Exploring the molecular mechanism of Licorice rose beverage anti-melasma based on network pharmacology, molecular docking technology and in vivo and in vitro experimental verification

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Abstract

Melasma is a pigmentation disease with refractory and high recurrence risk. Therefore, finding effective treatment has become the focus of research. The aim of this study was to reveal the mechanism of Licorice rose beverage (LRB) in treating melasma from the perspective of network pharmacology and in vitro and in vivo experimental techniques. Network pharmacological studies have shown that Isolicoavonol, quercetin, kaempferol are the main active components of anti-melasma and TYR is the main target. Molecular docking studies have shown that these compounds have a good affinity for these targets. In vitro tyrosinase inhibition experiments showed that LRB could significantly inhibit tyrosinase activity. In vivo studies showed that LRB could significantly improve skin damage and skin pigmentation, reduce the activities of serum and skin tyrosinase in model mice, increase the activity of SOD in serum, and reduce the content of MDA in mice, showing a good effect of anti-melasma. In conclusion, these findings reveal the molecular mechanism of LRB in treating melasma and provided the scientific basis for this product's development and clinical application.

1. Introduction

Melasma is a common pigmented skin disease, which is common in women(Handel et al. 2014). It has a long course and is easy to relapse. (Rigopoulos et al. 2007). In recent years, the incidence of melasma has been increasing year by year, and the prevention and treatment of melasma have attracted wide attention(Kwon et al. 2019). However, the pathogenesis of melasma has not been fully elucidated. Studies show that there are many pathogenic factors of melasma, among which ultraviolet radiation, endocrine dysregulation, oxygen free radicals, and improper use of drugs and cosmetics are the main causes of melasma(Passeron 2013). At present, there are many methods for the clinical treatment of melasma, but the overall efficacy is not ideal(Babbush et al. 2020). According to traditional Chinese medicine (TCM), the liver, spleen and kidney are closely related to melasma, which is caused by stagnation of the liver, dampness of the spleen and deficiency of the kidney(Zhang et al. 2019). Therefore, the treatment of melasma based on TCM theory will be a promising method.

Traditional Chinese medicine (TCM) is a medical system with a long history and unique theories and techniques(Conroy et al. 2020, Li et al. 2022). In recent years, the use of Chinese herbal medicine treatment of diseases associated with melasma has attracted wide attention. For example, Zhang et al. found that the cream containing camellia, mulberry, Dauphin oil and purslane could improve melasma by various mechanisms such as anti-inflammatory, anti-oxidant, improving microcirculation, inhibiting melanin production and improving the skin permeability barrier(Zhang, Tu, Gu, Sun, Wu, Man, Chen, Liu, Ma and He 2019). The Licorice rose beverage (LRB) includes Licorice, Rose flowers, Tangerine peel, Wolfberry, Poria cocos, Mulberry leaf and Cinnamon. Among them, Licorice is a very famous ancient herb and one of the most commonly used in TCM, such as antibacterial, antiviral, anti-inflammatory, antidiabetic, immunomodulatory, liver protection, etc.(Yang et al. 2017); In addition to glycyrrhizic acid, flavonoids, saponins, triterpenes, isoavones and chalcones, the major active ingredients in licorice also contain large amounts of iron (Fe), manganese (Mn) and cobalt (Co). These chemical elements are the reasons for the comprehensive utilization of glycyrrhiza in health(Icer 2017). The beauty and fragrance of rose flowers have been known since ancient times, known as the "gift of angels"(Mahboubi 2016). Rose flowers are rich in beneficial components, such as flavonoids (flavonols and anthocyanins) and aromatic components (essential oils), which can be used as disinfectants, anti-inflammatory agents and antioxidants, and are widely used in the food industry, perfume and cosmetics(Mileva et al. 2021); Tangerine peel is the mature peel of Rutaceae and its cultivated varieties. It is rich in phenolic compounds and carotenoids. It is an excellent source of dietary fiber and minerals. It is widely used in the food, pharmaceutical and cosmetic industries(Singh et al. 2020); Wolfberry has been used in TCM for thousands of years as a healthy food and a treatment for diseases. According to TCM theory and practice, wolfberry can function on both the liver meridian and kidney meridian, wolfberry's main health effects is to nourish the liver and kidney. According to China’s State Food and Drug Administration, medlar is one of 87 kinds of TCM ingredients that can be used as both normal food and functional food(Wenli et al. 2021); Poria cocos (Polyporaceae) is a saprophytic fungus that grows in different species of pine. TCM believes that Poria cocos have a diuretic, sedative and nourishing effect. Modern medical research shows that Poria Cocos has anti-inflammatory, anti-tumor and other pharmacological activities, widely used in medicine and the health food
field (Rios 2011); Mulberry leaves are very valuable food plants for nutrition and nutrient composition. In some Asian countries it is widely used as ethnic medicine and functional food, such as tea, drinks, noodles, etc., due to its biological and nutritional value (Zhang et al. 2022); Cinnamon is an eternal tree used in tropical medicine and is one of the most important spices used daily. Cinnamon contains manganese, iron, dietary fiber, cinnamaldehyde, cinnamic acid and polyphenols. It has antioxidant, anti-inflammatory, anti-diabetic, anti-bacterial and anti-cancer effects (Hariri and Ghiasvand 2016). LRB can regulate qi, tonify the spleen and kidney, promote blood circulation and regulate menstruation through the combined action of seven medicines, so as to play the role of beauty and beauty, skin nourishment and melasma removal. In general, although the pharmacological effects of these seven drugs are significant, the molecular mechanism of the combination of these drugs to treat melasma remains unclear.

Network pharmacology is an emerging discipline to explore the mechanism of action of TCM with multi-components, multi-targets and multi-pathways (Guo et al. 2022). It is helpful to study the active components, targets and molecular mechanisms of TCM in the intervention of diseases and provides direction for the development of new drugs. Molecular docking technology is a further analysis method based on the prediction of network pharmacology, which can explain the molecular mechanism of drug molecules acting on disease-related targets (Hu et al. 2022).

In order to reveal the mechanism of action of LRB in treating melasma, this study carried out a strategy based on TCM network pharmacology combined with calculation prediction and experimental verification. Firstly, network pharmacology and molecular docking technology were used to explore the molecular mechanism of LRB in the treatment of melasma, and in vitro experiments and mouse melasma model was used to verify it, so as to provide the scientific basis for the future development and clinical application of this product.

2 MATERIALS AND METHODS

2.1 Materials

Licorice rose beverage (LRB) were purchased from Guangzhou Tianzhiyuan Beauty & Health Products Co., Ltd.. The names and formulations of 7 herbs in LRB were provided (Table 1). Levodopa was purchased from Shanghai Aladdin Biotech Co., Ltd. Progesterone injection (20mg/mL) was purchased from Guangzhou Baiyunshan Mingxing Pharmaceutical Co., Ltd. Tyrosinase Reagent was purchased from Beijing Soleb Technology Co., Ltd. L-ascorbic acid, polyformaldehyde fixative, superoxide dismutase (SOD) and malondialdehyde (MDA) were purchased from Shanghai McLean Biochemical Technology Co., Ltd.

<table>
<thead>
<tr>
<th>Herbal name</th>
<th>Plant scientific name</th>
<th>Branch</th>
<th>Genus</th>
<th>Application area</th>
</tr>
</thead>
<tbody>
<tr>
<td>Licorice</td>
<td>Glycyrrhiza uralensis Fisch.</td>
<td>Leguminosae</td>
<td>Glycyrrhiza</td>
<td>Dried root</td>
</tr>
<tr>
<td>Rose flowers</td>
<td>Rosa rugosa Thunb.</td>
<td>Rosaceae</td>
<td>Rosa L.</td>
<td>Dried flower</td>
</tr>
<tr>
<td>Tangerine peel</td>
<td>Citrus Reticulata</td>
<td>Rutaceae</td>
<td>Citrus spp.</td>
<td>Dried bark</td>
</tr>
<tr>
<td>Wolfberry</td>
<td>Lycii Fructus</td>
<td>Solanaceae</td>
<td>Genus Lycium</td>
<td>Dried ripe fruit</td>
</tr>
<tr>
<td>Poria cocos</td>
<td>Poria Cocos(Schw.) Wolf.</td>
<td>Polyporaceae</td>
<td>Poria genus</td>
<td>Dried Mycorrhiza</td>
</tr>
<tr>
<td>Mulberry leaf</td>
<td>Mori Follum</td>
<td>moraceae</td>
<td>Morus genus</td>
<td>Dried leaf</td>
</tr>
<tr>
<td>Cinnamon</td>
<td>Cinnamomum cassia (L.) J.Presl</td>
<td>Lauraceae</td>
<td>Cinnamomum</td>
<td>Dried bark</td>
</tr>
</tbody>
</table>

2.2 Network pharmacology data analysis
The chemical constituents of licorice, rose, tangerine peel, wolfberry, poria cocos, mulberry leaves and cinnamon (Chou et al. 2013, Ngoc et al. 2009) in LRB were collected by means of the TCMSP database (http://tcmspw.com/tcmsp.php) and published literature. According to the pharmacokinetic characteristics given by the TCMSP data platform, oral bioavailability (OB ≥ 30%) and drug-like (DL ≥ 0.18) were selected as the screening parameters for the chemical components of traditional Chinese medicine (Gao et al. 2022, Tao et al. 2019). The two-dimensional structure of the compound is obtained from the PubChem database (www.pubchem.ncbi.nlm.nih.gov). Through GeneCards (https://www.genecards.org/) and DisGeNET (https://www.disgenet.org/) in the database for "melasma" related targets. These common targets were calculated using a Venn diagram (Figure S1). The interactions between LRB anti-melasma related targets were analyzed by the STRING database (https://string-db.org/). GO analysis and KEGG pathway enrichment analysis using the DAVID database (https://david.ncifcrf.gov/). Finally, the Cytoscape software (version 3.9.1) was used to construct the active compound-target network.

2.3 Molecular docking

TYR, the core target of network pharmacology, was used as receptor protein, and the top 3 compounds of H-C-T-P was used as ligand molecule. The three-dimensional structure of protein TYR (EC 1.14.18.1) and ligand molecule were obtained from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/)(Jumper et al. 2021). Among them, TYR is a protein-encoding gene. The gene can regulate the catalytic activity of tyrosine hydroxylase and dopa oxidase, regulate the formation of melanin and play a key role in the pathogenesis of melasma (Garcia and Fulton 1996, Hantash and Jimenez 2009). The Full Minimization module of Discovery Studio is used to minimize the energy of small molecules and set the CHARMM force field to be assigned to the structure. Molecular docking using the LibDock module on the Discovery Studio 2019 software. Make minor modifications according to the method reported. Among them, Libdockscore ≥ 90 indicates that the active component has a strong affinity with the target protein (Hu et al. 2022).

2.4 Tyrosinase inhibition assay

The inhibitory activity of LRB on tyrosinase in vitro was studied (Abbas et al. 2017). Briefly, the inhibition of tyrosinase was detected using the levodopa solution (L-DOPA) as the substrate (Sohretoglu et al. 2018). Glabridin was determined as a positive control. Add LRB of different concentrations (40µL) and substrate solution containing 40µL (0.453g/L) and 80µL potassium phosphate buffer (pH 6.8) to 96 hole plate, water bath at 37 °C for 10 minutes at a constant temperature, then add 200 U/mL tyrosinase 40µL in each hole, and shake and mix for 15 minutes under room temperature and light protection. Then, the absorbance was measured and recorded at 475 nm by Multiskan Go, and the inhibition rate was calculated 3 times in parallel.

2.5 Animal research

2.5.1 Modeling

A kind of melasma mouse model was established. Female mice from Kunming weighed about 20 grams each and all experiments were done at the Animal Laboratory Center of the Southern Medical University (Guangzhou, China, quality certificate number: SCXK [Yue] 20160041). The ambient temperature (25 ± 5 °C) and humidity (55 ± 5%) of mice remained stable during the experiment. During the study, mice were given standard diets and drinking water. After adapting to 2 days, 60 mice were randomly divided into 6 groups: blank, model, positive control, LRB high (1 g/mL), medium (0.5 g/mL), low concentration (0.25 g/mL), each group 10 mice. The melasma model mice were established by injecting progesterone (20 mg/kg) into the muscle of the hind leg and irradiating it with ultraviolet for 60 minutes. The control group was given Vitamin C (Vit C, 0.1 g/kg), and the other three groups were given an oral solution of the corresponding concentration for 30 consecutive days (1 dose per day).

2.5.2 Characterization of skin status and pathological sections

After 24 hours of the last dose, the skin condition of the back of the mice was carefully observed and photographed. The mice were anesthetized with pentobarbital sodium and weighed by electronic balance. Eyeball blood was collected, 3000rpm
was centrifuged for 30 minutes and serum was collected at -80 °C. Then the back skin of each mouse was taken into 2 parts. In addition, each mouse dorsal skin was taken 2 portions, fixed with polyformaldehyde fixative as hematoxylin-eosin (HE) staining material and frozen preserved as skin tissue homogenate material. None of the mice showed signs of toxicity throughout the experiment and all survived.

2.5.3 Effects on tyrosinase content in serum and skin tissue of melasma mice

Determination of tyrosinase in serum and skin tissues. The back skin of each mouse was homogenized in 0.9% NaCl cold solution (10%), centrifuged at 3 000 rpm for 15 minutes, and stored at -80°C. The serum was taken from "2.4.2". The determination of tyrosinase was carried out according to the operation requirements and steps of the tyrosinase kit. Finally, the tyrosinase content was calculated by measuring and recording the absorbance value at 450nm wavelength with a Multiskan Go enzyme micrograph.

2.5.4 Effects on serum antioxidant enzymes in mice

For serum biochemical analysis of mice, serum was taken from "2.4.2". The activity of SOD and the content of MDA was determined by the kit. The SOD activity was 450nm and the content of MDA was 532nm, respectively. The SOD activity and MDA content were measured by Multiskan Go.

2.6 Statistical analysis.

Data were expressed as mean ± standard deviation (SD). Pictures were drawn using GraphPad Prism 6.0. Statistical analysis was performed by one-way analysis of variance (ANOVA), and P < 0.05 was statistically significant.

3 Results and discussion

3.1 Network pharmacology analysis

3.1.1 GO and KEGG pathway enrichment analysis

Figure 1 shows the GO functional enrichment analysis of LRB anti-melasma related targets, including biological process (BP), cell composition (cellular component, CC) and molecular function (MF). The results of the GO analysis showed that the biological processes involved mainly include processes negative regulation of gene expression, negative regulation of transcription from RNA polymerase II promoter and positive regulation of transcription from RNA polymerase II promoter, positive regulation of gene expression, etc. The cellular components involved mainly include cytoplasm, cytosol, nucleus and nucleoplasm, etc. The molecular function involved mainly includes protein binding, enzyme binding, zinc ion binding, DNA binding and RNA polymerase II transcription factor activity, ligand-activated sequence-specific DNA binding, etc.

The KEGG pathway of 13 candidate targets was enriched by DAVID 6.8 database, and the first 10 signal pathways of LRB anti-melasma were obtained according to the P value, as shown in Fig. 2. The bubble area size represents the number of target genes, the bubble color represents enrichment significance, i.e., the P value, and the Y axis represents the pathway name. The main pathways related to melasma include Metabolic ways, Chemical Carcinogenesis-Receptor activation, Relaxin signaling pathway, etc. It can be found that the anti-melasma effect of LRB is realized by multi-pathway and multi-target.

3.1.2 "H-C-T-P" network construction and analysis

In order to better explore the molecular mechanism of LRB in the treatment of melasma, the "H-C-T-P" topological network with 227 nodes and 1320 edges was created using Cytoscape 3.9.1 software (Fig. 3). Nodes are made up of different colors and shapes. Edges are used to represent the correlation between different nodes. The active components in LRB interact with
different targets and pathways, which is the same as the concept of TCM multi-target and multi-way cooperative treatment of diseases.

The high degree values of the active ingredients Isolicoflavonol, quercetin, kaempferol, Norartocarpetin, Jaranol, Tetramethoxy luteolin, Quercetin der, Semilicoisoflavone B, Glepidotin A and Hedysarimcoumestan B (Table 2). It is suggested that these active ingredients play an important role in the anti-melasma process. The potential targets of LRB anti-melasma are TYR, ESR2, ESR1, AR, and EGFR, suggesting that these targets may be key targets for the treatment of melasma.

Table 2
The top ten potentially effective compounds in the prescription

<table>
<thead>
<tr>
<th>Pubchem ID</th>
<th>Compounds</th>
<th>Degree</th>
</tr>
</thead>
<tbody>
<tr>
<td>5318585</td>
<td>Isolicoflavonol</td>
<td>14</td>
</tr>
<tr>
<td>5280343</td>
<td>quercetin</td>
<td>14</td>
</tr>
<tr>
<td>5280863</td>
<td>kaempferol</td>
<td>13</td>
</tr>
<tr>
<td>5481970</td>
<td>Norartocarpetin</td>
<td>13</td>
</tr>
<tr>
<td>5318869</td>
<td>Jaranol</td>
<td>13</td>
</tr>
<tr>
<td>631170</td>
<td>Tetramethoxy luteolin</td>
<td>13</td>
</tr>
<tr>
<td>5316900</td>
<td>Quercetin der.</td>
<td>12</td>
</tr>
<tr>
<td>5481948</td>
<td>Semilicoisoflavone B</td>
<td>12</td>
</tr>
<tr>
<td>5281619</td>
<td>Glepidotin A</td>
<td>12</td>
</tr>
<tr>
<td>11558452</td>
<td>Hedysarimcoumestan B</td>
<td>11</td>
</tr>
</tbody>
</table>

3.1.3 PPI network construction and analysis

Intersection targets were uploaded to the STRING database and PPI networks were constructed (Fig. 4). The nodes represent proteins, the edges indicate the interaction relationship between proteins, and the more lines of the edges indicate the greater association between proteins. In PPI networks, the degree values of TYR, ESR1 and VEGFA are higher. It is found that TYR plays a central role in both H-C-T-P and PPI networks. Therefore, we speculate that LRB may affect downstream protein expression by regulating TYR.

3.2 Molecular docking verification

In order to verify the interaction between the components and targets screened by network pharmacology, and to explore the molecular mechanism of LRB in the treatment of melasma, molecular docking technology was used to verify the top three active components and target proteins. Table 3 shows the docking scores and energies of Isolicoflavonol, quercetin, kaempferol, glabridin and Vitamin C with TYR (EC 1.14.18.1) target proteins. The results showed that these compounds had a good binding activity with target proteins. The docks scores of Isolicoflavonol, quercetin and kaempferol with the target protein were all over 90, indicating that these three active compounds play a key role in LRB anti-melasma. Figure 8 shows the visualized molecular docking results of these active compounds with target proteins. It is found that the binding of these compounds to the target protein occurs mainly through hydrogen bonds, van der Waals forces and π bonds. Among them, the hydrogen atom on the hydroxyl group of the Isolicoflavonol compound acts as a hydrogen bond donor and the amino acid residues ASN and ARG on the TYR target protein to form hydrogen bond interactions with distances of 1.59 Å and 2.12 Å (Fig. 5a). The hydrophobic region of the benzene ring on the quercetin compound formed π bond interactions with amino acid residues HIS, GLN, ILE and VAL on TYR target protein with the spacing of 4.67 Å, 5.66 Å, 3.99 Å and 4.33 Å (Figure 5b). kaempferol compounds and amino acid residues GIU, PHE, ARG and SER on TYR target proteins form van der Waals forces
(Fig. 5c). The molecular docking results show that these compounds have a good affinity with target proteins, further verifying the rationality of network pharmacological prediction. In addition, glabridin and vitamin C also showed good docking activity with TYR target proteins, which could be used as positive drugs for experimental verification (Figure. 5d-e).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Protein</th>
<th>Libdock score</th>
<th>Binding Energy (kcal/mol)</th>
<th>Ligand Energy (kcal/mol)</th>
<th>Protein Energy (kcal/mol)</th>
<th>Complex Energy (kcal/mol)</th>
<th>Entropic Energy (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>quercetin</td>
<td>Tyrosinase (EC 1.14.18.1)</td>
<td>98.8798</td>
<td>66.3476</td>
<td>938.9846</td>
<td>-21533.3320</td>
<td>-20527.9998</td>
<td>19.4194</td>
</tr>
<tr>
<td>kaempferol</td>
<td>Tyrosinase (EC 1.14.18.1)</td>
<td>95.9171</td>
<td>65.0557</td>
<td>1233.1668</td>
<td>-21533.3320</td>
<td>-20235.1095</td>
<td>19.2817</td>
</tr>
<tr>
<td>glabridin (positive drug)</td>
<td>Tyrosinase (EC 1.14.18.1)</td>
<td>118.103</td>
<td>9.1556</td>
<td>49.1410</td>
<td>-21533.3320</td>
<td>-21475.0354</td>
<td>19.5861</td>
</tr>
<tr>
<td>Vitamin C (positive drug)</td>
<td>Tyrosinase (EC 1.14.18.1)</td>
<td>97.4644</td>
<td>30.9896</td>
<td>67.9329</td>
<td>-21533.3320</td>
<td>-21434.4122</td>
<td>17.7968</td>
</tr>
</tbody>
</table>

3.3 Inhibition of LRB on tyrosinase in vitro

Tyrosinase is a key enzyme in the skin melanin synthesis process, so it can directly affect the rate of melanin synthesis (Pillaiyar et al. 2017). By inhibiting tyrosinase, melanin production in cells is specifically inhibited because tyrosinase is produced only by melanocytes (Kumar et al. 2013). In this experiment, L-DOPA was used as a substrate, LRB as an effector, and glabridin was used as a positive control to determine its inhibition on tyrosinase (Sharif et al. 2015). As shown in Fig. 6, different concentrations of LRB can significantly inhibit tyrosinase activity, and with the increase of the concentration of LRB, the inhibition rate of tyrosinase increased gradually, showing a significant dose-dependence. The inhibition rate of tyrosinase at a high dose (1.0 g/mL) of LRB was similar to that of glabridin. This phenomenon suggests that the content of licorice in LRB may have a significant effect on tyrosine kinase inactivation. Therefore, this study shows that LRB can significantly inhibit tyrosinase activity, and its inhibition effect is positively correlated with concentration.

3.4 In vivo validation

3.4.1 Characterization of the skin condition of experimental animals

The effect of LRB on the skin of melasma model mice was evaluated by measuring the skin state of the mice. As shown in Fig. 7, after 30 days of intramuscular injection of progesterone and UV irradiation. Compared with the control group, the model group had obvious surface damage, redness, dryness, scab and coloration. However, compared with the model group, the skin surface of the positive group was smooth without skin pigmentation. Similarly, after administration of low, medium and high doses of LRBs, compared with the model group, the skin surface of mice in the administration group improved
significantly, and the effect increased with the increase of doses. The results showed that LRB could significantly improve the skin color, redness, dry peeling, sunburn and pigmentation of melasma model mice. It showed a good effect in preventing melasma.

### 3.4.2 Histopathological analysis of skin

The histopathological characteristics of the skin of mice with the melasma models were evaluated by observing the histopathological sections of mouse skin. As shown in Fig. 8, the epidermis and dermis of the blank group were intact without thickening, edema, inflammation, necrosis and so on. However, compared with the control group, the epidermal epithelial cells in the model group proliferated significantly, with more layers of cells, local necrosis, inflammatory cells, edema and obvious inflammatory phenomenon, hair and sebaceous glands proliferated obviously. Compared with the model group, the epithelial cells in the epidermis of the positive group arranged neatly without obvious hyperplasia or thickening, and most of them showed no obvious inflammation, necrosis, edema, etc. Similarly, compared with the model group, the epidermis and dermis of the mice in the treatment group were basically intact, without obvious necrosis, edema and inflammation, and the effect was more obvious with the increase of the dosage. The results showed that the LRB could repair the skin injury and skin pigmentation of melasma model mice, and improve the skin thickening, inflammation, necrosis and congestion of the model mice. In addition, the effective rate of skin pathology experiments was 100% by statistical analysis.

### 3.4.3 Effect of LRB on tyrosinase content in vivo

The inhibitory activity of LRB on tyrosinase in vivo was studied. As shown in Fig. 9, the tyrosinase activity in the serum and skin of the model group was significantly higher than that of the control group. Compared with the model group, tyrosinase activity in serum and skin decreased significantly in the positive group. Similarly, different doses of LRB were given after intervention. In comparison to the model group, the tyrosinase activity of serum and skin decreased, and the effect was more significant with the increase in dosage. Among them, LRBs inhibited the activity of serum tyrosinase strongly. The results showed that LRB could significantly inhibit the tyrosinase activity in the serum and skin of melasma model mice, especially the activity of serum tyrosinase. It is suggested that LRB can prevent and cure melasma by inhibiting melanin formation and precipitation.

### 3.4.4 Analysis of antioxidant enzymes in serum of LRB

SOD plays a vital role in the balance of oxidation and antioxidation (Johnson and Macdonald 2004). This enzyme can scavenge oxygen free radicals to protect cells from damage (Li et al. 2020). The activity of SOD indirectly reflects the ability to scavenge oxygen free radicals. The effect of LRB on SOD activity in the serum of melasma model mice is shown in Fig. 10a. Compared with the control group, the SOD activity in the serum of the model group was significantly lower, indicating that the model was basically successful in terms of SOD activity. Compared with the model group, the SOD activity of the LRB and Vit C positive control group were significantly enhanced, and the SOD activity of the high dose group was the most significant.

The content of MDA reflects the severity of the free radical attack (Ayala et al. 2014). When the body is attacked by free radicals, it will induce lipid peroxidation, and then MDA will be produced (Kapusta et al. 2018). The effect of LRB on serum MDA content in melasma model mice is shown in Fig. 10b. In comparison to the control group, the serum MDA content of the model group was significantly increased, indicating that the model was basically successful. Compared with the model group, the content of serum MDA in every dose group was significantly lower, and the content of serum MDA in the high dose group was lower than that in the positive control group Vit C.

In conclusion, LRB could significantly increase the activity of SOD and decrease the content of MDA in the serum of melasma model mice and the effect is more significant with the increase in the dose.

### 4 Conclusions
In this study, the molecular mechanism of LRB in the treatment of melasma was studied by network pharmacology, molecular docking and in vitro and in vivo experiments. Network pharmacological studies have shown that Isolicoflavonol, quercetin and kaempferol are the main active ingredients in LRB for the treatment of melasma. TYR is the core target of LRB drink anti-melasma. The molecular docking results show that the three representative compounds interact with TYR target proteins through hydrogen bonds and π bonds. In vitro tyrosinase assay showed that the activity of tyrosinase was inhibited by LRB, and the inhibition rate of high-dose LRB to tyrosinase was close to that of the positive control glabridin. In vivo animal experiments showed that LRB could significantly reduce the tyrosinase activity in the serum and skin of melasma model mice, increase the SOD activity and decrease the MDA content. The experimental results of skin state and pathological section showed that LRB had a good repair effect on skin damage and skin pigmentation in melasma model mice, and could significantly improve the symptoms of epidermal thickening, inflammation, necrosis and congestion in model mice, showing a good therapeutic effect. In conclusion, LRB can inhibit the tyrosinase activity of melanocytes and melanoma cells in local skin tissue by increasing the activity of the SOD enzyme and decreasing the content of MDA. LRB can promote the oxidation and reduction of skin cells, reduce the production of free radicals, inhibit the formation of melanin, and then effectively treat melasma.

**Abbreviations**

LRB - Licorice rose beverage  
SOD - superoxide dismutase  
MDA - malondialdehyde  
L-DOPA - levodopa solution  
TCM - traditional Chinese medicine

**Declarations**

**Ethical Approval and Consent to Participate**

All animal experiments were performed in accordance with the “Guiding Principles in the Care and Use of Animals” (China), and approved by the Ethics Committee of Southern Medical University (L2019036, date of approval: 13 April 2019).

**Consent for publication**

The manuscript is published with the consent of all authors.

**Conflict of Interest**

The authors declare no conflict of interest.

**Authors' contributions**

Dan Zhai and Yi Hu wrote the main manuscript text and Li Liu prepared figures. Zhuxian Wang, Peiyi Liang and CuiPing Jiang performed the experiment. Hui Li, Quanfu Zeng and Hongkai Chen performed the data analyses. Yufan Wu, Yinglin Guo and Yankui Yi helped perform the analysis with constructive discussions. Chunyan Shen, Hongxia Zhu and Qiang Liu contributed to the conception of the study. All authors reviewed the manuscript.
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Data Availability
Data supporting the results of this study may be obtained from the corresponding author upon reasonable request.

Supplementary information
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Effect of LRB on serum SOD activity (a) and MDA content (b) in melasma model mice (Note: # # p < 0.01 indicates extremely significant difference compared with the blank control group, * * p < 0.01 indicates extremely significant difference compared with the model group).

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