are not targeted at specific types of hair loss. Cyclophosphamide is mainly used for patients with hair loss caused by chemotherapy, and testosterone propionate is mainly used for androgenetic alopecia, but these hair loss models have causes that cannot fully simulate human hair loss [82], and the reason why two different species of mice were used in the experiment is that the literature found that C57BL6 mice, KM mice, and SD rats are commonly used in depilation animal models, with most females. Animal experiments of a variety of different species are more conducive to evaluating the effects of drugs and avoiding false positive results.

KM mice have a short hair growth cycle, which is suitable for short-term and rapid screening, and C57BL/6 mice skin has obvious periodicity and long hair growth cycle is suitable for long-term efficacy evaluation. C57BL/6 mice are in the growth phase when the skin is black. In the growth phase, the secondary hair embryos are activated to differentiate into hair follicles. The cells of the hair follicles are highly active and differentiate to form pigmented hair shafts. Hair follicle metabolism decreases during the anagen phase, and the cells gradually apoptosis, the skin color is gray at this time. At the end of the anagen phase, the cells of the hair follicle stop moving, and only a layer of differentiated cells remains on the surface of the hair papilla. The hair follicle forms a secondary hair embryo and enters the resting stage, and the skin of C57BL/6 mice turns pink [83]. Because of this feature of C57BL/6 mice, it has been widely used in hair growth animal experiments and skin diseases related fields [84]. In addition, when using C57BL/6 mice for experiments, all mice need to use sodium sulfide for hair removal. This is because the hair follicles of C57BL/6 mice are in the resting phase under normal physiological conditions and must rely on huge external stimuli (sulfide sodium treatment and plucking) can make the hair follicles of C57BL/6 mice enter the growth cycle [85], so the experiment using C57BL/6 mice in this article did not set a blank control group. Preliminary evaluation of efficacy using KM mice and re-evaluation of efficacy in C57BL/6 mice found that β-sitosterol, linoleic acid and vitamin E allowed mice to enter the hair growth phase earlier, increasing the length and weight of new hair. The effect of promoting hair growth is more obvious than other components. Although KM and C57BL/6 mice are used in this article, they still have limitations. The animal hair follicle cycle is different from the human hair growth cycle, and the hair follicles are collected after the experiment is completed. Changes in some related proteins during the growth cycle have not been discovered, so it is necessary to explore experimental models that are more in line with the causes of human hair loss and find animals that are closer to the human hair growth cycle for experiments. Through clinically consistent models, a variety of animal experiments, multi-time point sampling analysis, and more reasonable index evaluation methods reflect the effect of drugs on hair loss.

At present, the fact that the kernels of *P. mira* are not included in the *Pharmacopoeia of the People's Republic of China*(2020) has become a bottleneck restricting the cultivation and use of the kernels of *P. mira*, and the non-receipt by pharmaceutical factories has led to a sharp decline in usage. However, in the eyes of sellers in the medicinal material market, its appearance quality seems to be no weaker than that of peach kernels (the kernels of *Prunus persica* (L.) Batsch and *Prunus davidiana* (Carr.) Franch.). Author of this article visited the medicinal material market in Chengdu's International

Trade City and found that the drug sellers were willing to buy the kernel of *P. mira* at a price of about 40 yuan/kg. The peach kernels in the *Pharmacopoeia of the People's Republic of China* (2020) are derived from *Prunus persica* (L.) Batsch and *Prunus davidiana* (Carr.) Franch.[86], which has the functions of activating blood circulation and removing blood stasis and moistening intestines and defecating. It has been distributed in Hebei, Shaanxi, Yunnan, Gansu and Sichuan Province [87]. At present, HPLC has been used to study the fingerprints of the kernel of *P. mira* and peach kernels from many producing areas, and it is found that they have 12 common peaks, and the content of 3 peaks is different, indicating that the chemical components of the two are roughly similar [88]. In the future, various experimental animals such as mice, rats, rabbits, etc. can be used to create models of coagulation and bleeding, constipation, gastrointestinal tract movement, etc., to evaluate the effectiveness of the two systems and to combine quality control indicators and pharmacodynamic indicators. The Logistic algorithm evaluates and grades the quality of both [89]. In addition, it is necessary to evaluate the safety of acute toxicity in rats and mice, and long-term toxicity in rats. Comprehensive evaluation of the similarities and differences between the two through quality control indicators, safety evaluation, system effectiveness evaluation, etc., laid the foundation for its entry into the *Pharmacopoeia of the People's Republic of China* (2020). If the kernel of *P. mira* can enter the *Pharmacopoeia of the People's Republic of China* (2020), it can greatly promote the development of the acquisition, planting and processing of the kernel of *P. mira* in Tibetan areas, and make full use of the local medicinal resources, especially for the poverty alleviation in the deeply impoverished areas of the Tibetan area.

However, the chemical composition of the kernel of *P. mira* was complex, especially the fat-soluble components still have a large number of components that have not been quantitatively analyzed, but the fat-soluble components have the characteristics of low polarity and strong adhesion, making the test more difficult. When determining oleic acid and linoleic acid in this paper, the samples were first subjected to saponification treatment, so that there were fewer peaks in the spectrum, resulting in the unsaponifiable components in the kernel of *P. mira* that could not be determined [90]. Therefore, you can find new sample processing methods or establish new analysis conditions through literature review or use various instruments for further analysis. For example, the unsaponifiables such as sterols and triterpene alcohols in the kernel oil of *P. mira* are separated and dried by gel permeation chromatography [91], and then the content of sterols and triterpene alcohols is determined by LC-MS/MS. It can raise quality standards. The HPLC method was used to quantitatively analyze the kernel of *P. mira*, and the contents of oleic acid, linoleic acid and amygdalin in the kernel of *P. mira* were determined, which laid the foundation for improving the quality control of the kernel of *P. mira*. In the experiment, the chromatographic conditions and sample extraction methods were investigated to determine the best chromatographic conditions and extraction methods for oleic acid, linoleic acid and amygdalin. Under this condition, the measured oleic acid is 323.14 ~ 5170.20 μg/mL, linoleic acid is 50.76 ~ 812.14 μg/mL, and amygdalin has a good linear relationship in the range of 80.00 ~ 1280.00 μg/mL. The precision, repeatability and stability are good. The RSD% is less than 3%, and the established method is simple and accurate. Among the 12 batches of different origins, the content of oleic acid in the kernel of *P. mira* from Nixi Township, Shangri-La City, Yunnan Province, China and Xinlong County, Sichuan Province, China was significantly lower. The content of linoleic acid in Lixian and Shangri-La counties and cities is obviously low, and there is no significant difference between oleic acid and linoleic acid in other regions. The content of amygdalin in kernel of *P. mira* from Xinlong County, Kangding City and Jiulong County in Sichuan Province is higher than that in other regions. The three chemical components may be different due to the climatic conditions, sunlight time, rainfall, and soil conditions of the growing place. There are fewer peaks in the test samples for the determination of oleic acid and linoleic acid. It is possible that some unsaponifiable components in the kernel of *P. mira* have not been detected due to the pretreatment method, and unsaponifiables (such as sterols, Triterpene alcohol) content determination method detects more components in the kernel of *P. mira* and improves quality standards.

5. Conclusions

In the study of network pharmacology, it was found that there were 149 targets for the chemical composition and alopecia in the kernel of *P. mira*, and the "protein-protein network interaction diagram" and the network diagrams of "components in the kernel of *P. mira* - chemical -targets-hair loss" were constructed. KEGG enrichment resulted in 12 alopecia related pathways, involving 25 targets and 13 components. Furthermore, the average content of oleic acid, linoleic acid and amygdalin in 12 batches of the kernel of *P. mira* from different origins were determined. The determination method of oleic acid, linoleic acid and amygdalin in the kernel of *P. mira* was established by HPLC, which improved the quality standard. Vitamin E (3.125 mg/cm^2/d), β-sitosterol (0.061 mg/cm^2/d) and linoleic acid (0.156 mg/cm^2/d) in the kernel of *P. mira* can promote hair growth in mice, and its mechanism of action may be related to the Wnt/β-catenin pathway. The research results of this article provided a basis for revealing the research on the pharmacodynamic material basis and mechanism of the kernel of *P. mira* for the treatment of alopecia, and also provide clues for the development of new drugs for the treatment of alopecia.

Abbreviations

Akt/Pkb, Recombinant Protein Kinase B; β-catenin, Beta chain protein; C-myc, proto-oncogene protein; EGF, Epidermal Growth Factor; EGFR, epidermal growth factor receptor; GSK-3β, Glycogen synthase kinase 3; HE, Hematoxylin and eosin; HPLC, High Performance Liquid Chromatography; LEF 1, Lymphoid Enhancer-binding Factor 1; PI3K, phosphatidylinositol 3-kinase; RT-PCR, Real-time PCR; Tcf/Lef, T cell factor/Lymphoid enhancer factor; Wnt, Wingless/Integrated; IHC, Immunohistochemistry; GO, Gene oncology ; KEGG, Kyoto Encyclopedia of Genes and Genomes.

Declarations

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Author's Contributions

You Zhou and Jingwen Zhang contributed equally to this paper. You Zhou and Jingwen Zhang are mainly responsible for the research and the main work in this article; Wanyue Chen, Xiao li Li, Ke Fu, Weijun Sun, Yuan Liang, Min Xu, Jing Zhang, Gang Fan, Hongxiang Yin are responsible for assisting in the experiment. Zhang Wang functions as our communication author.

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Conflicts of Interest

All authors have no conflict of interest to disclose.

Consent for publication

Written informed consent for publication was obtained from all participants.

Ethical Approval and Consent to participate

Our animal experiments were approved by Chengdu Dashuo Biotechnology Co., Ltd.and bearing laboratory animal production license number SCXK (Chuan) 2015-030, Laboratory Animal Quality Qualification Certificate No.51203500006767, No.51203500009764, etc., and conformed to the guide for the Care and Use of Laboratory (approve number SYXK(Sichuan Province)2020-124).

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

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Figures

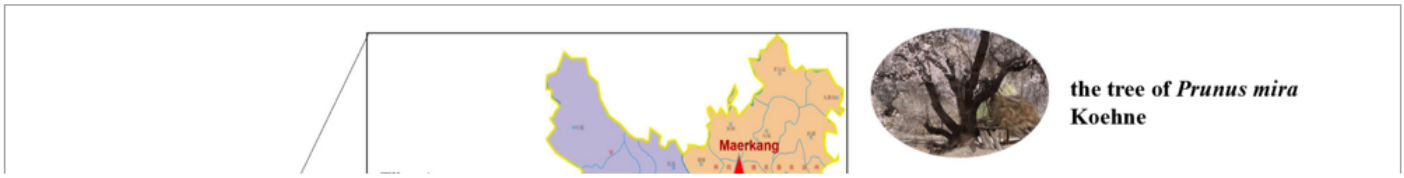

Figure 1

The distribution of *P. mira* in China

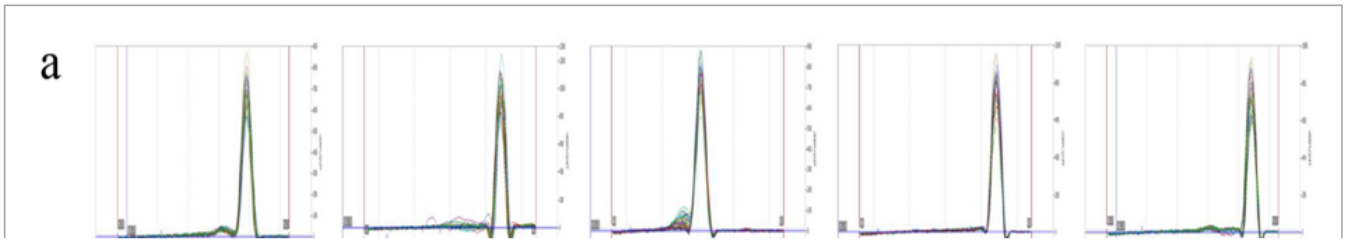

Figure 2

a: The dissolution curve of mRNA RT PCR products of four indicators in mice skin tissue. **b:** RT-PCR product amplification curves of four indicators in mice skin tissue

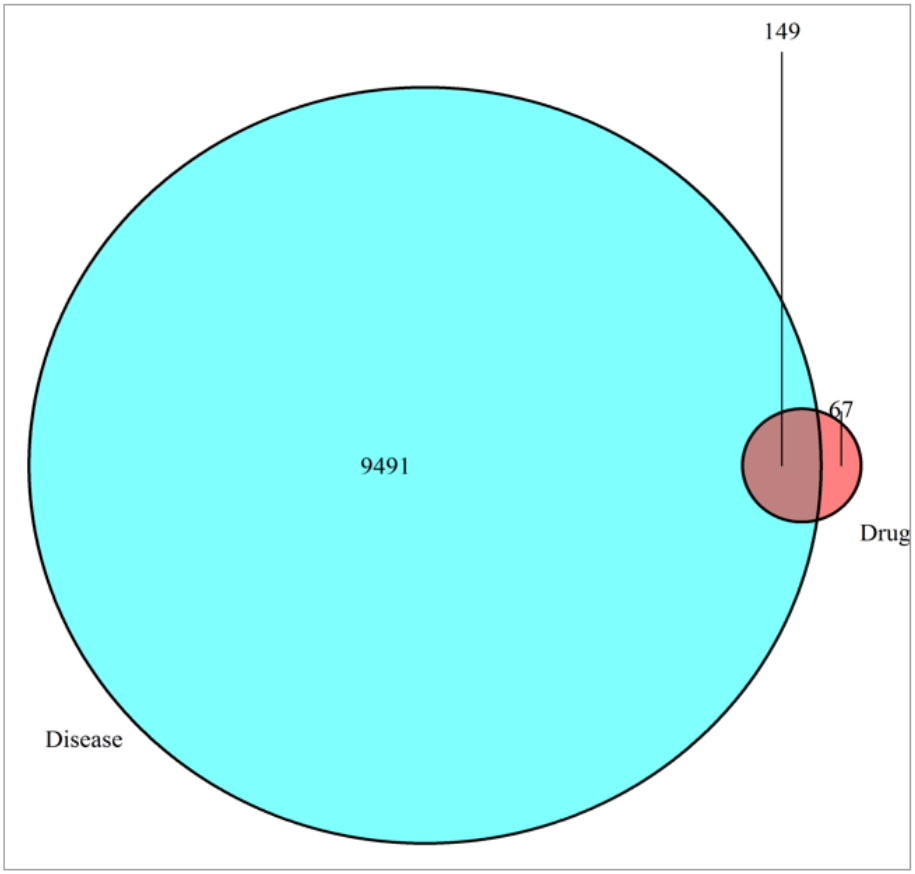

Figure 3

Venn diagram

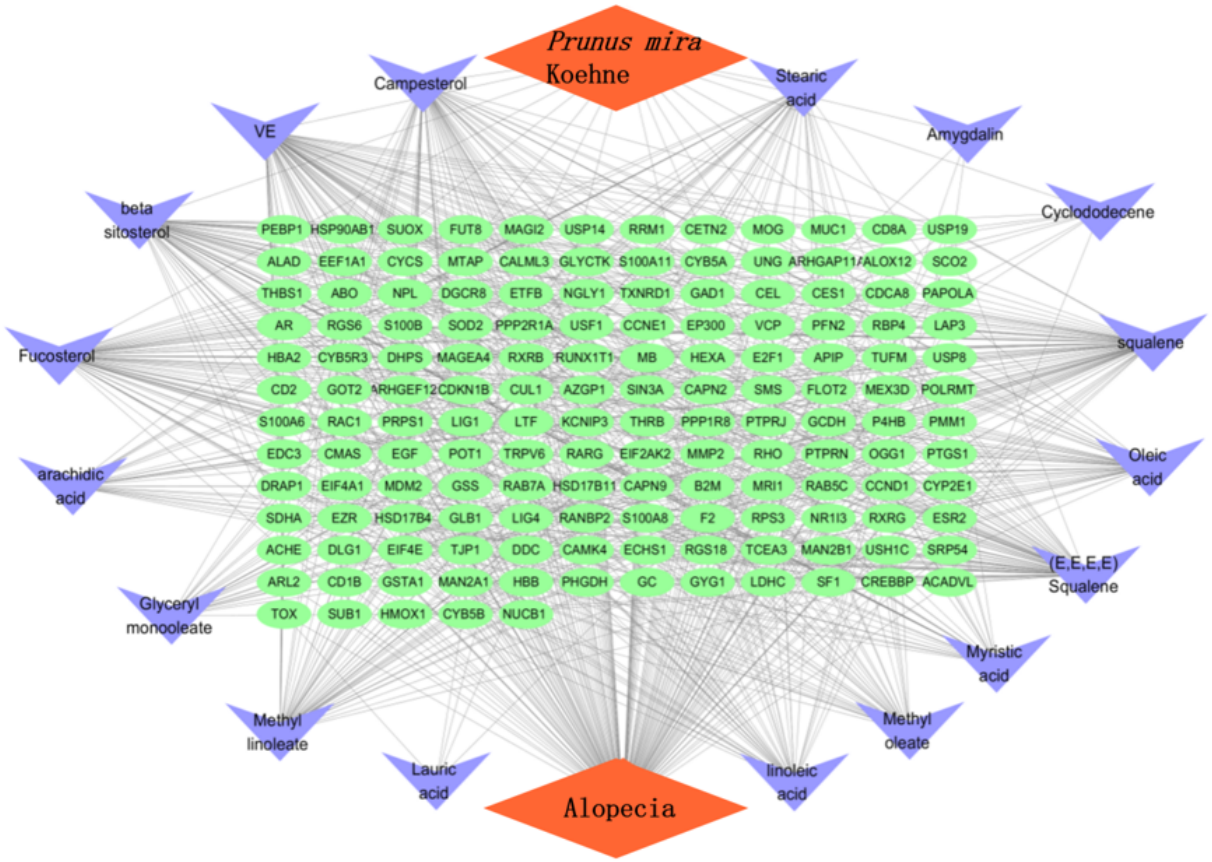

Figure 4

the kernel of *P. mira*-chemical composition-target-alopecia" network analysis diagram

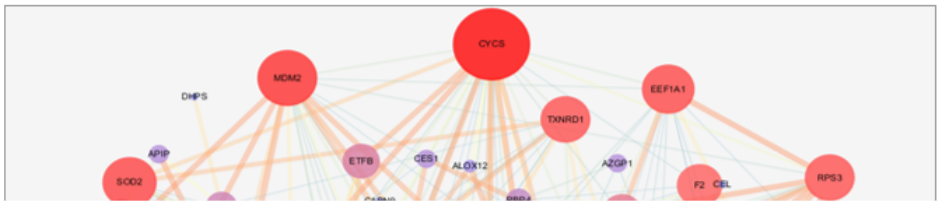

Figure 5

PPI diagram

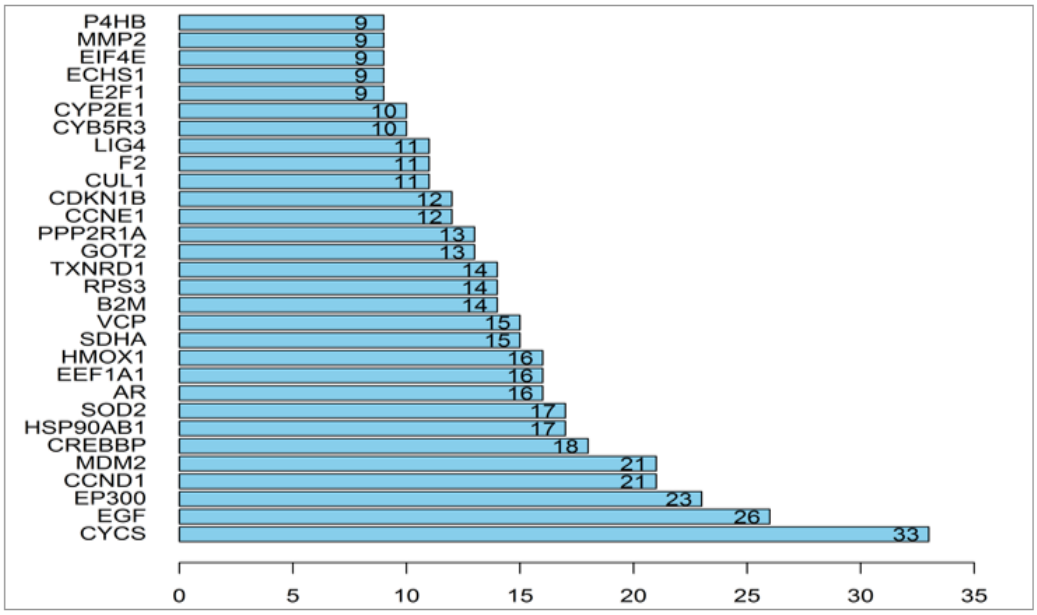

Figure 6

Degrees of freedom of the target

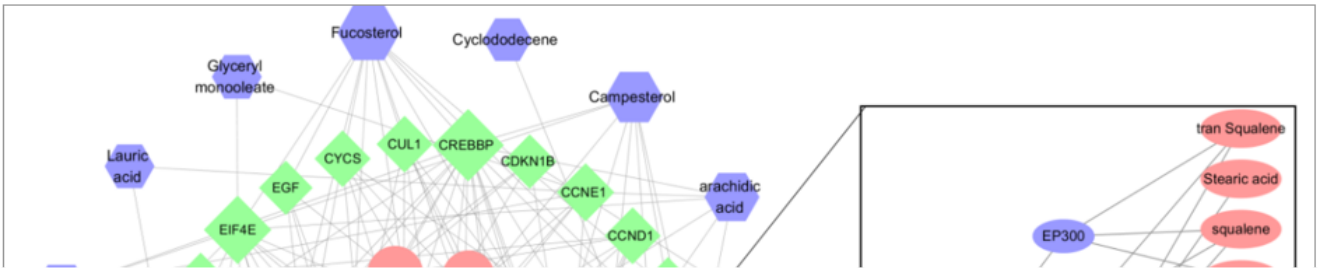

Figure 8

"the kernel of *P. mira* composition-target-signal pathway" network diagram

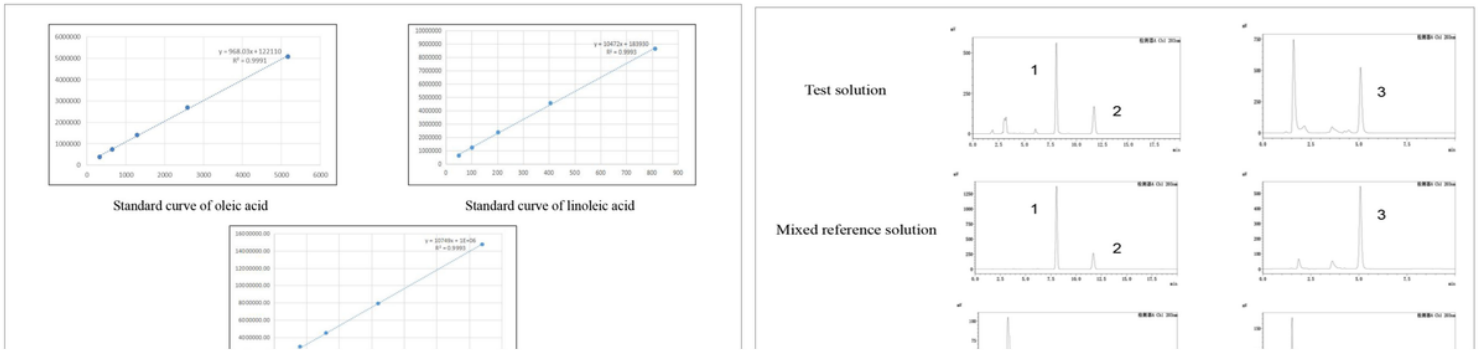

Figure 9

HPLC chromatograms of test solution, mixed reference solution and blank solvent

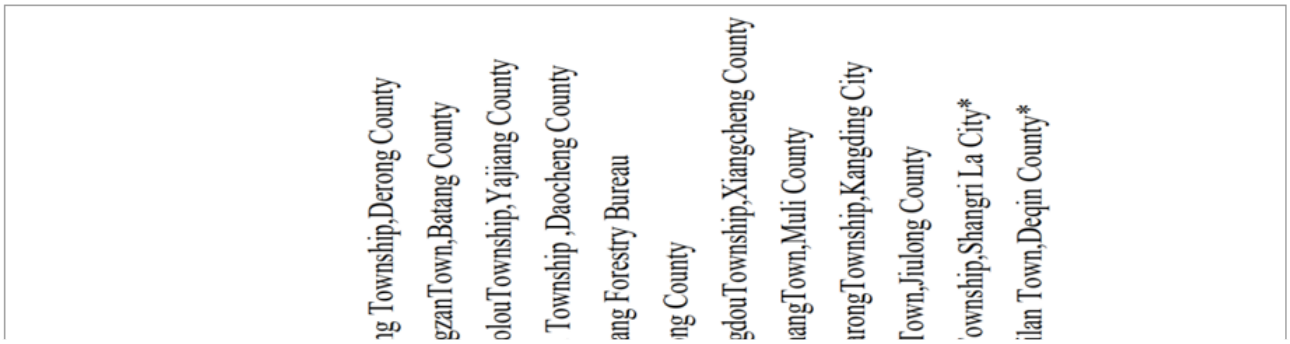

Figure 10

Heat map of the content of three components of the kernel of *P. mira* in 12 producing areas

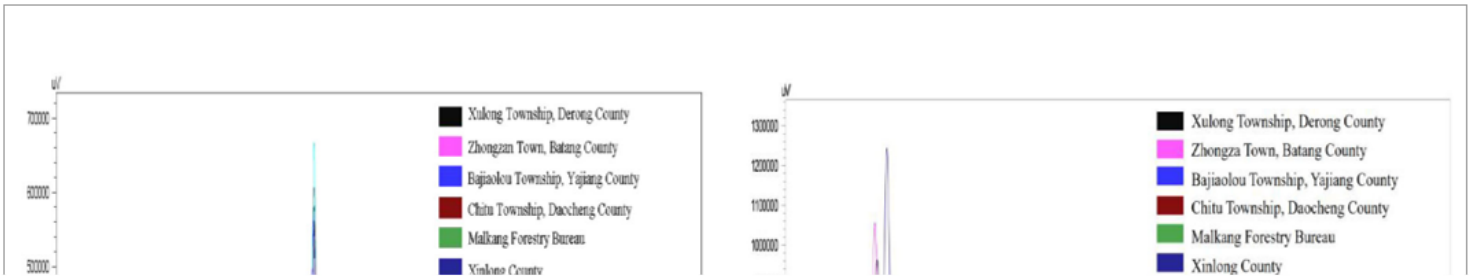

Figure 11

HPLC profile of comparison of content in 12 batches of the kernel of *P. mira* from different origins

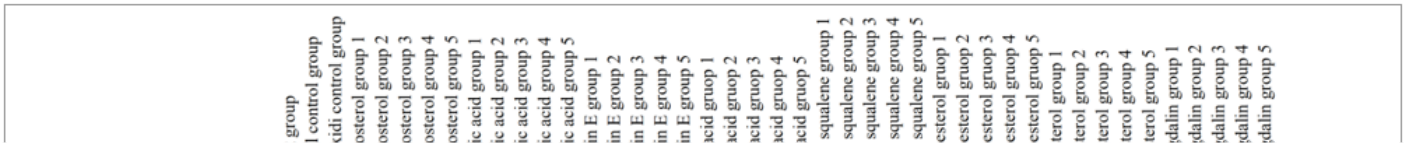

Figure 12

Heat map of the degree of influence of components in the kernel of *P. mira* on KM mice after depilation induced by sodium sulfide

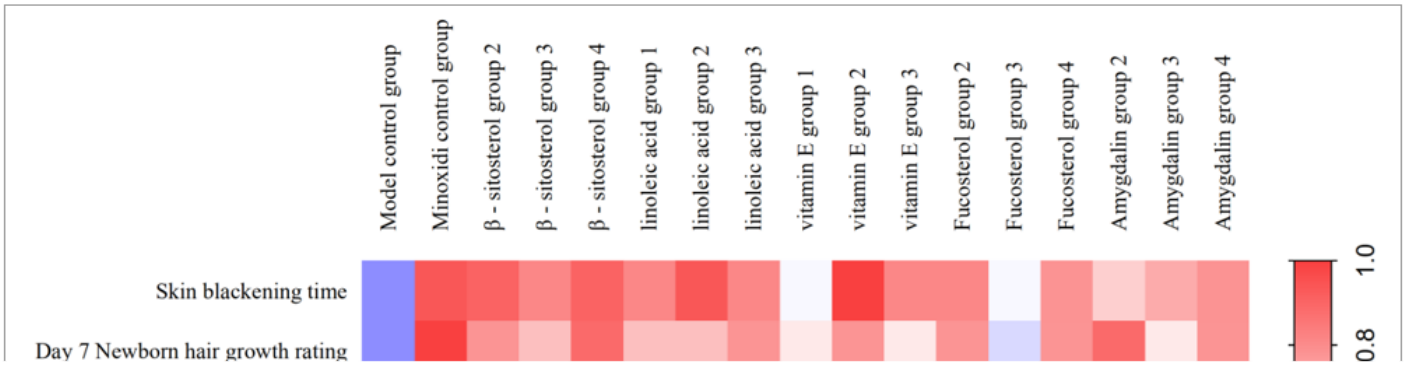

Figure 13

Heat map of the degree of influence of components of the kernel of *P. mira* on the depilation of C57BL/6 mice induced by sodium sulfide. (Note: The closer the color is to red, the more obvious the promoting effect of the test substance on hair growth)

Figure 15

Number of hair follicles and dermal thickness (× 100)

Figure 16

Immunohistochemistry diagram (× 400)

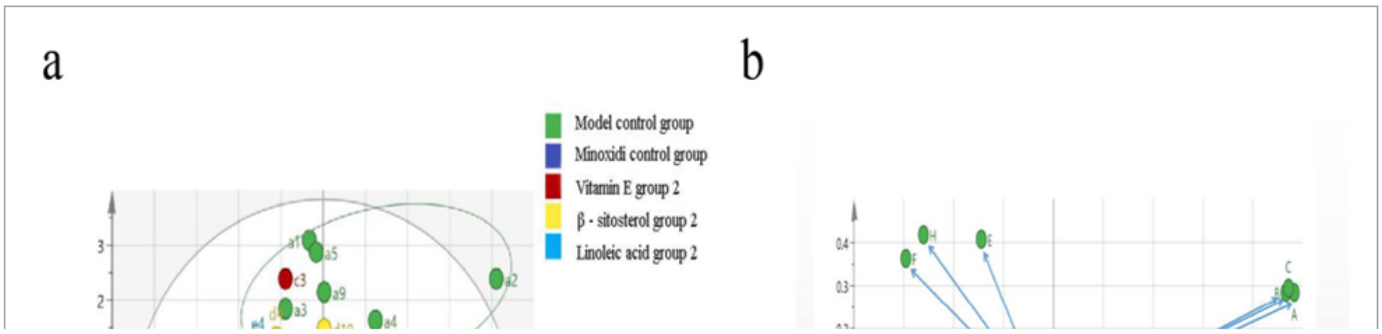

Figure 17

a: PCA-X score diagram. **b:** Contribution rates of indicators (A: Total number of hair follicles; B: Number of primary hair follicles; C: Number of secondary hair follicles;D: dermal thickness;E: mRNA expression of β-catenin; F: mRNA expression of GSK-3β;G: Cyclin D 1 mRNA expression; H: mRNA expression of LEF 1;I: protein expression of β-catenin; J: protein expression of GSK-3β;K: protein expression of Cyclin D 1; L: protein expression of LEF 1)

Figure 18

Wnt / β - catenin signaling pathway