



***Quercus infectoria* and *Terminalia chebula* decrease melanin content and tyrosinase activity in B16/F10 cell lines**

[*Quercus infectoria* y *Terminalia chebula* disminuyen el contenido de melanina y la actividad tirosinasa en las líneas celulares B16/F10]

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Abstract

Context: One of the most complained skin cares in ethnic skin like Asian women is hyperpigmentation, and lightening preparations have been long-standing desired. Due to the side effects of current drugs, medicinal plants have attracted more attentions as a source of novel drugs. Mazo (*Quercus infectoria* galls) and *Terminalia chebula* fruits have been suggested in Persian Traditional Medicine as a safe treatment for hyperpigmentation.

Aims: To evaluate the cytotoxicity and the effect on melanin synthesis in B16/F10 melanoma of *Q. infectoria* and *T. chebula* extracts.

Methods: After collection and scientific authentication, plants were extracted by maceration method with methanol and were standardized based on total phenolic content. MTT assay and colorimetric method were used for cytotoxicity and determination of melanin content and tyrosinase activity in B16/F10 cells, respectively. Kojic acid was used as a reference compound.

Results: Total phenolic content of *Q. infectoria* and *T. chebula* was determined as 287.34 ± 4.21 and 172.61 ± 8.67 mg gallic acid equivalent/g dried extract, respectively. Both plants decreased cell viability at 100 $\mu\text{g/mL}$ and significantly reduced intercellular melanin to 66.25% and 71.1%, respectively in comparison to kojic acid (56.63%) at 50 $\mu\text{g/mL}$. In the same concentration, 65.7% and 71.2% tyrosinase activity was inhibited by *Q. infectoria* and *T. chebula*, which significantly were different from control ($p < 0.001$).

Conclusions: These findings suggest that both plants especially *Q. infectoria* could inhibit melanogenesis in non-toxic concentrations and would be a good candidate for further studies.

Keywords: B16/F10 melanoma cells; depigmentation; melanin; *Quercus infectoria*; *Terminalia chebula*.

Resumen

Contexto: Uno de los cuidados de la piel más reclamados en la piel étnica de las mujeres asiáticas es la hiperpigmentación, y se han deseado, desde hace mucho tiempo, preparaciones de aclaramiento. Debido a los efectos secundarios de los fármacos actuales, las plantas medicinales han atraído más atenciones como fuente de nuevos fármacos. Mazo (agallas de *Quercus infectoria*) y frutos de *Terminalia chebula* han sido sugeridos en la medicina tradicional persa como un tratamiento seguro para la hiperpigmentación.

Objetivos: Evaluar la citotoxicidad y el efecto sobre la síntesis de melanina en células de melanoma B16/F10 de los extractos de *Q. infectoria* y *T. chebula*.

Métodos: Después de la recolección y la autenticación científica, las plantas se extrajeron por método de maceración con metanol y se normalizaron en base al contenido fenólico total. El ensayo MTT y el método colorimétrico se utilizaron para la citotoxicidad y la determinación del contenido de melanina y la actividad tirosinasa en células B16/F10, respectivamente. Se usó ácido kójico como compuesto de referencia.

Resultados: El contenido fenólico total de *Q. infectoria* y *T. chebula* se determinó como $287,34 \pm 4,21$ y $172,61 \pm 8,67$ mg equivalente de ácido gálico/g de extracto seco, respectivamente. Ambas plantas disminuyeron la viabilidad celular a 100 $\mu\text{g/mL}$ y redujeron significativamente la melanina intercelular a 66,25% y 71,1%, respectivamente, en comparación con el ácido kójico (56,63%) a 50 $\mu\text{g/mL}$. En la misma concentración, *Q. infectoria* y *T. chebula* inhibieron el 65,7% y el 71,2% de actividad tirosinasa, que fueron significativamente diferentes del control ($p < 0,001$).

Conclusiones: Estos hallazgos sugieren que ambas plantas, especialmente *Q. infectoria*, podrían inhibir la melanogénesis en concentraciones no tóxicas y sería un buen candidato para estudios posteriores.

Palabras Clave: células de melanoma B16/F10; despigmentación; melanina; *Quercus infectoria*; *Terminalia chebula*.

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INTRODUCTION

Melanogenesis is mainly responsible for skin and hair colors and plays an important protective role against the harmful effects of UV radiation. Overproduction of melanin creates various skin dermatological disorders like age spots, sites of actinic damage or other hyperpigmentation (Briganti et al., 2003; Lam et al., 2010). Due to catalytic role of tyrosinase in melanin production, compounds with tyrosinase inhibitory effect have been used in cosmetic products for skin lightening (Zhu et al., 2013). However, the concerns for the side effect of these preparations led to paying more attention to natural products especially medicinal plants. In Persian Traditional Medicine (PTM), different treatments have been proposed for skin lightening such as Mazo (*Quercus infectoria* galls) and Halila (*Terminalia chebula* fruits). In this study, cytotoxicity and anti-tyrosinase activity of a standardized extract of these medicinal plants have been examined for melanin production in B16/F10 murine melanoma cell line as a suit model for investigation on a human melanoma.

Quercus infectoria Olivier (oak) (Fagaceae family) is an endemic plant to Iran, Greece and different regions of Asia and is a gall-bearing small tree, which is found in Zagros forests at a high altitude of about 2500 m a.s.l. Zagros forests consist of a variety of leafy trees especially oak ones and cover a vast area of Zagros mountains in Iran ranges from Fars province to Western Azarbaijan province, northwest to southeast of Iran and roughly paralleling the country's western border (Olfat and Pourtahmasi, 2010). The gall of the plant is known in Persian Traditional Medicine (PTM) as "Mazo" with Arabic name of "Afis". Two types of Mazo have been used, the green, hard and heavy one and the other with yellow color with less hardness and weight. The first one has been used as anti melasma in PTM and folk medicine. A combination of vinegar and coarse particle of plant galls (1:4) with a small part of olive oil has been recommended for night topical use on skin. The second type has been used as hair color. In the literature, the plant galls have been reported to have anti-candida, analgesic and hepatoprotective

effects (Pithayanukul et al., 2009; Fan et al., 2014; Baharuddin et al., 2015). The major phytochemical of galls are from phenolic acids especially gallic acid and tannins (Shrestha et al., 2014). The fruits of *Terminalia chebula* Reitz. from Combretaceae family have been known with Persian name of "Halila" and common name of chebulic myrobalan. It grows throughout central Asia and some other parts of the world (Kapoor, 2000). Plant is native to Nepal, India, and west China and is called the King of Medicine because of its extraordinary power of healing. *T. chebula* has been used in PTM as astringent and for the treatment of cut, burns and "Ghorhe", which contains a broad variety of scars. In PTM, an aqueous extract of *T. chebula* (which has been grounded with pestle and mortar and levigates to give a fine particle powder) has been proposed topically for melasma (Razi, 2000). Traditional healers also use the *T. chebula* as an astringent and for the treatment of burning especially as a demulcent and for the treatment of pigmented area after burning. Anti-hepatitis C, analgesic, antioxidant and anticarcinogenic effects of the plant have been studied (Mahesh et al., 2009; Ajala et al., 2014; Rekha et al., 2014; Kumar et al., 2015). Plant fruits are composed of tannins, triterpenoids and phenolic acids such as gallic acid (Li et al., 2014; Zhang et al., 2015). Both *Q. infectoria* and *T. chebula* have exhibited strongly tyrosinase inhibitory activity in previous studies (Khazaeli et al., 2009; Ansari et al., 2011; Sharififar et al., 2012). This work aimed to evaluate the cytotoxicity and inhibitory effect of *Q. infectoria* and *T. chebula* on melanin synthesis in B16/F10 melanoma cells.

MATERIAL AND METHODS

Reagents

Fetal bovine serum (FBS) and Dulbecco's Modified Eagle's Medium (DMEM) and L-DOPA were purchased from Gibco-Invitrogen (USA). B16/F10 melanoma ATCCR CRL-6475TM cells were prepared from Pasteur Institute (Iran). Streptomycin - penicillin and trypsin were provided from Biosera, England. Mushroom tyrosinase, trichloroacetic acid (TCA), dimethyl sulfoxide (DMSO) and all other organic solvents were purchased from Sigma (USA).

Plant materials

Galls of *Q. infectoria* were gathered from Kermanshah in July 2015 and fruits of *T. chebula* were provided from the market. A voucher specimen of *Q. infectoria* was deposited in Herbarium Center of Razi, Agriculture Faculty (968RUH) and Faculty of Pharmacy (KF 1234). Authentication of the plant was done in Kerman University of Medical Sciences by Dr. Mirtdzadini.

Extraction

An amount of 500 g of *T. chebula* fruits and *Q. infectoria* galls were extracted with 500 mL of methanol 80% by percolation method separately. The extracts were concentrated under vacuum in rotary evaporator (Heidolph WB 2000, Germany), completely dried in an oven (Behdad, Iran) under 40°C and kept at -20°C until experiment.

Total phenolic content

Total phenolic content of tested plants was measured based on the gallic acid calibration curve. The λ_{\max} of gallic acid was determined by spectrophotometric method. Briefly, 0.1 g accurately weighed gallic acid was dissolved in 100 mL deionized water. Serial dilutions of gallic acid were prepared from stock solution. In the first order derivative method, the solutions were scanned at 200-400 nm by spectrophotometric method. The obtained spectrum was reprivatized from first to fourth order. First order derivative ($n=1$) spectrum showed good sensitivity and linearity hence the zero crossing wavelengths, and 291 nm was used for more analysis. The calibration curve was prepared by plotting absorbance of each standard solution versus its concentration at 291 nm by a UV-Visible spectrophotometer (Lambda 25, Perkin Elmer, USA). Each sample was tested three times, and the results were reported as mean \pm SD. All measurements were made at room temperature and pH 6.8. Validation parameters such as linearity, precision, and accuracy were determined. The correlation between concentration and absorbance was determined for linearity measurement. The intra-day and inter-day precision were determined by measuring the absorbance in the replicate standard samples at three concentration levels (40, 80 and 120

mg/L). For the intra-day assay precision, five replicates of the samples at each concentration were assayed all at once within a day. RSD% <5 was regarded as a criterion for acceptable precision. Accuracy was measured by the calculation of the error percent. For assaying the total phenolic content of the plant, 0.3 g of each dried plant extract was dissolved in deionized water. Absorbance was measured at 291 nm.

Cell studies

Cell culture

In the present study, B16/F10 melanoma cell line has been used that had the ability to produce melanin. The cells were maintained in 100 μ L Dulbecco's Modified Eagle's medium (DMEM) containing penicillin 100 unit/mL, FBS (10%) and streptomycin 100 μ g/mL and were cultured at 37°C in a humidified atmosphere (5% CO₂) (Nepco, Japan). After 24 hours, cells were treated with the extract, and after nine days extra and intracellular melanin was measured. Kojic acid (100 μ g/mL) was used as a positive standard. Plant extracts were added at concentrations of 10, 50, 100, 500 and 1000 μ g/mL to the cells.

MTT assay

Potential cytotoxic effects of the plant extracts on B16/F10 cells were evaluated by measuring cell survival by MTT method. MTT (tetrazolium salt, 3, 4, 5 dimethylthiazol-2, 5 diphenyl tetrazolium bromide), is a yellow tetrazole, which reduces to insoluble purple formazan in living cells. This method is used measured at 450- 600 nm by a spectrophotometer (UV-Vis Shimadzu 1240, Japan). The melanoma cell line was harvested in the exponential phase, seeded separately into 96-well plates (20,000 cell/well) and was allowed for 24 h until adhesion. Different concentrations of plant extract were added and incubated for 24 h. Then, 150 μ L DMEM containing MTT (5 mg/mL in PBS) was added to each well, incubated for 4 h at 37°C. The medium was removed, cells were incubated at 25°C for 1 h to dry and 150 μ L of DMSO was added and optical density was determined at 490 nm. MTT assay was performed in three replicates for each experiment (Doyle and Bryan, 1998).

Evaluation of melanin content

For the measurement of intracellular melanin, the cells were washed twice with 100 mL phosphate buffer saline (PBS), cells isolated from the bottom of the plate with trypsin 25%, EDTA (30 μ L), and PBS (100 μ L). Each well poured within a micro tube and was centrifuged at 1500 rpm for 10 min (Micro-centrifuge, Eppendorf, Germany). The supernatant was discarded, 0.5 mL distilled water was added to each tube. The cells lysed by double freeze-thaw and were centrifuged at 1500 rpm for 10 minutes. The sediment was washed and centrifuged three times (5 min) by 1% trichloroacetic acid (TCA) and twice with ethanol:ether (3:1) and dried on exposure to air. A volume of 0.3 mL KOH (0.85 N) was added and left at 100°C for 50 minutes. Absorbance was measured at 475 nm with spectrophotometer. All samples were run in triplicate.

Tyrosinase activity

The cell samples (2×10^5 cells) were incubated for 24 h at 37°C in a humidified atmosphere (5% CO₂). After 24 hours, the cells were treated with the extract and were washed twice with PBS. An amount of 300 mL PBS containing 1% Triton-X100 was added to each well and freeze-thawed. The mixture was then transferred into the micro tubes and centrifuged at 1500 rpm for 10 minutes. A volume of 90 μ L supernatant was added to 90 μ L L-

DOPA (2 mg/mL), and finally, the kinetic change in absorbance was studied at 475 nm over 60 min by Elisa reader (Bio-Tek Instruments, Winooski, USA) while incubated (Incubator, Iran Zaeim, Tehran, Iran) at a constant temperature of 37°C (Hunt et al., 1994). The correction was made for L-DOPA auto oxidation. All samples were run in triplicate.

Statistical analysis

Data were reported as the mean \pm SD derived from three replications. Comparison between the control and the test group was carried out using non-parametric test (Mann-Whitney test) by using SPSS 16.0 statistical software. The comparison between groups was performed using ANOVA, Duncan test. Differences were statistically significant at $p < 0.05$.

RESULTS

Total phenolic content (TPC) of plant extracts

Calibration curve of gallic acid was provided and TPC of each plant was determined as mg/g gallic acid equivalent (GAE) using equation of $Y = 0.0472X + 0.0953$, $R^2 = 0.9956$ (Fig. 1A). TPC of *Q. infectoria* and *T. chebula* was determined as 287.34 ± 4.21 and 172.61 ± 8.67 mg GAE/g extract) respectively. UV spectrum of gallic acid and plant extracts has been shown in Fig. 1B.

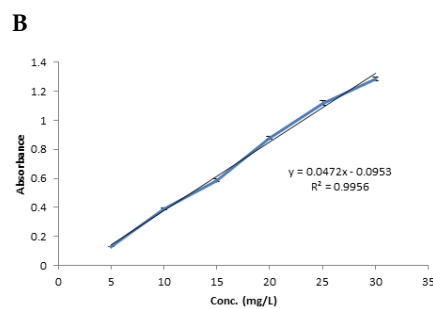
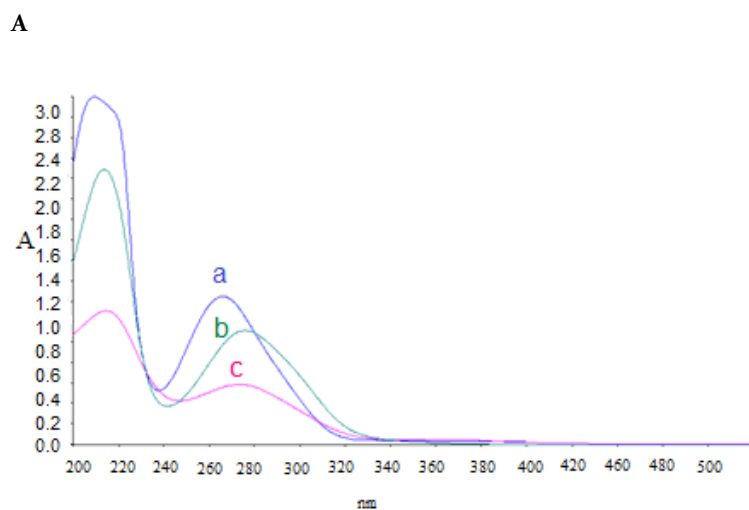


Figure 1. UV spectrum of gallic acid, *Q. infectoria* and *T. chebula* extracts and calibration curve of gallic acid in maximum wavelength

A: UV spectrum of forth derivation of UV spectrum of (a) gallic acid, (b) *Q. infectoria* and (c) *T. chebula*; **B:** Calibration curve of gallic acid in deionized water at room temperature (mean \pm SD, $n=5$)

Cytotoxicity against B16/F10 cell lines

The cells of B16/F10 were treated with different concentrations of *Q. infectoria* and *T. chebula* to assess safety of the tested extracts. Results indicated on concentration-dependent cytotoxicity of both *Q. infectoria* and *T. chebula* extracts, however this effect was not significant from control at 10 µg/mL ($p > 0.05$). *Q. infectoria* extract exhibited maximum toxicity at 100 µg/mL, which decreased cell viability to 82.92% in comparison to control. This plant was completely nontoxic at concentrations of 1 and 10 µg/mL. Due to low solubility, it was not possible to use concentrations more than 100 µg/mL. Kojic acid decreased live cells to 55.24% at 100 µg/mL. *T. chebula* did not exhibit toxic effect on B16/F10 cells at a concentration of 10-50 and 100 µg/mL while could reduce cell viability about 70.21% and 39.83% at 500 and 1000 µg/mL, respectively (Fig. 2).

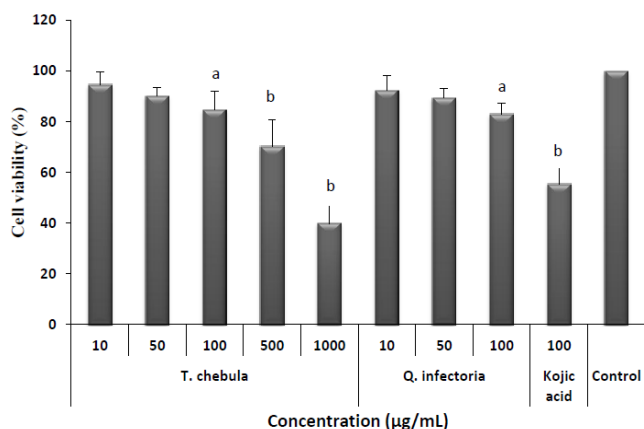


Figure 2. Cytotoxic effect of *Q. infectoria* and *T. chebula* extracts in B16/F10 murine melanoma cells by MTT test.

B16/F10 cell lines were incubated with different concentrations of plant extracts for 48 h and live cells were measured by MTT assay. Data were expressed as mean \pm SD of three independent experiments. ^a $p < 0.05$ and ^b $p < 0.001$ statistically significant from control.

Melanin content in B16/F10 cells

As shown in Fig. 3, by concentration increasing, inhibition of intracellular melanin was increased. Melanin content was not significantly different from control at 10 µg/mL of each extract ($p > 0.05$). Among the non-toxic concentrations, *Q. infectoria* at 50 and 100 µg/mL exhibited a significant decrease in cellular melanin content in comparing to control (66.25% and 36.99%, respectively). This was comparable with 56.63% melanin content of kojic acid at

100 µg/mL. *T. chebula* inhibited melanin synthesis in B16/F10 cells in non-toxic concentrations. This plant decreased melanin content about 89.56%, 77.1% and 55.18% at 10, 50 and 100 µg/mL. The greatest melanin reduction was observed at a toxic concentration of 1000 µg/mL (17.15%).

Non-toxic concentrations of *Q. infectoria* extract did not show any significant difference in reducing of intracellular melanin in comparison to control. But toxic concentrations of plant extracts exhibited potent inhibition of melanin production in cultured cells at 500 and 1000 µg/mL.

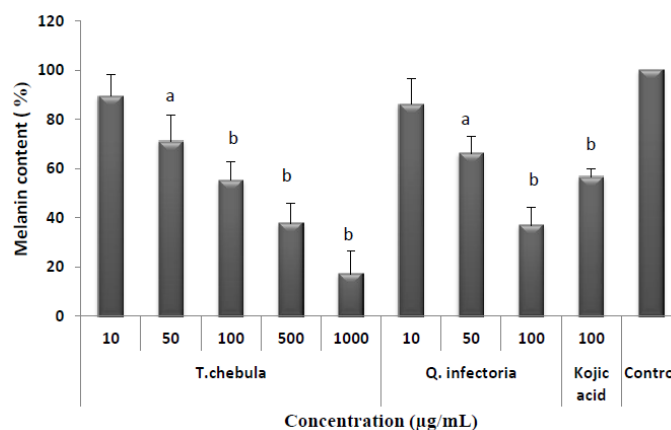


Figure 3. Effect of *Q. infectoria* and *T. chebula* extracts on melanin content in B16/F10 murine melanoma cells.

B16/F10 cell lines were incubated with different concentrations of plant extracts for 48h. Absorbance was measured at 490 nm with microplate reader. Data were expressed as mean \pm SD of three independent experiments. ^a $p < 0.05$ and ^b $p < 0.001$ statistically significant from control.

Tyrosinase activity in B16/F10 cells

Enzyme inhibition was carried out at different intervals of 10, 20, 30, 40, 50 and 60 minutes after adding substrate in the presence of different concentrations of plant extracts (Fig. 4). At primary times regarding the extent of enzyme activity, there was no significant difference between control, kojic acid, and tested plants. Obtained results indicated that after 50 minutes incubation, the enzyme inhibition reached the maximum. *Q. infectoria* caused maximum 59.3% tyrosinase inhibition at 100 µg/mL, which was significantly different from control ($p < 0.001$). This activity was concentration-dependent. *T. chebula* also decreased tyrosinase activity in both nontoxic and toxic concentrations. Maximum inhibition of enzyme activity was due to 1000

µg/mL (49.6% inhibition). In comparison to kojic acid as a reference compound, *Q. infectoria* (100 µg/mL) and *T. chebula* (100, 500 and 1000 µg/mL) could inhibit tyrosinase activity.

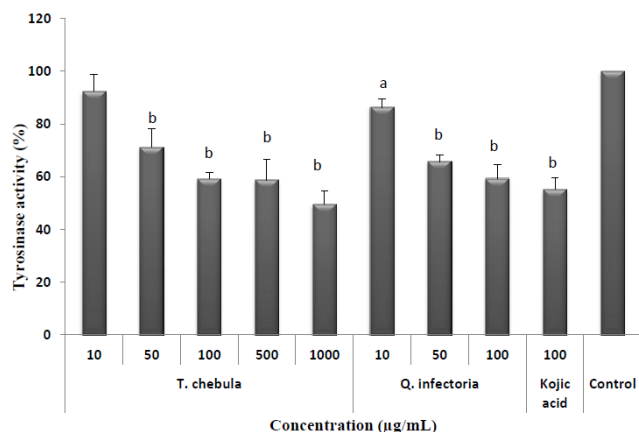


Figure 4. Tyrosinase inhibitory effect of *Q. infectoria* and *T. chebula* extracts in B16/F10 murine melanoma cells.

Tyrosinase activity in B16/F10 cell lines was determined in the presence of different concentrations of *Q. infectoria* and *T. chebula* extracts. Cell lines were treated with extracts for 24 h and after adding L-DOPA change in absorbance was studied at 475 nm over 60 min. Data were expressed as mean \pm SD of three independent experiments. ^a $p < 0.05$ and ^b $p < 0.001$ statistically significant from control.

DISCUSSION

Despite different uses of medicinal plants in traditional and/or folk culture of each country, people are still worried about efficacy and nonhazardous effects of these drugs. Health care system practitioners recommend that people should be beneficiaries of completely safe and regulated traditional treatments. So many investigations have been focused on various dimensions of safety, toxicity, quality, efficacy and rational use of medicinal plants. Today, more attention has been paid to safety of cosmetics because of permanent use of skin preparations, which makes them more prone for inducing adverse effects. Current drugs such as hydroquinone cause side effects like skin rash, scaling and contact dermatitis and cannot fulfill requirements of an ideal anti pigmentary agent. So, in continuing of search for novel drugs, with a special view to medicinal plants with a broad spectrum of phytochemicals, the aim of this work was to evaluate the toxicity, melanin content and tyrosinase inhibitory potential of *Q. infectoria* and *T. chebula* extracts, which have been proposed in PTM. Recent

works indicate that these two plants have potential-ly inhibit tyrosinase in *in vitro* models (Khazaeli et al., 2009; Ansari et al., 2011; Dugaheh et al., 2013). Most of antimelanogenesis compounds are from phenolics, so total phenolic content of *Q. infectoria* and *T. chebula* was determined as 287.34 ± 4.21 and 172.61 ± 8.67 mg gallic acid equivalent/g dried extract, respectively. Gallic acid could suppress melanogenesis in melanocytes and inhibits mice skin hyperpigmentation induced by UVB radiation (Panich et al., 2012; Su et al., 2013).

In all, phenolics especially flavonoids and catechins have been known as antioxidant phytochemicals that are able to reduce melanin production (Lee et al., 2002; Zheng et al., 2012). Considerable amounts of phenolic compounds such as phenolic acids and tannins have been reported in different species of *Quercus* and *Terminalia* species (Saleem et al., 2002; Ansari et al., 2011; Hapidin et al., 2012). However, for promoting the investigation about these plants, it is needed to warrant about safety and lack of cytotoxicity of these plants on melanocytes. At first, cell survival was studied in the presence of plant extracts. Based on the results of MTT assay, both tested plants showed no significant toxicity on B16/F10 cells at concentrations of 10, 50 µg/mL. A decrease about 10-21% in live cells was considered at 100 µg/mL concentration of these plants. The higher concentrations of *T. chebula* caused a significant decrease in cell viability. Kojic acid was completely toxic in the concentration of 100 µg/mL (Fig. 2).

Evaluation of inhibitory effects on melanin synthesis indicated that *Q. infectoria* and *T. chebula* could reduce melanin content about 33.7% and 28.9%, respectively in non-toxic concentrations in comparison to control ($p < 0.05$). In higher concentrations, both plant extracts decreased melanin content in comparison to kojic acid. At concentration 100 µg/mL, 73.0% and 44.8% melanin reduction was resulted by *Q. infectoria* and *T. chebula*, respectively while kojic acid caused 43.4% decrease in melanin content at 100 µg/mL. Difference in melanin inhibition by *Q. infectoria* and kojic acid was significant ($p < 0.05$). Toxic concentrations of *T. chebula* could decrease melanin up 83.0% (Fig. 3).

Tyrosinase activity was also affected by different concentrations of the plant extracts. As shown in Fig. 4, *Q. infectoria* could inhibit tyrosinase activity

in both nontoxic and toxic concentrations. This plant significantly exhibited 14.0% and 34.3% enzyme inhibition ($p < 0.05$ and $p < 0.001$, respectively) at 10 and 50 $\mu\text{g/mL}$. Maximum inhibition was considered at 100 $\mu\text{g/mL}$ (41.7% inhibition versus control). *T. chebula* caused about 7.6% and 28.8% tyrosinase inhibition at 10 and 50 $\mu\text{g/mL}$ concentrations (Fig. 4). Maximum inhibition was exhibited at 1000 $\mu\text{g/mL}$ (51.4% inhibition). Kojic acid caused 44.7% tyrosinase inhibition at 100 $\mu\text{g/mL}$.

Anti-tyrosinase activity has been demonstrated by various plant extracts (Lee et al., 2002; Momtaz et al., 2008; Zheng et al., 2012; Dugaheh et al., 2013). The presence of phenolic acids and tannins in these two plants might be responsible for the antityrosinase effect. Hydroxyl groups of phenolic compounds can form a hydrogen bond with the active site of the enzyme and lead to some changes in enzyme conformation or hindrance and finally enzyme inactivation (Kubo et al., 2003; Momtaz et al., 2008; Khan, 2012).

It was found that phenolic compounds separated from the extracts of *Morus australis*, *Morus alba* and *Pulsatilla cernua* inhibit tyrosinase enzyme and thus could control the biosynthesis of melanin (Lee, 2002). Both plants studied here, especially *Q. infectoria* could inhibit tyrosinase and decrease melanin synthesis in non-toxic concentrations. The obtained results qualify the traditional antimelasma claim of *Q. infectoria* and *T. chebula* and indicate that topical preparations of these plants (even in combination) could be effective and safe for cosmetic purposes. However, more trials are needed to confirm this.

CONCLUSIONS

In this work, cytotoxicity and antityrosinase effect of *Q. infectoria* and *T. chebula* extracts has been studied and the results confirm that these two plants can inhibit melanogenesis in non-toxic concentrations.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Author contribution:

Contribution	Jamshidzadeh A	Shokri Y	Ahmadi N	Mohamadi N	Sharififar F
Concepts or ideas					X
Design					X
Definition of intellectual content	X				X
Literature search	X	X	X		X
Experimental studies	X	X			X
Data acquisition	X	X			X
Data analysis	X	X			
Statistical analysis	X				
Manuscript preparation			X	X	X
Manuscript editing					X
Manuscript review					X

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