

## Research Article

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

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**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 VL 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 VL (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>

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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 anesthetic,<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 methannolic 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 72 h. The extract was concentrated under vacuum to give a dried mass.

### Fractionation

An amount of 300 g of dried extract was suspended in 400 ml 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 100 g of completely dried methanolic fraction was dissolved in 150 ml warm distilled water. The aqueous solution was partitioned sequentially with petroleum ether ( $5 \times 150$  ml); petroleum ether: chloroform (4:1) ( $5 \times 150$  ml); petroleum ether: chloroform (1:1) ( $5 \times 150$  ml); petroleum ether: chloroform (1:4) ( $5 \times 150$  ml); chloroform ( $5 \times 150$  ml); chloroform:ethyl acetate (1:1) ( $5 \times 150$  ml); chloroform:ethyl acetate (7:3) ( $5 \times 150$  ml); chloroform: ethyl acetate (1:9) ( $5 \times 150$  ml); ethyl acetate ( $5 \times 150$  ml); ethyl acetate: methanol (9:1)

( $5 \times 150$  ml); ethyl acetate: methanol (4:1) ( $5 \times 150$  ml); ethyl acetate: methanol (3:2) ( $5 \times 150$  ml); ethyl acetate: methanol (1:1) ( $5 \times 150$  ml); ethyl acetate:methanol (2:3) ( $5 \times 150$  ml) ; ethyl acetate: methanol (3:7) ( $5 \times 150$  ml); ethyl acetate: methanol (1:4) ( $5 \times 150$  ml); ethyl acetate methanol (1:9) ( $5 \times 150$  ml); methanol ( $5 \times 150$  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  $\mu$ l of L-tyrosine (0.5 mM) was added to wells containing 50  $\mu$ l of sample (in different concentrations) and incubated for 4 minutes in room temperature (25–27 °C). Seventy  $\mu$ l 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  $\pm$  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).

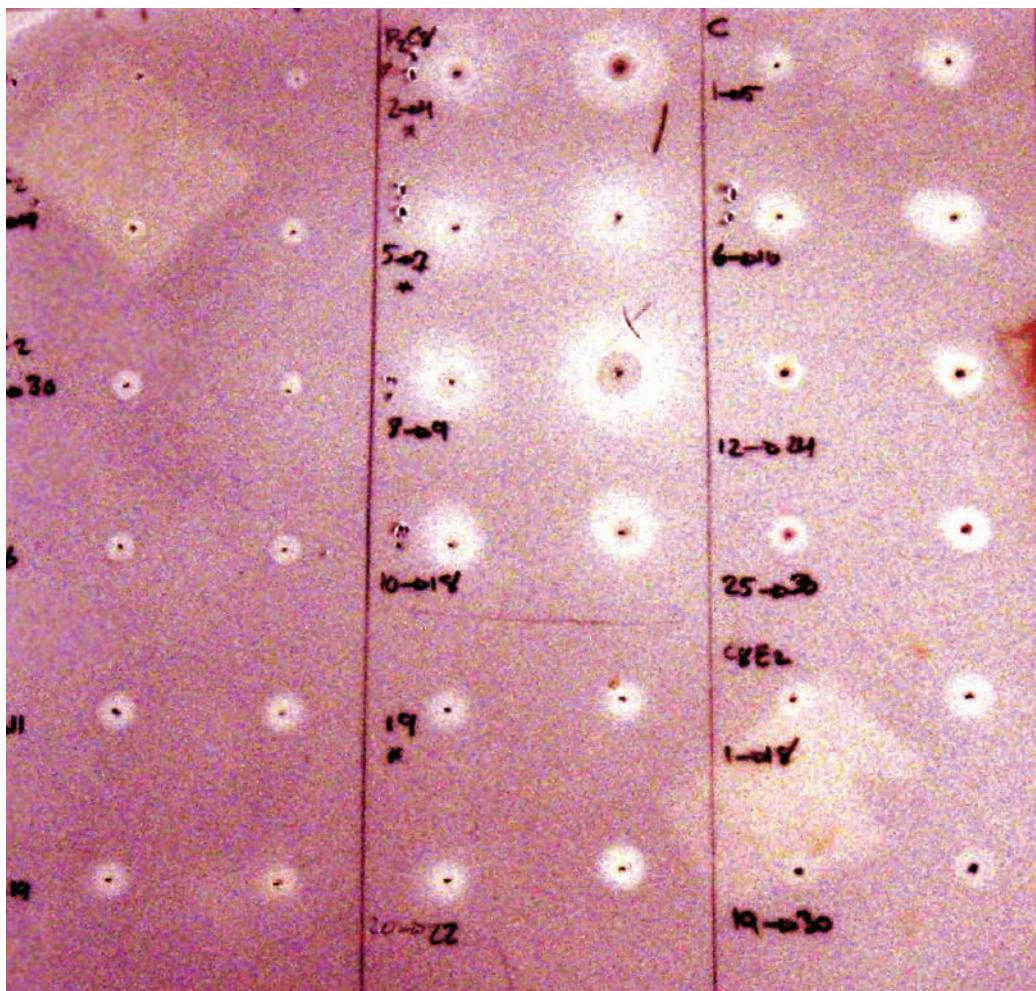
#### Bioautography of separated fractions

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

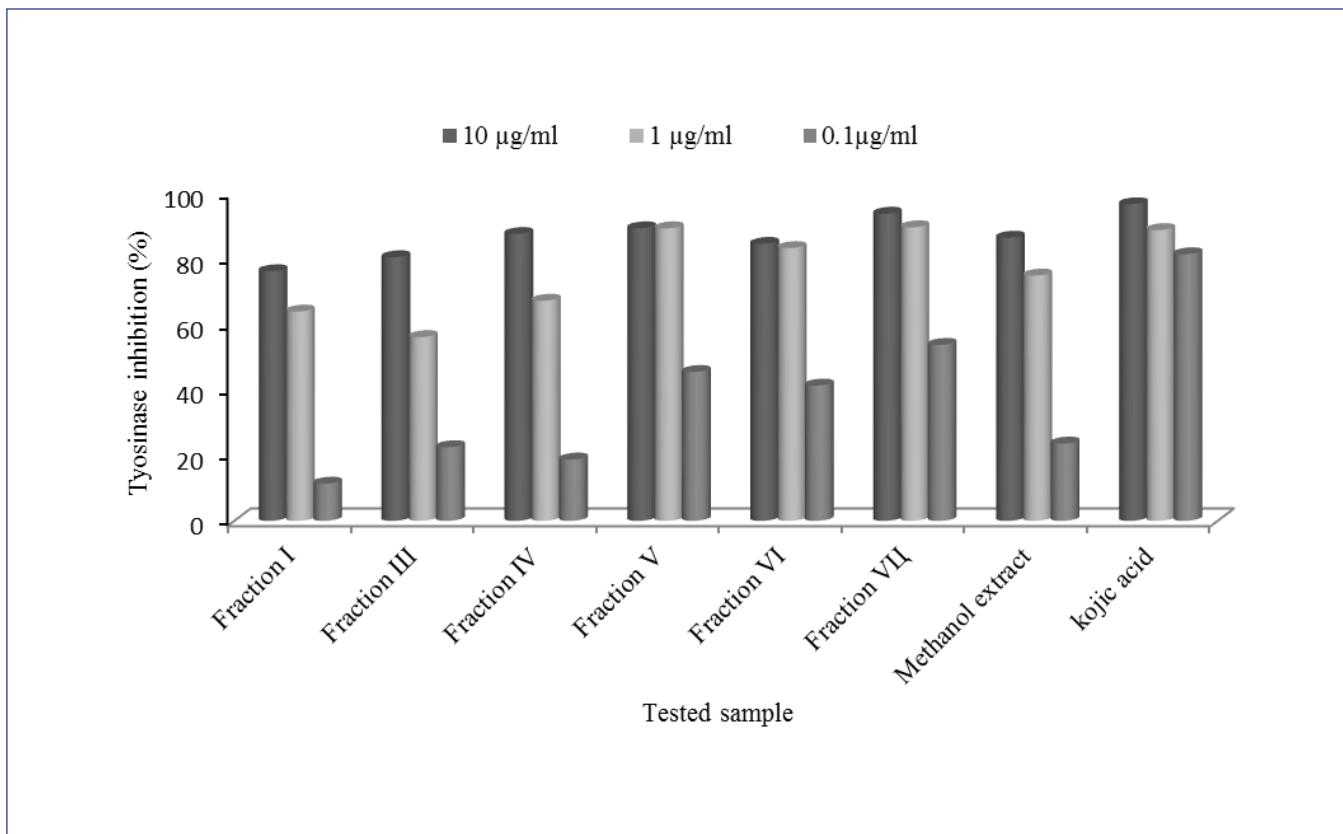
background (Figure 2). The information of six more potent fractions have given in Table 1.

#### 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  $\mu\text{g}/\text{ml}$  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<sub>50</sub> and percent of tyrosinase inhibition by spectrophotometry method**

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

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

IC<sub>50</sub>: 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<sub>50</sub> values also were

due to these fractions (IC<sub>50</sub> values = 12.0 and 8.0 µg/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 extarct 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 ( $IC_{50} = 12.0 \mu\text{g}/\text{ml}$ ), and fraction VII showed high potency tyrosinase inhibition ( $IC_{50} = 8.0 \mu\text{g}/\text{ml}$ ), 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.

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