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Inhibitory effects of the extracts of *Ziziphora clinopodioides* Lam. on mushroom tyrosinase activity

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ABSTRACT

Ziziphora clinopodioides Lam. has been used in traditional Uyghur medicine for a long time. In order to illustrate the pharmaceutical activity of Z. clinopodioides as skin-whitening agents, The petroleum ether, chloroform, ethyl acetate, water extracts, and hydrodistilled essential oil from Z. clinopodioides were evaluated for their inhibitory effects on tyrosinase. The inhibitory effects on tyrosinase were weak but show a dose-dependent type. We also studied preliminary phytochemical and showed differences were exist between different extract. As rosmarinic acid is a major active compound in Z. clinopodioides, the result of tyrosinase activity indicated rosmarinic acid have both inhibit and activate effect at low and high does, respectively. A 3D model showed a good docking result between rosmarinic acid and tyrosinase. The weak effects may reveal that the concentration was too low to inhibit this enzyme or tyrosinase inhibitor and activator exist in Z. clinopodioides at the same time.

Key words: tyrosinase, inhibition, *Ziziphora clinopodioides* Lam., preliminary phytochemical, rosmarinic acid, molecule docking.

INTRODUCTION

Traditional herbs and plants are widely used for their medicinal properties. Their extracts are rich of bioactive compounds and have been shown to exert various biological activities and pharmacological effects. *Ziziphora clinopodioides* Lam., a perennial plant belonging to the Lamiaceae, is a well known traditional Uyghur medicinal herb from Xinjiang, China. It is used for the treatment of hypertension, fever, edema, heart disease, neurasthenic, insomnia, tracheitis, lung abscess and hemorrhoids [1,2] Other ethnobotanical uses of *Ziziphora* species have been recorded in Iranian and Turkish folk medicines as stomachic, carminative and wound healing material [3,4]. In folk society, the fresh and dry *Z. clinopodioides* was also used as a kind of aromatic ingredient in noodle and tea. Previous pharmacological investigations have determined antimicrobial [5], antifungal [6], antioxidant [7], and anti-hypertension [8] due to the essential oils. Phytochemical investigations of this genus described it as a source of flavonoids, polyphenols, polysaccharides, fatty acids and sterols [9]. The herb is able to produce some phenolic compounds such as caffeic acid, rosmarinic acid, and flavonoid derivatives including luteolin, linarin, diosmin, and thymonin [10, 11]. Moreover, this aromatic plant is rich in essential oil. Pulegone, trans-isopulegone, menthol, 1,8-cineole and limonene were found to be the major components of *Z. clinopodioides* essential oil [12].

Tyrosinase (EC 1.14.18.1) also known as polyphenol oxidase, catechol oxidase, It is a copper- containing aerobic enzyme which has complex structure of the multisubunit copper oxidoreductase [13]. It is widely distributed in animals, plants, microorganisms and human body. It plays the role of key enzyme and ratelimiting enzyme in the synthesis of melanin, involved in determining the color of mammalian skin and hair [14]. The role of melanin is to protect the skin from ultraviolet (UV) damage by absorbing UV sunlight and removing reactive oxygen species (ROS). But various dermatological disorders, such as melasma, ephelide, age spots and sites of actinic damage [15], arise from the accumulation of an excessive level of epidermal pigmentation. Great interest has been shown in the

involvement of melanins in malignant melanoma, the most life-threatening skin tumors.

Plant sources rich in bioactive chemicals, mostly without harmful side effects, are attracting increasing efforts on screening for cosmetic products. Some phenolic compounds, such as ellagic acid, tannic acid, and quercetin, act as potent tyrosinase inhibitors [16]. The essential oil obtained from some plants are currently used as natural preservatives in cosmetic and food products for their anti-tyrosinase properties, such as lavender and peppermint essential oil [17]. Recently, safe and effective tyrosinase inhibitors have become important for their potential applications in improving food quality and preventing pigmentation disorders and other melanin-related health problems in humans [18]. Furthermore, tyrosinase inhibitors are also important in cosmetic applications for skin-whitening effects, because many men and women prefer a lighter skin colour. Since plants are a rich source of bioactive chemicals, and are mostly free of harmful side effects, there is an increasing interest in using them as a source of natural tyrosinase inhibitors.

In this work, we study for the first time to reveal the effect of tyrosinase activity of volatile oil, nonvolatile parts (different polar extracts) from *Z. clinopodioides* and one of its major active compounds, namely rosmarinic acid. Using mushroom tyrosinase as a convenient model, in view of the applications of these natural substances either as ingredients of skin-whitening cosmetics or as additives for food products.

EXPERIMENTAL SECTION

1.1 Plant materials

The samples were collected in Xinjiang and identified by Yonghe Li, a chief apothecary of the Chinese Medicine Hospital of Xinjiang, China. All *Ziziphora clinopodioides* Lam. were cut into small pieces and dried in shade. All were separately powdered in a blender, screened using a 200 mesh sieve.

2.2 Chemicals, enzymes and instrument

Mushroom tyrosinase (Sigma, USA), L-DOPA, kojic acid (Aladdin Industrial Corporation), rosmarinic acid (China Pharmaceutical and Biological Products, China), dimethyl sulfoxide, sodium dihydrogen phosphate, disodium hydrogen phosphate, ultra-pure water and other reagents were of analytical grade; bioluminescence instrument (Molecular Devices, Inc., USA), 96-well plates (Costar company), precision analytical balance (Switzerland Mettler).

2.3 Preparation of different extracts of *Z. clinopodioides*.

The *Z. clinopodioides* were weighed accurately (218.54 g). The powdered plant material was firstly immersed with 500 mL of 80% ethanol at room temperature for 2 hours and then extract via ultrasonic method (80 W, 25 °C, 30 min) for 3 times. Combined all the extract and filtered through filter paper and evaporated in reduced pressure until dryness to give the crude extracts. Then extract with petroleum ether, chloroform, ethyl acetate, and distilled water, progressively. Each obtained extraction was concentrated by a rotary evaporator under reduced pressure, and the condensed extract was frozen at －70 °C in an ultra-low temperature freezer, and then pulverized in a freeze dryer for 24 h. The resulting powder was used for this study. Yields (w/w) of the prepared extracts were obtained as follows; petroleum ether extract (PEE): 9.38 g; chloroform extract (CE): 13.65 g; ethyl acetate extract (EAE): 12.23 g; water extract (WE): 16.79 g.

The air-dried *Z. clinopodioides* were extracted via a hydrodistilled in a Clevenger apparatus for 3 hours. The essential oil were collected and stored at 4°C, in air-tight vials, shielded from light.

2.4 Determination of tyrosinase inhibition activity

Mushroom tyrosinase (EC 1.14.18.1) was used for the bioassay because it is readily available. Since the mode of inhibition depends on the structure of both the substrate and inhibitor, L-DOPA was used as the substrate in this experiment, unless otherwise specified. Therefore, inhibitors discussed in this paper are inhibitors of diphenolase activity of mushroom tyrosinase, and their effect on the enzyme was determined by spectrophotometry, based on dopachrome formation at 490 nm. All the samples were first dissolved in dimethyl sulfoxide (DMSO) and used for the experiment at 30 times dilution. The assay was performed as previously described [19], with some modifications. L-DOPA solution (100 μL, 4.5 mM) was mixed with 60 μL of 20 mM phosphate buffer (pH 6.8), and incubated at 37 °C for 10 min. Then, 20 μL of various concentrations of sample solutions followed by 20 μL of the aqueous solution of mushroom tyrosinase (120 U/mL) was added to the mixture and the enzyme reaction was monitored by measuring the change in absorbance at 490 nm (at 37 °C), corresponding to the formation of dopachrome, for 25 min at 1 min intervals. Kojic acid was used as reference standard inhibitor for comparison. Tyrosinase activity inhibition rate of samples were calculated according to the following formula:

Inhibition rate% = [(A1 – A2) – (A3 – A4)] / (A1 – A2) × 100%

In this formula: A1 = A490 – sample + substrate + enzyme;

A2 = A490 – sample – substrate + enzyme;
A3 = A490 + sample + substrate + enzyme;
A4 = A490 + sample – substrate + enzyme.

As rosmarinic acid is a major active compound in *Z. clinopodioides*, the same activity inhibition test was also study at the same time. Tyrosinase was treated with various does of rosmarinic acid as the process mentioned above.

2.5 Preliminary phytochemical screening and TLC assay

The various extracts of *Z. clinopodioides* were subjected to qualitative chemical examination by Yu et al. [9], including tests of alkaloids, carbohydrates, steroids, flavonoids, tannins and phenolic compounds, saponins, proteins and amino acids.

A high-performance thin-layer chromatographic method was applied for the evaluation of different *Z. clinopodioides* extracts. TLC was performed on silica gel GF254 TLC plates (5 cm × 10 cm). Before use, the plates were activated for 10 min at 105°C. The samples (5 μL) and the standards were spotted on the plates as bands, 6 mm wide (distance between tracks 9.0 mm, first application position 15.0 mm, distance from the lower edge of the plate 8.0 mm). Plates were developed as reported by Zhang et al. [20], with ethyl acetate-formic acid-acetic acid-water (15:1:1:1.5, v/v) as the mobile phase in a twin-trough chamber, Before development, the chamber was lined with filter paper and presaturated with mobile phase vapor for 20 min. The development distance from the lower edge ascended to 90 mm of the plate. The temperature and relative humidity were 21–24°C and 40–45%, respectively, measured by use of an Acurite instrument. After development the plate were removed from the chamber and dried. Profile pictures were taken in CAMAG documentation visualize attached with DXA252 camera under UV 254 nm, 365 nm and in visible light (derivatization if necessary).

2.6 In silico docking of tyrosinase and rosmarinic acid

An available 3D model of human tyrosinase was builded by homology modeling based on the X-ray crystal structure of hemocyanin (PDB code 3NQ1) [21], which was obtained from a protein data bank (http://www.rcsb.org). Polar hydrogens were added to a macromolecule by using AutoDock, after which the structure was saved in PDBQT file format that contains a protein structure with hydrogen in all residues. For ligand, the 3D structure of rosmarinic acid was searched in the PubChem database (http://pubchem.ncbi.nlm.nih.gov/). The SDF file of ligand was converted into a PDB file by using Open Babel software. The structure of rosmarinic acid was minimized by computing gasteiger changes and the structure was saved in PDBQT file format via AutoDock.

We used the automated version of AutoDock program (AutoDock Vina) [22] in the structure-based virtual screening of tyrosinase inhibitors. The selected values for grid dimensions and the center were 25 × 25 × 25 and x = -9.685, y = -18.675, and z = 6.986, respectively. The predicted binding affinity (kcal/mol), which indicates how strongly a ligand binds to a receptor, was calculated on the basis of the scoring was identified using the binding affinity scores. Docking was performed with default values selected for the energy grid forcefield, and 'Minimizer' was selected for the minimization algorithm. The preferable orientation of the protein with compound was then presented. To present molecular docking results, PyMOL software was used to show the homology structure of human tyrosinase with rosmarinic acid.

RESULTS AND DISCUSSION

3.1 Inhibitory Effect of *Z. clinopodioides* extracts on Mushroom Tyrosinase Activity

The nonessential and essential extracts from *Z. clinopodioides* were tested for their inhibitory activity using mushroom tyrosinase. DOPA oxidase activity was assayed according to standard procedure, monitoring DOPA chrome formation at 490 nm.

Both water extract (figure 1A) and essential oil (figure 1B) of *Z. clinopodioides* had the inhibition effect on tyrosinase, although the inhibition rate was strong at a high concentration or a weak inhibition at a low concentration. And the inhibition rates were decreased with the concentration of extracts reduced. That means the inhibition has a relation of concentration dependence. The maximal inhibitory effect obtained for water and essential oil was 88.48 % at 100 mg/ mL and 118.85 % at 0.8 g/mL, respectively. The effects of PEE, CE and EAE were not showed because of weak inhibition.

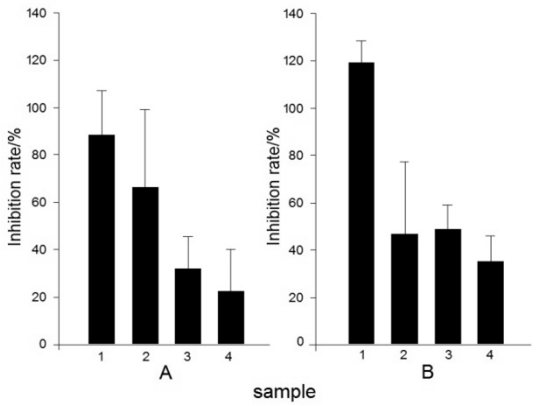

Fig. 1 Inhibition effect of *Z. clinopodioides* extracts with different doses on tyrosinase activity

A is the water extract with the dose of 100, 50, 25, 12.5 mg/mL from 1 to 4. B is the essential oil with the dose of 0.8， 0.4， 0.2， 0.1 g/mL from 1 to 4. Data are expressed as mean ±SEM; n=3.

3.2 Preliminary phytochemical screening of *Z. clinopodioides*.

Biological activity and pharmacological effects have a close relation with the chemical compounds of drug or herb material. Different phytoconstituents were confirmed from the preliminary phytochemical screening and TLC test of *Z. clinopodioides*. In table 1, major plant secondary metabolite was concentrate in EAE and WE parts. Flavonoids, tannins and phenolic compounds were the most significant. The HPTLC profile of the PEE (T1), CE (T2), EAE (T3), WE (T4) parts of *Z. clinopodioides* was showed under UV 365 nm (Fig. 2A), UV 365 nm with derivatization of 10% ethanolic−sulphuric acid (Fig. 2B), and white light (Fig. 2C). In figure 2A, the red band was striking at Rf = 0.98 in T1 and T2 and different blue bands were distributed homogeneously between the Rf value of 0 to 0.30 in T3 and T4 which means rich chemical component exist in EAE and WE parts. However, less and weak band showed in this range to T1 and T2. Figure 2B showed the details of TLC plate after derivatization. T3 showed two totally different bands to other extracts, a yellow band at the Rf value of 0.46 and a blue band at 0.76. These two bands were obvious and indicated that there was a high content distributed in EAE. More details about TLC *Z. clinopodioides* were showed in Table 2.

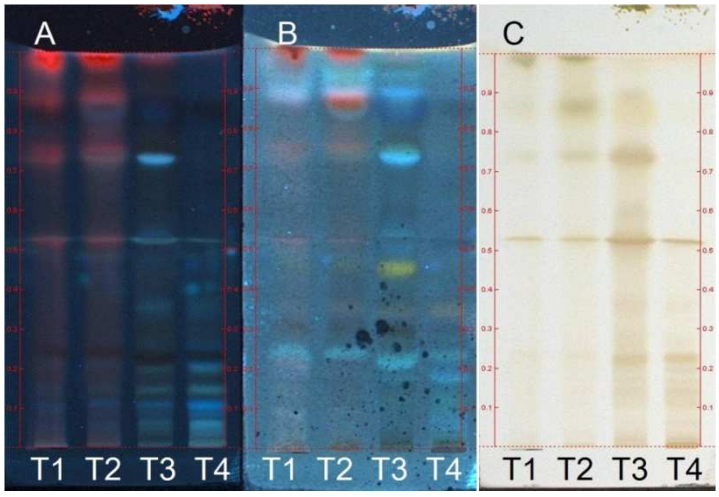

Fig. 2 (HP)TLC details of different polar extracts of *Z. clinopodioides*

A is under UV 365 nm; B is under UV 365 nm and sprayed with 10% ethanolic−sulphuric acid; C is under white light. T1: petroleum ether extract, T2: chloroform extract, T3: ethyl acetate extract, T4: water extract.

Table 1 Preliminary phytochemical screening of *Z. clinopodioides*

Phytoconstituents	Extract			
	PEE	CE	EAE	WE
Alkaloids	−	−	−	−
Carbohydrates	−	−	−	+
Steroids	−	−	+	+
Flavonoids	−	−	−	++
Tannins & phenolic compounds	+	+	+++	+++
Saponins	−	−	+	+
Triterpenes	−	−	−	+
Proteins & amino acids	−	−	−	−

+ Indicates presence and − indicates absence.

Table 2. TLC details of *Z. clinopodioides* extracts

		UV 365nm				UV 365nm & derivative				White light			
No.	**Rf**	**T1**	**T2**	**T3**	**T4**	**T1**	**T2**	**T3**	**T4**	**T1**	**T2**	**T3**	**T4**
1	**0.02**			black									
2	**0.06**			light blue	blue							light brown	light brown
3	**0.11**	light blue	light blue	blue	blue								
4	**0.14**	light green	light green	light blue	blue								
5	**0.18**								blue				light brown
6	**0.20**			light blue	blue								light brown
7	**0.24**	dark red	dark red	black	black	blue	blue	blue	blue	light brown	light brown	brown	brown
8	**0.36**			light blue			light orange	light orange	orange			light brown	light brown
9	**0.46**						light yellow	yellow					
10	**0.52**	red	red	blue	blue	pink	pink	blue		brown	brown	brown	brown
11	**0.58**											light brown	
12	**0.73**	pink	pink	blue			yellow	blue					
13	**0.76**	pink	pink	black		pink	pink	green		light green	green	brown	
14	**0.84**	black	light blue	dark blue	black		light blue	dark blue		light green	dark green	brown	
15	**0.88**					pink	red	blue		light green	dark green	brown	
16	**0.89**	pink	pink			light blue	light blue	blue					
17	**0.94**						light blue	light blue					
18	**0.98**	red	red	pink		red	red			dark green	dark green		

T1: petroleum ether extract, T2: chloroform extract, T3: ethyl acetate extract, T4: water extract

3.3 Inhibitory effect of rosmarinic acid on mushroom tyrosinase activity

Rosmarinic acid (Fig. 3A) is a phenolic compound commonly found in various plants of the Lamiaceae family. Rosmarinic acid exhibits different biological activities such as antioxidant [23], anti-inflammatory [24], anticancer [25], antimicrobial [26] activities. As one of the bioactive compounds in *Z. clinopodioides*, rosmarinic acid was also study to effect tyrosinase activity. As shown in Figure 2, we found that the tyrosinase inhibition rate negative correlated with the dose of rosmarinic acid within the range of 500−62.5 μg/mL. On the contrary, it showed a inhibition effect when the dose of rosmarinic acid was 0.1 and 0.05 μg/mL, Two opposite effects exist to one compound, higher dose negative inhibit tyrosinase and showed a negative correlation between dose and inhibition, while lower dose inhibit tyrosinase activity and maybe it is a positive correlation. Kojic acid was used as reference standard inhibitors for comparison and its inhibition was 94.76% when the dose was 500 μg/mL. This result indicated that rosmarinic acid was a tyrosinase activator or inhibitor with different dose level.

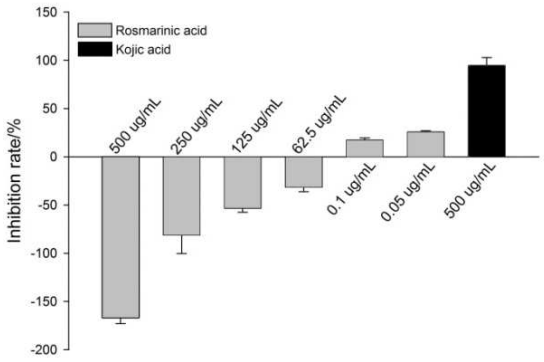

Fig. 2 Tyrosinase activity effect with various doses of rosmarinic acid and kojic acid was used as reference inhibitor at 500 μg/mL

3.2 Molecular docking study

In this literature, it is assumed that many tyrosinase inhibitors act by complexing the two copper ions, present in the active site of the enzyme [27]. To explore potential binding interactions of rosmarinic acid with human tyrosinase, we carried out molecular docking studies with a focus on the di-copper binding site. We then carried out docking study of rosmarinic acid in the active site of human tyrosinase with the AutoDock Vina program that can perform flexible-ligand docking using the LGA algorithm. The proposed favorable binding orientations of rosmarinic acid with the lowest binding free energy estimated by AutoDock. As shown in Figure 3B, the two hydroxyl groups of rosmarinic acid can form hydrogen bonds with residues Gly216, Gly46, and Asn57, with a bonding energy of -5.4 kcal/mol. Importantly, one of the ortho-dihydroxyl groups in rosmarinic acid makes metal-coordination interactions with the copper ions. Such coordination interactions are consistent with key binding interactions between monophenol or ortho-diphenol oxidases and their phenol substrates [28]. Based on this model, we suggest that the reduced effect of rosmarinic acid on human tyrosinase may be because it bind the active site as indicated by AutoDock.

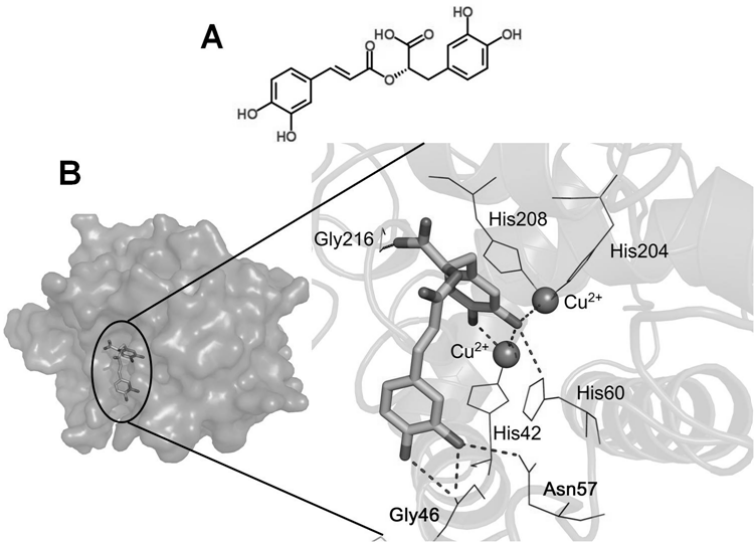

Fig. 3 (A) Compound structure of rosmarinic acid. (B) The proposed binding modes of rosmarinic acid in the active site of human tyrosinase

The inhibitor molecule is showed as sticks and the residues are showed as lines. The dashed lines show hydrogen-bonding or metal-coordination interactions. The docking models were generated using the AutoDock program and optimized by MD simulation and energy minimization.

CONCLUSION

In this study, the inhibitory effects of *Z. clinopodioides* volatile, nonvolatile parts and rosmarinic acid on tyrosinase and were evaluated. We have demonstrated for the first time that the essential oils and water extract of *Z. clinopodioides* exhibit a weak inhibitory effect on mushroom tyrosinase activity but the observed enzymatic inhibition was dose-dependent. The weak effects may reveal that the concentration was too low to inhibit this enzyme or tyrosinase inhibitor and activator exist in *Z. clinopodioides* at the same time. Analysis of their

preliminary phytochemical revealed each polar part were different. Rosmarinic acid showed activated in high dose level and inhibited in low dose. Result indicated it was both tyrosinase inhibitor and activator. Our findings suggest that extract and rosmarinic acid of *Z. clinopodioides* may be potential candidates for the development of skin-whitening and/or anti-food-browning agents. Further research will focus on the investigation of their compounds effect on tyrosinase independently and interaction between each compound, as well as on the isolation and characterization of their active constituents.

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REFERENCES

[1] Y.M. Liu. Pharmacography of Uighur, Xinjiang Science & Technology & Hygiene Publishing House, Urumqi, **1999** pp. 446-449.
[2] F. Senejouxa, C. Girarda, P. Kerramb, H.A. Aisac, A. Berthelota, F. Bévalota and C. Demougeota, *J. Ethnopharmacol.*, **2010**, 132, 268-273.
[3] G.E. Meral, S. Konyalioglu, B. Ozturk, *Fitoterapia*, **2002,** 73, 716-718.
[4] S. Ozturk and S. Ercisli, *Food Control* **2007,** 18, 535-540.
[5] Z.H. Ji, Q. Yu, X.Y. Zhou and H. Upur, *J. Xinjiang Med. Univ.*, **2012,** 35, 1031-1034.
[6] J. Behravan, M. Ramezani, M.K. Hassanzadeh, M. Eskandari, J. Kasaian and Z. Sabeti, *J. Essent. Oil Bear. Pl.*, **2007**, 10, 339-345.
[7] S.G. Tian, Y. Shi, X.Y. Zhou, L. Ge and H. Upur, *Pharmacogn. Mag.*, **2011**, 7, 65-68.
[8] Y.T. Guo, W. Lan, Y.N. Wu and Z. Geng, *J. Xinjiang Med. Univ.*, **2014**, 7, 257-260.
[9] Q. Yu, Y. Shi, S.N. Yuan and S.G. Tian, *J. Xinjiang Med. Univ.*, **2012,** 35, 301-305.
[10] X.J. Yang, N. Li, D.L. Meng, W. Li and X. Li, *J. Shenyang Pharma. Univ.*, **2008**, 25, 456-458.
[11] S.G. Tian, Q. Yu, L.D. Xin, Z.S. Zhou and H. Upur, *Nat. Prod. Commun.*, **2012,** 9, 1181-1184.
[12] W.H. Ding, T. Yang, F. Liu and S.G. Tian, *Pharmacogn. Mag.*, **2014,** 37, 1-5.
[13] F. Solano, S. Briganti, M. Picardo, G.H. Ghanem. *Pigm. Cell Res.*, **2006**, 19, 550-571.
[14] M.P. Germano, F. Cacciola, P. Donato, P. Dugo, G. Certo, V. D'Angelo, L. Mondello, A. Rapisarda. *Fitoterapia*, **2012**, 83, 877-882.
[15] E. Frenk. Treatment of melasma with depigmenting agents. In: Melasma: New Approaches to Treatment, Martin Dunitz, London, **1995**, pp. 9-15.
[16] M. Pitchaon, H.G. Michael. *Food Chem.*, **2009**, 117, 332-341
[17] D. Fiocco, D. Fiorentino, L. Frabboni, S. Benvenuti, G. Orlandini, F. Pellatic, A. Galloned. *Flavour Fragr. J.*, **2011**, 26, 441-446.
[18] Y.J. Kim, H. Uyama. *Cell Mol. Life Sci.*, **2005,** 62, 1707-1723.
[19] C.W. Zhang, Y.H. Lu, L. Tao, X.Y. Tao, X.C. Su, D.Z. Wei. *J. Enzym. Inhib. Med. Chem.*, **2007**, 22, 91-98.
[20] X.J. Zhang, J. Li, H. Upur, S.G. Tian. *J. Planar Chromatogr.*, **2014** 27, 186-191.
[21] M. Sendovski, M. Kanteev, B.V. Shuster, N. Adir, A. Fishman. *J. Mol. Biol.*, **2011**, 405, 227-237.
[22] O. Trott, A.J. Olson. *J. Comput. Chem.*, **2010**, 31, 455-461.
[23] B. Tepea, O. Eminagaoglub, H.A. Akpulata, E. Aydin, *Food Chem.*, **2007**, 100, 985-989.
[24] X. Chu, X. Ci, J. He, L. Jiang, M. Wei, Q. Cao, M. Guan, X. Xie, X. Deng, J. He, *Molecules* **2012**, 17, 3586-3598.
[25] D.O. Moon, M.O. Kim, J.D. Lee, Y.H. Choi, G.Y. Kim, *Cancer Lett.*, **2010,** 288, 183-191.
[26] S. Moreno, T. Scheyer, C.S. Romano, A.A. Vojnov, *Free Radical Res.*, **2006**, 40, 223-231.
[27] A. Sanchez-Ferrer, J.N. Rodriguez-Lopez, F. Garcia-Canovas, F. Garcia-Carmona. *BBA-Protein Struct. M.*, **1995**, 1247, 1-11.
[28] Y. Matoba, T. Kumagai, A. Yamamoto, H. Yoshitsu, M. Sugiyama. *J. Biol. Chem.*, **2006**, 281, 8981-8990.