

Inhibitory Mechanism of Salidroside on Tyrosinase

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Abstract Tyrosinase is a key rate-limiting enzyme in melanin synthesis which can be blocked to reduce melasma and freckles by inhibiting the activity of tyrosinase. Salidroside showed certain inhibitory effects on tyrosinase by enzyme kinetics with inhibition of 33.04%. Besides it was also a competitive inhibitor of tyrosinase which served as a substrate analogue to combine with enzyme to produce quinones with stronger light absorption. Furthermore, docking results implied that inhibitory mechanisms might be attributed to the weak interactions between salidroside and tyrosinase such as hydrogen bonds and van der Waals. Salidroside is a main kind of plant flavonoids from *Rhodiola rosea*, walnut and so on. Studying inhibitory mechanism of salidroside on tyrosinase is not only helpful for developing anti-freckle products in which salidroside is one of the most important components, but also beneficial for making better use of plant flavonoids to seek for a new generation of natural skin care cosmetics.

Keywords: tyrosinase, salidroside, inhibitor, mechanism, flavonoid

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1. Introduction

Tyrosinase (EC 1.14.18.1) also known as polyphenol oxidase (PPO) is a multifunctional copper-containing oxidase which is widely distributed in plants, animals and microorganisms [1]. Melanin, the major pigment in skin, plays an important role in protecting the skin against ultraviolet rays owing to its ability to absorb ultraviolet light and scavenge oxidative free radicals [2,3,4,5]. However, excessive melanin formation and accumulation of melanin can cause skin hyperpigmentation such as melasma and freckles [6]. The biosynthetic pathway of melanin includes following steps: Tyrosine is gradually oxidized to dopamine and dopamine quinone by tyrosinase. Then after rearrangement and a series metabolic process, they are aggregated into 5,6-dihydroxyindole and indole quinone. Eventually, melanin is formed [7,8]. Throughout the course of producing melanin, tyrosinase which is the main rate-limiting enzyme in formation process of melanin is both served as hydroxylase of tyrosine and dopa oxidase and oxidase of 5,6-dihydroxyindole [9,10]. The inhibition of melanin in skin mainly contains intracellular and extracellular inhibition of melanocyte. The inhibition of tyrosinase belongs to intracellular inhibition. According to different mechanisms of inhibition, the inhibition of tyrosinase can be divided into destructive and nondestructive inhibition. The former is achieved by inhibiting tyrosinase synthesis or replacing substrates of tyrosinase to block the formation of melanin, while the latter is realized by modifying and transforming the tyrosinase [11]. The mechanism related to lots of anti-freckle products is generally connected with

the ability of products to decrease melanin synthesis by inhibiting activity of tyrosinase, formation of melanin, and transference of melanosomes from melanocytes to keratinocytes [12,13]. At present, there are some compounds that also can inhibit the activity of tyrosinase, such as kojic acid and arbutin [14,15]. Nevertheless, the compounds have side effects, particularly low clinical efficiency and skin toxicity [16,17]. Therefore, it is necessary to develop new natural anti-freckle products among which phenolic and polyphenolic compounds of plant origin are likely to be another effective and safe skin care cosmetics [18,19,20].

Salidroside, a compound with the chemical structure of phenol glycosides, is an important kind of flavonoids extracted from the root of *Rhodiola rosea* [21], existed in seed coat of walnut and so on [22,23]. Besides roles in anti-inflammation [24], anti-oxidation [25], scavenging free radical [20], salidroside also can reduce ischemia-mediated myocardial damage [26], moreover, it has been shown that salidroside has ability to inhibit the activity of tyrosinase which is the main rate-limiting enzyme in the process of melanin synthesis [20]. So far there have been some cosmetics added to extracts in which salidroside is the main ingredient, however, until now no researches have been carried to explore the inhibitory mechanism of salidroside on tyrosinase. Thus the aim of this paper is to study the inhibitory mechanism of salidroside on tyrosinase in order to make better use of salidroside or extracts in which salidroside is the main ingredient to develop new anti-freckle products.

2. Materials and Methods

2.1. Material and Chemicals

Salidroside used in this study was purchased from Xi'an Kailai biological engineering Co. Ltd. (China). L-3,4-Dihydroxyphenylalanine (L-DOPA) was acquired from Xi'an Huilin Biological technology Co. Ltd. (China). CuSO₄ was from Tianjin Fengchuan Chemical Reagent Co. Ltd. (China). Dimethylsulfoxide (DMSO) was provided by MP Biomedicals LLC (U.S.). Mushroom tyrosinase (EC 1.14.18.1) was the product of the Worthington Biochemical Corporation (U.S.). The specific activity of the enzyme was 845 U/mg. All other reagents were of analytical grade. Ultrapure water was used for the whole process.

2.2. Tyrosinase Activity Assay

The tyrosinase activity assay was performed as previously described with some modifications [27,28,29]. The reaction media (3 ml) for tyrosinase activity assay contained 0.5 mM L-DOPA in 50 mM sodium phosphate buffer (pH 6.8), 0.1 ml of different concentrations of inhibitor that was dissolved in DMSO of which the final concentration in the test solution was 3.3%, and 0.1 ml of mushroom tyrosinase of which the eventual concentration was 6.67 µg/ml. Throughout the process, firstly 0.1 ml of different concentrations of inhibitor dissolved in DMSO solution was added into the test tube. Then 2.8 ml L-DOPA in 50 mM sodium phosphate buffer was also put into the test tube, and at the same time the temperature was controlled at 30°C. After incubation for 10 min, 0.1 ml of the aqueous solution of mushroom tyrosinase was added, immediately the solution was monitored for 1 min for the formation of dopachrome by measuring the linear increase in absorbance at 475 nm accompanying the oxidation of the substrates with a molar absorption coefficient of 3700 (M⁻¹•cm⁻¹) by ultraviolet spectrophotometer. The inhibition of tyrosinase activity was calculated as follows:

$$\text{Inhibition(\%)} = \left(1 - \frac{A_3 - A_4}{A_1 - A_2}\right) \times 100\%$$

Where A₁ was the absorbance at 475 nm with tyrosinase and substrate, A₂ was only with tyrosinase, A₃ was the absorbance at 475 nm with tyrosinase, substrate and inhibitor, A₄ was also with tyrosinase and inhibitor finally.

Keep concentration of substrate unchanged and measure the catalytic activity of mushroom tyrosinase at different enzyme concentrations and different concentrations of inhibitor in order to research that whether the inhibition of salidroside on tyrosinase is reversible or not. Then remain unchanged enzyme concentration and study the catalytic activity of mushroom tyrosinase at different concentrations of substrate and different concentrations of inhibitor so as to determine the inhibitory type of salidroside on tyrosinase by lineweaver-burk plots [29].

2.3. UV-VIS Spectra

The ultraviolet-visible spectroscopy was used to study the chelation of salidroside and copper ions in the active center of tyrosinase. Prepare three test tubes which were signed with 1, 2, 3, and respectively added to 2.8 ml ultrapure water and 0.1 ml salidroside that was dissolved

in DMSO. Then the first test tube was added to 0.1 mL tyrosinase which was dissolved in sodium phosphate buffer, and the second was put in 0.1 mL CuSO₄ solution, meanwhile the third was added to 0.1 mL sodium phosphate buffer. Keep temperature of 30°C for 10 min. Finally, Scan ultraviolet-visible spectrum of each mixture respectively in the wavelength of 200-600 nm in the reaction of 0, 10, 20, 30, and 40 min [30,31].

2.4. Model Building and Molecular Docking

Molecular docking was performed to investigate the binding mode of salidroside and tyrosinase by means of Autodock Vina version 1.1.2 [32]. The three-dimensional coordinate of the copper-bound tyrosinase (PDB ID: 2ZWG) was downloaded from Protein Data Bank (<http://www.rcsb.org/pdb/home/home.do>) and was used as the input structure for docking calculations because of the similarity between salidroside and 3,4-dihydroxyphenylalanine. The AutoDock Tools version 1.5.6 package (<http://mgltools.scripps.edu>) was employed to generate the docking input files. Ligand structure was prepared for docking by merging non-polar hydrogen atoms and defining rotatable bonds. The search grid of tyrosinase was identified as center_x: -14.594, center_y: -16.832, and center_z: 22.285 with dimensions size_x: 15, size_y: 15, and size_z: 15. In order to increase the docking accuracy, the value of exhaustiveness was set to 20. For Vina docking, the default parameters were used as described in the Autodock Vina manual unless otherwise specified. The top ranked pose as judged by the Vina docking score was subject to visually analyze using PyMOL software (<http://www.pymol.org/>).

3. Results and Discussion

3.1. Effects of Salidroside on the Activity of Tyrosinase

Through studying the reaction media (3 ml) for tyrosinase activity assay that contained 2.8 ml of 0.5 mM L-DOPA in 50 mM sodium phosphate buffer (pH 6.8), 0.1 ml of different concentrations of inhibitor and 0.1 ml of 6.67 µg/ml mushroom tyrosinase, the effects on the oxidation of L-DOPA of salidroside at different concentrations by mushroom tyrosinase were tested. Salidroside showed inhibitory effects on tyrosinase activity (Figure 1). What's more, when the concentration of salidroside was up to 0.1 mM, the highest inhibition rate of tyrosinase by salidroside reached 33.04%. Whereas, when the concentration of salidroside was over 0.1 mM, with the increasing concentration of inhibitor, the inhibition of tyrosinase by salidroside was reduced. According to the following study, salidroside was served as substrate analogue to react with tyrosinase. When concentrations of inhibitor became too high, the inhibitors competed with each other to combine with the enzyme, which might lead to the change of spatial structure of enzyme. However, the change might be helpful for the combination between substrate and enzyme, so the inhibition rate of salidroside on tyrosinase was reduced. In a word, the result suggested that salidroside had ability to inhibit the activity of tyrosinase, and also could reduce melasma and freckles to some extent.

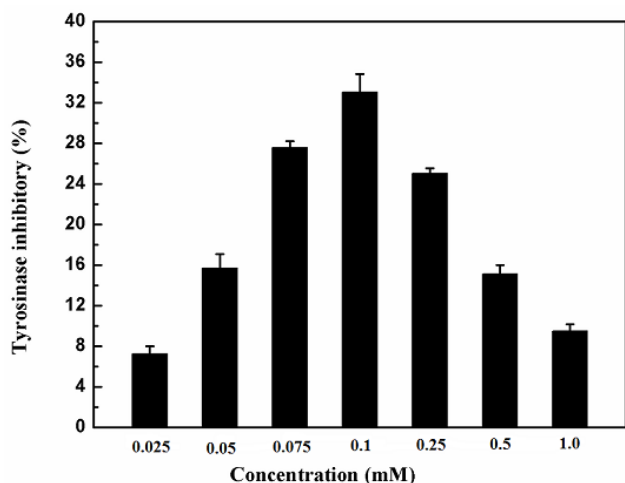


Figure 1. Inhibition of tyrosinase by different concentrations of salidroside

3.2. Inhibitory Type of Salidroside on Tyrosinase

The inhibitory type of mushroom tyrosinase of L-DOPA by salidroside was also studied. The relationship between enzyme activity and different concentrations of salidroside was showed in Figure 2. The plots of the remaining enzyme activity versus enzyme concentrations at different concentrations of salidroside formed a series of straight lines which all passed through the origin. Increasing the concentration of inhibitor resulted in a descent of the line slope, indicating that the inhibition by salidroside on the activity of mushroom tyrosinase was reversible. So the effector reduced catalytic efficiency of enzyme by inhibiting enzyme activity, which led to the decrease of enzyme activity, not by reducing the amount of enzyme. Thus salidroside was a reversible inhibitor of mushroom tyrosinase for the oxidation of L-DOPA.

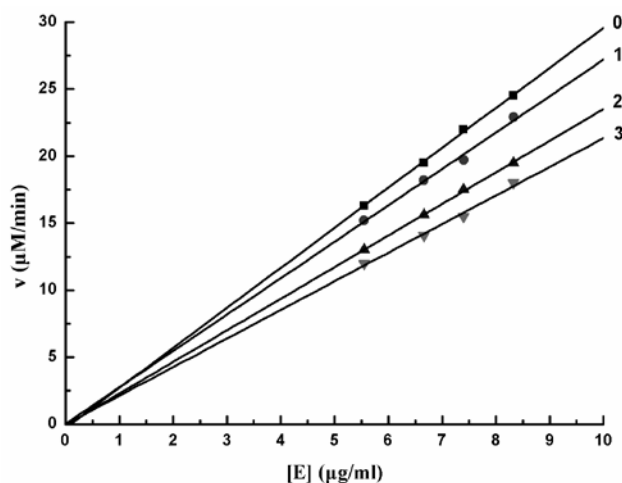


Figure 2. The catalytic activity of tyrosinase at different enzyme concentrations and different concentrations of salidroside. Concentrations of salidroside for curves 0-3 were 0, 0.1, 0.25, and 0.5 mM, respectively

The kinetic behavior of mushroom tyrosinase during the oxidation of L-DOPA was investigated, and the oxidation of L-DOPA by mushroom tyrosinase followed Michaelis–Menten kinetics. Lineweaver–Burk plot yielded a series of straight lines intersecting at the ordinate (Figure 3). With increasing concentration of salidroside, K_m increased,

while V_{max} was constant. Thus salidroside belonged to competitive inhibitor, and indicated that salidroside competed to the substrate with the free enzyme. The equilibrium constant for inhibitor binding with free enzyme, K_i , was obtained from a plot of the apparent Michaelis–Menten constant (K_m^{app}) versus the concentration of salidroside, which was linear as shown in the inset. The obtained constant was 0.882 mM.

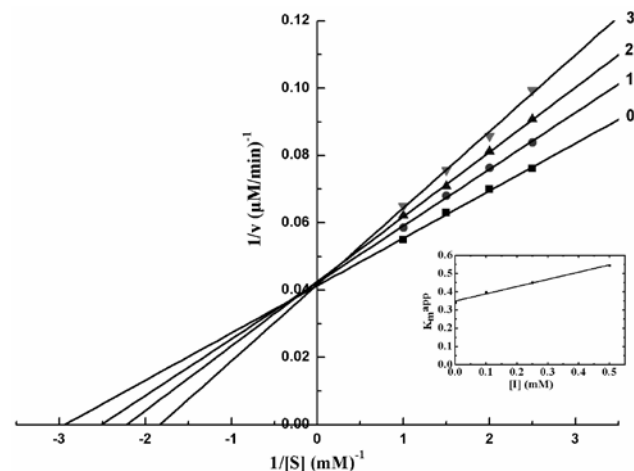


Figure 3. Lineweaver–Burk plots for the catalysis of L-DOPA by tyrosinase at 30°C, pH 6.8. Concentration of salidroside for plots 0-3 was 0, 0.1, 0.25, and 0.5 mM, respectively. The inset represented the plot of apparent Michaelis constant (K_m^{app}) versus the concentration of salidroside for determining the inhibition constant K_i

3.3. Analysis of UV Spectrum of Tyrosinase with Salidroside

Tyrosinase including two copper ions located on helical bundle (CuA and CuB) in its active center belonged to the copper metalloprote. Particularly the active center had a high conservation, thus it played an important role in the catalytic process of tyrosinase [33]. In ultraviolet visible range of wavelengths, small molecule inhibitors with characteristic absorption would chelate with tyrosinase, which could enhance internal conjugation of inhibitors and ultraviolet absorption peak of inhibitors got red-shifted [30,31].

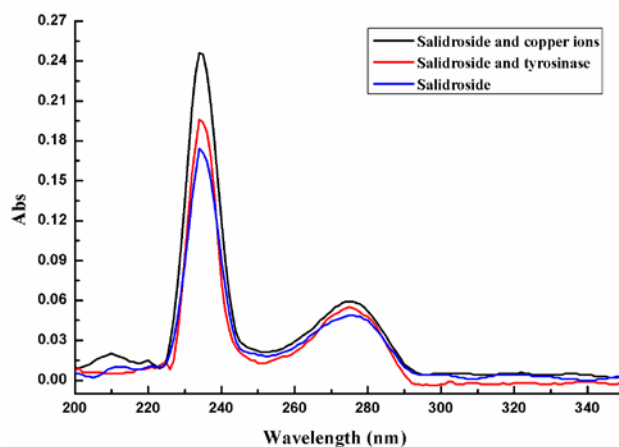


Figure 4. Ultraviolet-visible spectrum of each mixture in the wavelength of 200–600 nm. The three plots represented Ultraviolet-visible spectrum of each mixture only with salidroside, added tyrosinase and copper ions, respectively

The paper studied the ultraviolet absorption spectrum of salidroside when $CuSO_4$ solution or tyrosinase was added.

As Figure 4 shown, salidroside had the maximum absorption at 234nm and 275nm. After putting excess copper ions into salidroside, the wavelength of characteristic absorption didn't change, which indicated that salidroside didn't chelate with copper ions. Similarly, when tyrosinase was added to the solution of salidroside, the wavelength of characteristic absorption still didn't change, which further illustrated that salidroside didn't chelate with copper ions in active center of tyrosinase.

Scanning the ultraviolet-visible spectroscopy in the reaction of 0, 10, 20, 30, and 40 min found that only when test tube was added to copper ions or sodium phosphate buffer, the ultraviolet absorption spectrum of salidroside didn't change over time. However, as Figure 5 shown, only when test tube was added to tyrosinase without substrate, salidroside could be catalyzed to produce reaction products which significantly enhanced the absorption in the area of ultraviolet and visible light, whereas, the product had significant absorption peak at 234 nm and 275 nm, so it might be quinones. With the time going from 0 to 40 min, the absorption of ultraviolet and visible light became stronger and stronger, and it meant that the reaction products were accumulated gradually. Salidroside had phenolic hydroxyl groups similar to tyrosine, thus perhaps enzymatic oxidation of hydroxyl group lead to the intensive absorption of light. Hence salidroside served as substrate analog of tyrosinase to react with tyrosine to product quinones which could enhance the light absorption [34].

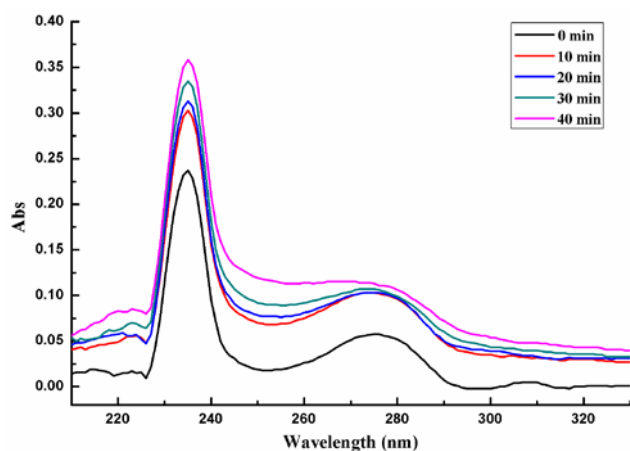


Figure 5. Ultraviolet-visible spectrum of each mixture of tyrosinase in the wavelength of 200-600 nm at different times. The five plots represented Ultraviolet-visible spectrum of each mixture in the reaction of 0, 10, 20, 30, and 40 min, respectively

When seeking and developing a kind of anti-freckle product, not only should we consider that whether it had a mild and significant effect on skin or not, and at the same time if it was safe and reliable, but also we should consider its own economic and comprehensive benefits. Though some extracts in which salidroside was the main ingredient were low-purity, the methods of extraction and separation could be optimized to improve yield. The monomers related to plants such as salidroside could be artificially synthesized by the means of organic synthesis, which not only decreased complexity of manufacturing, but also reduced costs. Meanwhile some monomers related to plants had ability to anti-microbial, anti-lipid peroxidation, antihypertension and so on [24,25]. Hence the functions could be maximized by combining with

modern biotechnology to achieve a kind of anti-freckle product with high comprehensive performance.

3.4. Molecular Modeling for the Binding of Salidroside with Tyrosinase

According to the result of modeling docking, salidroside was docked into the binding pocket of the tyrosinase. Detailed analysis showed that phenyl group fitted into the aromatic-rich binding pocket, surrounded by the aromatic side Phe59, Trp184, His54, His38 and His97 (Figure 6). Moreover, the phenyl ring of salidroside was vertical to the aromatic ring of residue Trp184, giving rise to a positive CH- π interaction. The glucose moiety of salidroside was located in the entrance of binding pocket of tyrosinase and made a high density of van der Waals when it contacted with the around residues. Importantly, the 2-OH, 3-OH, 4-OH and 6-OH of glucose formed hydrogen bond interactions with residues His97, Asn191, Ala202 and Thr203, respectively, which was main binding affinity between salidroside and tyrosinase. All in all, docking results implied that the possible inhibitory mechanisms may be attributed to the weak interactions between salidroside and tyrosinase such as hydrogen bonds and van der Waals.

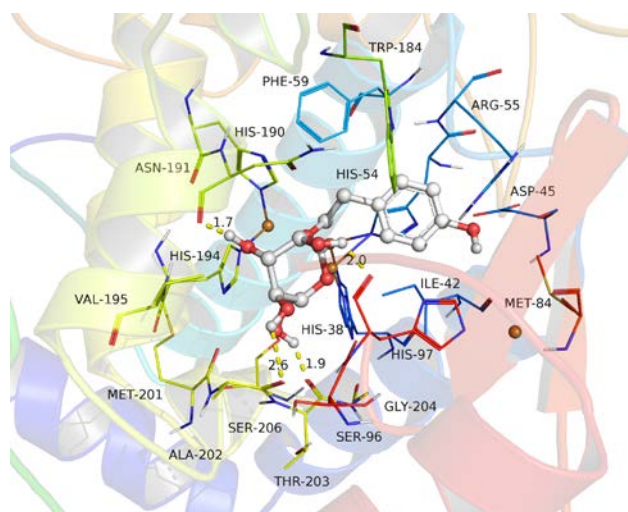


Figure 6. Detailed analysis of interactions between salidroside and tyrosinase

L-DOPA and salidroside had similar molecular structures, and according to the previous experimental results, it could be inferred that salidroside functioned as substrate analogs to react with tyrosinase. From the molecular structure point of view, L-DOPA was chelated with copper ions located in the active center of tyrosinase on account of the o-phenolic hydroxyl group which was in a plane, while salidroside was only with phenolic hydroxyl group, so as to salidroside couldn't chelate with copper ions. Furthermore, even if the glucose moiety of salidroside contained o-phenolic hydroxyl group, it also wasn't able to chelate with copper ions due to retrorse spatial structure and flexible tetrahydropyran rings which were adverse to chelation. In a word, The results based on molecular docking further validated that salidroside didn't react with copper ions located in the active center of tyrosinase, which was in accordance with the conclusion achieved by UV spectrum. In addition, Donghyun Kim et al. had found that some flavonoids had a high inhibition to

tyrosinase, which was related to the ability to form chelate with copper ions [35]. However, in this experiment, the inhibition of salidroside on tyrosinase was only up to 33.04%. Thus, it could be speculated the reason that salidroside didn't have too high inhibition to tyrosinase was that the inhibitor couldn't react with copper ions located in the active center of tyrosinase. So far many researches had showed that flavonoids had a high inhibition to tyrosinase such as quercetin due to the similar structures to L-DOPA, and others such as rutin also had a great inhibition to tyrosinase which mainly reacted with copper ions located in the active center of enzyme [35,36]. In conclusion, it could be conjectured that the inhibition ability to inhibitors of tyrosinase was not only related to the similarity of substrates, but also associated with the extent of chelation between inhibitor and copper ions located in the active center of tyrosinase.

4. Conclusions

So far there had been some cosmetics added to extracts in which salidroside was the main ingredient, however, until now no researches had been carried to explore the inhibitory mechanism of salidroside on tyrosinase. Here, we found that salidroside showed certain inhibitory effects on enzyme activity by enzyme kinetics, moreover the highest inhibition rate was up to 33.04%. Besides salidroside was also a competitive inhibitor of tyrosinase which served as a substrate analogue to combine with enzyme to produce quinones that could enhance the light absorption in ultraviolet-spectral analysis. Salidroside was a main kind of plant flavonoids from *Rhodiola rosea*, walnut and so on. Studying the inhibitory mechanism of salidroside on tyrosinase was not only helpful for developing anti-freckle products in which salidroside was one of the most important components, but also beneficial for making better use of plant flavonoids to seek for a new generation of natural skin care cosmetics. In addition, molecular docking showed that the weak interactions between salidroside and tyrosinase contained hydrogen bonds and van der Waals, and it also gave us rational explanation of interaction between salidroside and tyrosinase, which provided valuable information for further study of tyrosinase inhibitors. Furthermore, according to the result of molecular docking, we conjectured that the inhibition ability to inhibitors of tyrosinase was associated with the similarity of substrates and the extent of chelation between inhibitor and copper ions located in the active center of tyrosinase. Thus, we could consider using molecular docking to optimize the molecular structure of salidroside or design structural analogues of salidroside so as to obtain some monomers with high inhibition rate of tyrosinase, which provided a new way to study the anti-freckle products.

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