

## Research Article

# Tyrosinase Inhibitory Activity of Major Fractions of *Quercus Infectoria* Galls

Fariba Sharififar<sup>1</sup>, Gholamreza Dehghan-Nudeh<sup>2</sup>, Zeinab Raeiat<sup>3</sup>, Bagher Amirheidari<sup>1</sup>, Mandan Moshrefi<sup>4,5</sup>, Amin Purhemati<sup>\*4,5</sup>

<sup>1</sup>Traditional and Herbal Medicines Research Center, Faculty of Pharmacy, Kerman University of Medical Sciences, Kerman, Iran

<sup>2</sup>Pharmaceutics Research center, Faculty of Pharmacy, Kerman University of Medical Sciences, Kerman, Iran

<sup>3</sup>Student Research Committee, Faculty of Pharmacy, Kerman University of Medical Sciences, Kerman, Iran

<sup>4</sup>Department of Plant Protection, Faculty of Agriculture, Shahid Bahonar University of Kerman, Kerman, Iran

<sup>5</sup>American Chemical Society, Washington, DC 20036, USA

**ABSTRACT: Objective:** Our previous studies have shown high tyrosinase inhibitory effect of *Quercus infectoria* galls, an endemic plant to Iran. Considering the potency of tyrosinase inhibitors in cosmetic as a skin depigmentation and lightening agent, we have studied the *Q. infectoria* galls, and its major fractions for tyrosinase-inhibitory activity. **Materials and methods:** A methanolic extract of *Q. infectoria* galls was evaporated in vacuum. The resulting residue was suspended in water and extracted successively with a combination of petroleum ether, chloroform, ethyl acetate and methanol in increasing order of polarity. As a result, fractions 1–18 were obtained. The fractions were initially screened for the O-diphenolase inhibitory activity of tyrosinase using L-tyrosine as a substrate on TLC plate by the bioautography method. All the active inhibitors from the first test were dissolved in methanol to give different concentrations. 80 microliter of L-tyrosine (0.5mM) was added to wells containing a 50µl sample, and incubated for 10 minutes at room temperature. Seventy µl of mushroom tyrosinase (1000 units/ml) was added and incubated again for 10 min at 35°C. The enzyme reaction was monitored by measuring the change in absorbance at 475 nm (at 37°C) for 10 and 20 min after incubation. The percent of inhibition of the enzyme was measured and IC<sub>50</sub> values of each sample were calculated by probit analysis. **Results:** Amongst the separated fractions, fractions V and VI separated in ethyl acetate-methanol exhibited the highest inhibition of mushroom tyrosinase (89.2% and 93.65% inhibition respectively) in comparison to kojic acid (96.54% inhibition). The least IC<sub>50</sub> values were due to fractions of V and VI (IC<sub>50</sub> values of 12.0 and 8.0µg/ml respectively). The total extract induced 86.34% inhibition with IC<sub>50</sub> value of 42.0µg/ml (kojic acid with IC<sub>50</sub> = 2.7 µg/ml). **Conclusion:** Our findings indicated that the fractions with potent antityrosinase effect were extracted in the ethyl acetate-methanol fraction, so may have semi-polar nature like gallic acid and/or ellagic acid or might be from flavonoids. The confirmation of this needs further fractionation which is the subject of further study in our laboratory.

**KEYWORDS:** Fractionation, *Quercus infectoria* galls, Tyrosinase inhibition, Phenolic acids

## INTRODUCTION

Skin has an important physiological role in human and its pigmentation is one of the defense mechanisms against UV radiation hazards. Melanin is a pigment which is

secreted by melanocytes in epidermis of basal cells and is considered as an important defense mechanism against UV radiation.<sup>[1]</sup> Tyrosinase is a monooxygenase enzyme which catalyzes two reactions of hydroxylation of (L) tyrosine to L-DOPA followed by its oxidation leading to melanin biosynthesis. L-DOPA oxidation results a reactive product.<sup>[2]</sup> This is a copper-containing membrane-bound enzyme. Irregular and excessive melanin production or its abnormal distribution leads to hyperpigmentation of the skin. Many approaches such as competitive or non-competitive inhibition of activity of tyrosinase or reducing the enzyme stability have been used for control of melanogenesis.<sup>[3]</sup>

\*Correspondence

Amin Purhemati

Tel.: +98 913 343 1841; Fax: +98 341 244 8696

E- mail: a.purhemati@gmail.com

DOI: 10.5530/pc.2013.1.6

Galls of *Quercus infectoria* (Fagaceae), a plant found in Iran, Greece and Asia Minor, have been used in Iranian medicine as an astringent, for the treatment of throat pain, and as antidiarrhea.<sup>[4]</sup> Different medicinal values have been reported for plant galls in the literature, for example for wound healing,<sup>[5]</sup> as a local anaesthetic,<sup>[6]</sup> an anti-inflammatory,<sup>[7,8]</sup> and antibacterial agent.<sup>[9]</sup>

Our previous studies have proven the high tyrosinase inhibitory effect of methanolic extract of *Q. infectoria* galls.<sup>[10,11]</sup> Considering the potency of tyrosinase inhibitors in cosmetic as a skin depigmentation and lightening agent, we aimed to separate major fractions with antityrosinase effect from *Q. infectoria* galls as a first step in isolation of the active components.

## MATERIALS AND METHODS

### Chemicals

Mushroom tyrosinase, L-tyrosine and kojic acid were purchased from Sigma. Hydrogen phosphate monosodic dihydrogen phosphate monosodic and the different solvents were prepared from Merck, Germany. The other materials were analytical grade.

### Plant materials

The galls of *Q. infectoria* were purchased from a local market and authenticated by Dr. Mirtadzadini, Bahonar University, Botany Department, Kerma, Iran. Plant materials were milled and extracted with 80% methanol for 72h. The extract was concentrated under vacuum to give a dried mass.

### Fractionation

An amount of 300g of dried extract was suspended in 400ml warm distilled water and extracted successively with the same volume of petroleum ether, chloroform, methanol in increasing polarity order.

### Liquid-liquid extraction (LLE) of active extract

The methanolic fraction which showed the most tyrosinase inhibition was used for more fractionation by LLE method. An amount of 100g of completely dried methanolic fraction was dissolved in 150 ml warm distilled water. The aqueous solution was partitioned sequentially with petroleum ether (5×150 ml); petroleum ether: chloroform (4:1) (5×150 ml); petroleum ether: chloroform (1:1) (5×150 ml); petroleum ether: chloroform (1:4) (5×150 ml); chloroform (5×150 ml); chloroform:ethyl acetate (1:1) (5×150 ml); chloroform:ethyl acetate (7:3) (5×150 ml); chloroform: ethyl acetate (1:9) (5×150 ml); ethyl acetate (5×150 ml); ethyl acetate: methanol (9:1)

(5×150 ml); ethyl acetate: methanol (4:1) (5×150 ml); ethyl acetate: methanol (3:2) (5×150 ml); ethyl acetate: methanol (1:1) (5×150 ml); ethyl acetate:methanol (2:3) (5×150 ml); ethyl acetate: methanol (3:7) (5×150 ml); ethyl acetate: methanol (1:4) (5×150 ml); ethyl acetate methanol (1:9) (5×150 ml); methanol (5×150 ml).

### Bioautography screening of separated fractions against tyrosinase activity

The different extracts (petroleum ether, chloroform, and methanol extracts) and isolated fractions were initially screened for the O-diphenolase inhibitory activity of tyrosinase using L-tyrosine as substrate on TLC plate by bioautography method.<sup>[12]</sup>

### Spectrophotometric determination of inhibitory effect of fractions against tyrosinase activity

Active fractions from the bioautography test were further studied for tyrosinase inhibition by spectrophotometry method with some modifications.<sup>[13]</sup> Each sample was dissolved in methanol to give at least 4 different concentrations. 80 µl of L-tyrosine (0.5 mM) was added to wells containing 50 µl of sample (in different concentrations) and incubated for 4 minutes in room temperature (25–27 °C). Seventy µl of mushroom tyrosinase (1000 units/ml) was added and incubated again for 10 min at 35°C. The enzyme reaction was monitored by measuring the change in absorbance at 475 nm (at 37 °C) after 10 and 20 min incubation. For each sample, a blank solution containing all ingredients except tyrosinase was used. Kojic acid in different concentrations and methanol were used as positive and negative controls respectively. The percent of tyrosinase inhibition was measured as following:

$$\text{Percent of inhibition} = (A_{\text{cont}} - A_{\text{sam}}) / A_{\text{con}} \times 100$$

Where in,  $A_{\text{cont}}$  = absorbance of control  $A_{\text{sam}}$  absorbance of sample.  $IC_{50}$  values of each sample were calculated by Probit analysis. The same method was used for evaluation of tyrosinase inhibitory effect of fractions resulted from LLE which were active in bioautography method.

### Statistical analysis

All samples were assayed in triplicate and the results were reported as mean ± SD.  $IC_{50}$  value of samples was calculated by Probit analysis.

## RESULTS

### Extraction results

The yield of total extract of dried and unprocessed galls of *Q. infectoria* was about 70.2% (w/w). The total extract

was completely dried to give a brittle pale brown colored extract. Amongst the different extracts, the methanolic extract obtained from fractionation of total extract by different solvents gave the highest percent yield (86.34%) (Table 1). The results of LLE lead to separation of 18 major fractions from which 13 fractions were selected on the basis of bioautography for spectrophotometry method (Figure 1).

### Bioautography of separated fractions

The majority of the tested fractions were active against tyrosinase activity in the bioautography assay. Amongst the 18 tested fractions by bioautography method, 13 fractions inhibited tyrosinase enzyme and potentially caused discoloration of the plate in a brown color

background (Figure 2). The information of six more potent fractions have given in Table 1.

### Spectrophotometry method for determination of tyrosinase inhibition

Thirteen active fractions were studied for quantitative measurements by the spectrophotometry method. As shown in Table 1, the maximum of inhibition and  $IC_{50}$  values of total and methanolic extracts and six active fractions have given in comparison to kojic acid. The total extract and the methanolic extract exhibited 83.2% and 86.34% tyrosinase inhibition respectively ( $IC_{50}$  value = 67.0 and 42.0  $\mu\text{g/ml}$  respectively). Amongst the separated fractions by LLE method, six fractions could potentially inhibit tyrosinase enzyme. The other

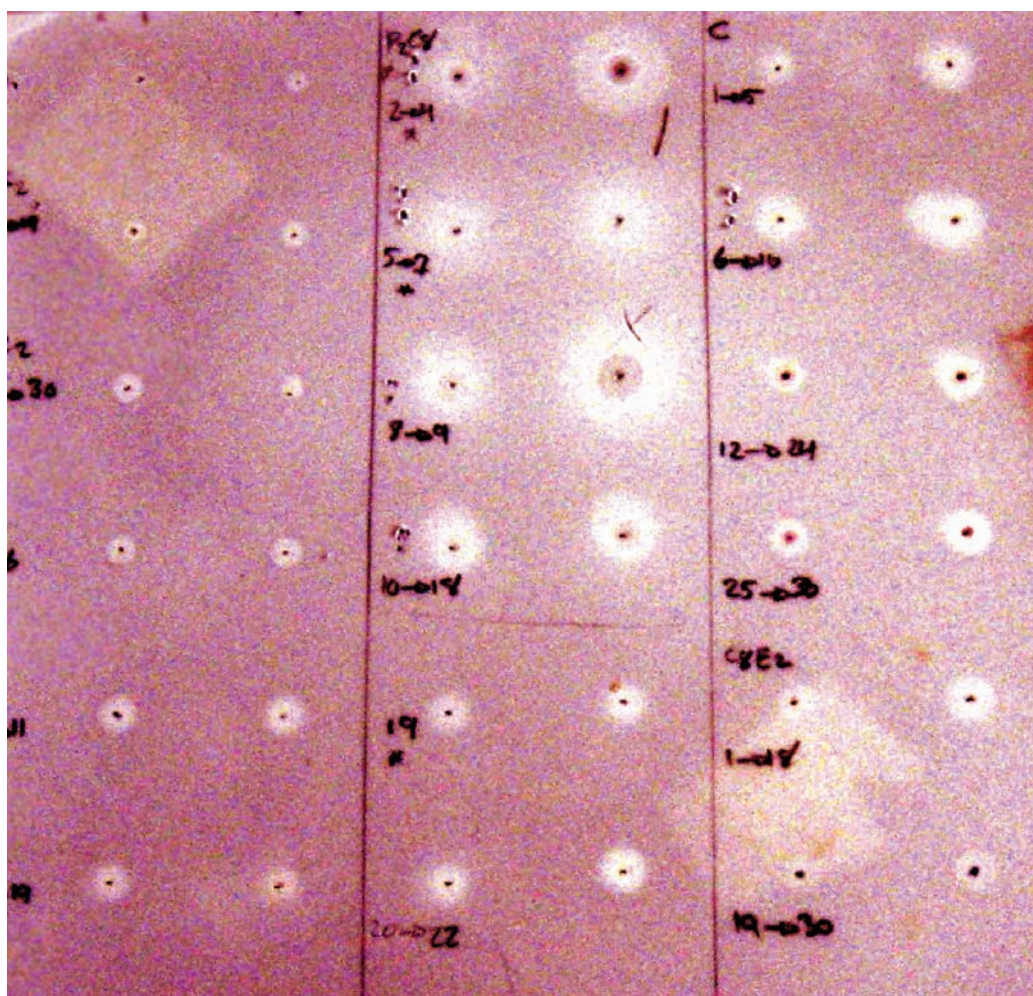
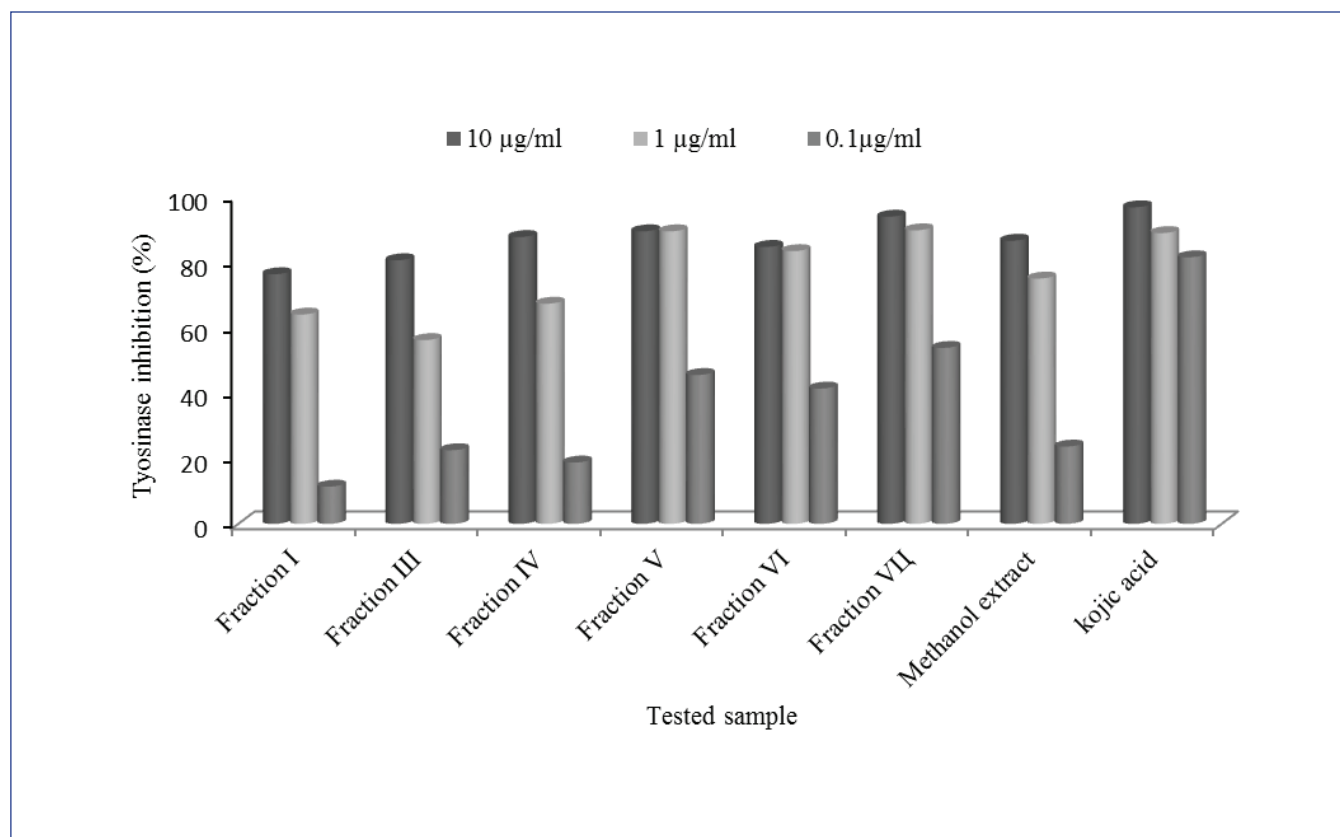


Figure 1: A demonstration of bioautography of 18 isolated fractions (in two concentration) from LLE separation of *Q. infectoria* galls.



**Figure 2:** Tyrosinase inhibitory (%) of different fractions isolated by liquid-liquid extraction in different concentration in comparison to kojic acid.

**Table 1: Results of extraction, fractionation and determination of  $IC_{50}$  and percent of tyrosinase inhibition by spectrophotometry method**

Sample	Percent of extraction (w/w)	Maximum tyrosinase inhibition (%)	$IC_{50}$ ( $\mu\text{g/ml}$ )
Total extract	70.2	83.2 $\pm$ 1.7	67.0 $\pm$ 1.2
Petroleum ether extract	6.18	ND	ND
Chloroform extract	4.22	ND	ND
Methanolic extract	87.6	86.34 $\pm$ 2.	42.0 $\pm$ 0.7
Fraction chloroform: ethylacetate; 5:5 (I)	4.2	76.12 $\pm$ 3.1	88 $\pm$ 2.7
Fraction chloroform: ethylacetate ; 1:9 (III)	25.5	80.38 $\pm$ 2.7	97 $\pm$ 1.9
Fraction ethyl acetate (IV)	5.53	87.48 $\pm$ 1.9	84 $\pm$ 1.4
Fraction ethyl acetate:methanol; 9:1(V)	16.8	89.2 $\pm$ 2.6	12 $\pm$ 0.4
Fraction ethyl acetate:methanol; 8:2 (VI)	19.1	84.44 $\pm$ 2.3	23 $\pm$ 0.3
Fraction ethyl acetate:methanol; 7:3 (VII)	16.8	93.65 $\pm$ 3.7	8.0 $\pm$ 0.3
Kojic acid	–	96.54 $\pm$ 2.1	2.7 $\pm$ 0.4

Each sample was tested in triplicate and the results are mean of three measurements.

$IC_{50}$ : concentration which inhibits 50% of tyrosinase.

ND: not determined.

fractions exhibited weak activity (data have not shown). All of these six fractions exhibited more than 76% tyrosinase inhibition (Table 1). Fractions V and VII separated in ethyl acetate-methanol, exhibited the highest inhibition of mushroom tyrosinase (89.20% and 93.65% inhibition respectively) in comparison to kojic acid (96.54% inhibition). The least  $IC_{50}$  values also were

due to these fractions ( $IC_{50}$  values = 12.0 and 8.0  $\mu\text{g/ml}$  for fraction V and VII respectively).

Tyrosinase inhibitory effect of fractions obtained by the LLE method after 10 and 20 min incubation are shown in Figure 1. The greatest inhibition was shown by fraction VII (93.65% inhibition). The tyrosinase inhibitory effect

of this fraction was dose-dependent and decreased over the time.

## DISCUSSION

Many of current drugs with antityrosinase effects such as arbutin and cyaniding have plant origins<sup>[14]</sup>. We have previously reported the antityrosinase effect of methanolic extract of *Q. infectoria*,<sup>[10,11]</sup> so the isolation of active compounds from this plant would be helpful for accessing new therapeutic agents. In the present study, we aimed to isolate the active fractions and compare their antityrosinase effect. Initial extraction was done using different solvents in order of increasing polarity. The results of bioautography of the different extracts indicated that the extract inhibited tyrosinase to a much greater extent than the other extracts and active compounds of the plant. The methanolic extract was used for subsequent fractionation which lead to separation of 18 fractions. Six fractions showed greater than 76% tyrosinase inhibition in spectrophotometry method (Table 1). Out of these, 3 fractions inhibited tyrosinase stronger than the methanolic extract (Table 1). Fractions IV and VI inhibited tyrosinase strongly (87.48% and 84.44% inhibition respectively) when compared with kojic acid (96.54% inhibition). Fraction V exhibited potent tyrosinase inhibition ( $IC_{50} = 12.0 \mu\text{g/ml}$ ), and fraction VII showed high potency tyrosinase inhibition ( $IC_{50} = 8.0 \mu\text{g/ml}$ ), which is nearly three times weaker than kojic acid which might be to the presence of a complex combination of different secondary metabolites in plant extracts and also in the isolated fractions. So the active compounds are expected even to be more active than kojic acid in equal concentrations.

The active fractions of *Q. infectoria* galls were extractable with a combination of methanol and ethyl acetate. Ethyl acetate is a specific solvent for flavonoids, thus the active fractions may contain flavonoids. There are many reports in the literature which show the anti tyrosinase effect of flavonoids.<sup>[15-17]</sup> Galls of *Q. infectoria* are composed mostly from phenolic acids including gallic acid, ellagic acid and tannic acid.<sup>[18,19]</sup> An ellagic acid rich pomegranate extract has previously been shown to have tyrosinase inhibition in both *in vitro* and *in vivo* methods.<sup>[20]</sup> Conversely, most phenolic acids have exhibited antityrosinase activity, so the separated fractions also might contain phenolic acids.<sup>[21-23]</sup>

## CONCLUSION

In our study, the suitable solvent for extraction of anti tyrosinase agent was found to be a combination of methanol and ethyl acetate. It is suggested that the active

agents of *Q. infectoria* galls for tyrosinase inhibition may be phenolic compounds, especially flavonoids and/or phenolic acids, although further work is required to confirm this and to isolate the active constituents.

## ACKNOWLEDGEMENTS

This work was supported by the Vice Chancellor for research, Kerman University of Medical Sciences, Kerman. This article also has been derived from the thesis of Pharm.D student, Zeinab Raeiat in Kerman University of medical Sciences, Faculty of Pharmacy, Kerman, Iran.

## REFERENCES

1. Burns T, Breathnach S, Cox N, Griffi C. Rook's Textbook of Dermatology: Wiley-Blackwell 2010; 1:51-2,514,3:2703-2709.
2. Katiyar S, Saify K, Rai M, Sharma A, Singh S, Singh N. A Systemic Review on Melasma: A review. Int J Cur Bio Med Sci. 2011;1:63-8.
3. Yoshimura K, Sato K, Aiba-Kojima E, et al. Repeated Treatment Protocols for Melasma and Acquired Dermal Melanocytosis. Dermatol Surg. 2006;3: 365-71.
4. Zargari A. Medicinal Plants. Volume 2. Tehran: Tehran University Pub.; 1972.
5. Umachigia S, Jayaveerab K, Kumarc CA, Kumard G, swamye BV, Kumar DK. Studies on Wound Healing Properties of *Quercus infectoria*. Trop J Pharm Res. 2008; 7:913-19.
6. Hussein G, Miyashiro H, Nakamura N, Hattori M, Kakiuchi N, Shimotohno K. Inhibitory effects of Sudanese medicinal plant extracts on hepatitis C virus protease. Phytother Res. 2000; 14:510-16.
7. Kaur G, Hamid H, Ali A, Alam M, Athar M. Antiinflammatory evaluation of alcoholic extract of galls of *Quercus infectoria*. J Ethnopharmacol 2004; 90:285-92.
8. Aroonrerk N, Kamkaen N. Anti-inflammatory activity of *quercus infectoria*, *Glycyrrhiza uralensis*, *kaempferia alanga* and *Coptis chinensis*, the main components of thai herbal remedies for aphthous ulcer. J Health Res 2009; 23:17-22.
9. Dar M, Ikram M, Fakouhi T. Pharmacology of *Quercus infectoria*. J Fharni Sci. 1976; 65:1791-4.
10. Ansari M, Etelaei F, Shariffar F. Tyrosinase kinetic in the presence of three inhibitory medicinal plants. Int J Green Pharm. 2011; 5:103-6.
11. Khzaeli P, Goldoozian R, Shariffar F. An evaluation of extracts of five traditional medicinal plants from Iran on the inhibition of mushroom tyrosinase activity and scavenging of free radicals. Int J Cosmetic Sci. 2009; 31:375-81.
12. Wangthong S, Tonsiripakdee I, Monhaphol T, Nonthabenjawan R, Wanichwecharungruang SP. Post TLC developing technique for tyrosinase inhibitor detection. Biochemical Chromatography. 2007; 21:94-100.
13. Jo Y-H, Seo G-U, Yuk H-G, Lee S-C. Antioxidant and tyrosinase inhibitory activities of methanol extracts from *Magnolia denudata* and *Magnolia denudata* var. *purpurascens* flowers. Food Nutr Res. 2011; In press.
14. yamakoshi J, Otsuka F, Sano A, et al. Lightening effect on ultraviolet-induced pigmentation of guinea pig skin by oral administration of a proanthocyanidin-rich extract from grape seeds. Pigment Cell Res. 2003;16:629-38.
15. Shimizu K, Kondo R, Sakai K. Inhibition of tyrosinase by flavonoids, stilbenes and related 4-substituted resorcinols: structure-activity investigations. Planta Med. 2000; 66:11-5.
16. Kim D, Park J, Kim J, et al. Flavonoids as Mushroom Tyrosinase Inhibitors: A Fluorescence Quenching Study. J Agric Food Chem. 2006;54:935-41.
17. Saewan N, Koysoomboon S, Chantrapromma K. Anti-tyrosinase and anti-cancer activities of flavonoids from *Blumea balsamifera* DC. Journal of Medicinal Plants Research 2011;5:1018-25.

18. Hamid H, Kaur G, Abdullah S, Ali M, Athar M, Alam M. Two new compounds from the galls of *quercus infectoria* with nitric oxide and superoxide inhibiting ability. *Pharm Biol.* 2005;43:317–23.
19. Ghafour N, Aziz H, alMolla R. Determination of Some Chemical Constitutes of Oak Plants (*Quercus* spp) in the Mountain Oak Forest of Sulaimani Governorate. (JZS) *Journal of Zankoy Sulaimani.* 2010;13:129–42.
20. Yoshimura M, Watanabe Y, Kasai K, yamakoshi J, Koga T. inhibitory effect of an ellagic acid-rich pomegranate extract on tyrosinase activity and ultraviolet-induced pigmentation. *Biosci Biotechnol Bioche.* 2005;69:2368–73.
21. Sangsrichan S, Ting R. Antioxidation and Radical Scavenging Activities and Tyrosinase Inhibition of Fresh Tea Leaves, *Camellia Sinensis.* *Sci J UBU.* 2010;1:76–81.
22. Alam N, Yoon K, Lee T. Evaluation of the antioxidant and antityrosinase activities of three extracts from *Pleurotus nebrodensis* fruiting bodies. *African Journal of Biotechnology* 2011;10:2978–86.
23. O'zer O, Mutlu B, Kivc B. Antityrosinase Activity of Some Plant Extracts and Formulations. Containing Ellagic Acid. *Pharm Biol.* 2007;45:519–24.