Evaluation of anti-melanogenic activity of Ziziphus jujuba fruits obtained by two different extraction methods

M. Salimi¹, P. Sarkhail²*, P. Sarkheil², H. Mostafapour Kandelous¹, M. Baeeri²

¹Pharmacology & Physiology Department, Pasteur Institute of Iran, Tehran, Iran. ²Pharmaceutical Sciences Research Center, Tehran University of Medical Sciences, Tehran, Iran.

Abstract
Background and objectives: Dried pulps and peels of Ziziphus jujuba fruits are commonly applied as food because of their high nutritional value. It has been widely used in traditional medicine as laxative, tonic, wound healing agent and appetizer. The aim of this study was to evaluate the anti-melanogenic effects of Z. jujuba fruit. Methods: Fruit extracts were obtained by two different extraction methods, percolation (cold extraction) and soxhlet (hot extraction) using methanol 80% as the solvent. The total phenolic and flavonoid contents, DPPH radical scavenging activity and anti-tyrosinase capacity of the MeOH extracts from Z. jujuba fruits were evaluated in vitro. In addition, the effects of fruit extracts on the melanin content and cytotoxicity on human melanoma SKMEL-3 cells were determined after 72 hours. Results: The amount of total phenolic and flavonoid contents of the cold extract were found higher in comparison to the hot extract. Moreover, the antioxidant (SC₅₀ =1.40 mg/mL) and anti-tyrosinase activities (IC₅₀ = 0.54 mg/mL) of the cold extract were significantly stronger than the hot extract. At the dose of 500 μg/mL, the cold extract showed weaker toxicity to the melanoma cells than the hot extract. Melanin content of the cold extract was reduced to 30% at this concentration, while the hot extract had no inhibitory effect on melanin formation. Conclusion: The results showed that the percolation method was more suitable for extraction of the (poly)phenolics from Z. jujuba fruits. In addition, the results of tyrosinase activity and melanin content assays suggested that the cold extract of Z. jujuba fruit can be considered as a dermatological whitening agent in skin care products.

Keywords: anti-tyrosinase, cytotoxicity, DPPH, melanin, Ziziphus jujuba

Introduction
Edible fruits of Ziziphus jujuba Mill. (Chinese/Korean Date, Jujube), a member Rhamnaceae family, is known as “annab” in Persian. This species is distributed in the subtropical and tropical regions of Europe, Australia and Southeast Asia [1]. It is commonly used in Iranian, Indian and Chinese Traditional Medicine as laxative, tonic, wound healer and appetizer and has shown positive effects in diabetes, insomnia, fever, colds and flu [2]. Dried pulps and peels of jujube are commonly applied as food, food additives and flavoring agents [1]. Some pharmacological studies have confirmed that Z. jujuba fruits can act as antimicrobial,
antiulcer, anti-inflammatory [3], sedative [4], antioxidant, immune-stimulant [5], anticancer [6] and wound healing agents [7]. A controlled clinical trial has indicated that the fruits were useful for chronic constipation [8]. In another clinical trial, it was shown that Z. jujuba fruit was effective in controlling dyslipidemia in obese adolescents [9]. These different properties can be due to various functional components in Z. jujuba fruit such as phenolics, amino acids, vitamins, flavonoids, organic acids, polysaccharides, saponins, terpenoids and high levels of fiber [2]. For example, the pectic polysaccharide, a main efficient compound in Z. jujuba fruit, has been shown to stimulate the rat spleen cell proliferation activity [10], while in another investigation, the polysaccharides have exhibited the anti-proliferation activity on melanoma cells in a dose-and time-dependent manner [11]. Cheng et al. [12] have confirmed that the flavonoids from Z. jujuba fruits could be responsible for its sedative activity. Several studies have revealed that the antioxidant activity of the fruits is due to the different phenolic or non-phenolic acid compounds, including gallic acid, chlorogenic acid, caffeic acid, \( \rho \)-hydroxybenzoic acid, protocatechuic acid and ascorbic acid (vitamin C). In addition, some isolated flavonoids from the fruits of Z. jujuba, such as procyanidin B2, epicatechin, rutin and hyperoside have shown antioxidant properties [7].

Melanin, a pigment produced by cells called melanocytes, is responsible for coloring mammalian hair and skin. Tyrosinase, a multifunctional copper-containing enzyme oxidase, is the key enzyme concerned in the biosynthesis of melanin through enzymatic reactions. Hyper activity of tyrosinase leads to hyperpigmentation, which causes some dermatological disorders such as age spots, acne and melasma [13,14]. Numerous studies have confirmed that some plant phenolic or polyphenolic antioxidants protect the living organisms against damages caused by the reactive oxygen species (ROS) [15]. Antioxidants can improve the treatment of hyperpigmentation disorders via their scavenging power. Many reports have demonstrated that synthetic depigmenting agents in drug formulations show poor skin penetrations and induce side effects including irritant and allergic contact dermatitis, cytotoxicity and mutagenicity [13]. Accordingly, this study was designed to find out a new and safe herbal whitening agent. To the best of our knowledge, the anti-melanogenic activity of Z. jujuba fruits has not yet been investigated. As Z. jujuba fruit is rich of polyphenols, it can be involved in free radicals scavenging and inhibition of tyrosinase activity. Therefore, in the present study, we have evaluated the anti-melanogenic and antioxidant activity of the extracts from Z. jujuba fruits that had been prepared by two different extraction methods (percolation and soxhlet).

**Experimental**

*Chemicals and reagents*

Mushroom tyrosinase, butylated hydroxytoluene (BHT), 1,1-diphenyl-2-picrylhydrazyl (DPPH), kojic acid, quercetin, dimethyl sulfoxide (DMSO), sodium carbonate, potassium dihydrogen phosphate, di-potassium hydrogen phosphate and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma, USA. Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin and trypsin-EDTA were purchased from Gibco BRL, USA. Folin–Ciocalteu reagent was obtained from Fluka (Switzerland). Human melanoma (SKMEL-3) cells were provided from Pasteur Institute of Iran. All other chemicals and solvents used in the experiments were of analytical grade and were obtained from Merck (Germany).

*Plant material and the extracts preparation*

Fresh fruits of *Ziziphus jujuba* Mill. were purchased from the local market, Tehran, Iran in spring 2012 and were identified by the corresponding author. A specimen was deposited at the Pharmaceutical Sciences Research Center
laboratory, Tehran University of Medical Sciences, Tehran, Iran. The fruits were crushed and the seeds were separated. The pulps and peels were dried at 40 °C and 100 grams of the dried powder was extracted using two different extraction methods. In the first method, Z. jujuba fruit were extracted with methanol/water (80:20) using a percolator for 7 days at room temperature and the second method was performed using soxhlet apparatus with MeOH 80% for 4h at 80 °C. The extracts were evaporated in vacuum (Buchi type Rotavapor). The dried extracts were stored at 4 °C for experimental studies.

**Determination of the total phenolic content**
The total phenolic content of the extracts was determined according to the Folin-Ciocalteu method [15]. 100 μL of Folin-Ciocalteu reagent (diluted 10 times) was added to the sample and shacked for 5 min. The mixture was further mixed with 0.75 μL of sodium carbonate 6% (w/v). After incubation for 30 min, the absorbance was measured at 750 nm using an ELISA reader (Synergy HT, BIO-TEK, USA). The results were expressed as milligrams of gallic acid equivalents per gram of the extract (mg of GA/g of extract).

**Determination of total flavonoid content**
The total flavonoid content of the extracts was estimated according to the aluminum chloride colorimetric method with some modifications using 96-well plate [16]. The extract (20 μL) was mixed with water (80 μL) followed by the addition of NaNO₂ (15%, 6 μL). After shaking the mixture for 5 min, 6 μL of AlCl₃ (10%), 80 μL NaOH (4%) and (80 μL) distilled water were added. After 15 min, the absorbance was measured at 510 nm with an ELISA reader. Total flavonoids contents were calculated as quercetin equivalents per gram of the extract.

**Determination of the scavenging effect on DPPH**
The technique using 96-well microplate was applied to determine the DPPH free radical scavenging activity of the extracts [17]. Different concentrations of the extracts (0.3125-2.5 mg/mL in DMSO, 160 μL) were mixed with 40 μL of DPPH solution (150 μM). After incubation at 25 °C for 30 min in a dark place, the absorbance was recorded at 517 nm using an ELISA reader. Quercetin was used as the positive control.

**Determination of mushroom tyrosinase inhibitory activity**
Tyrosinase inhibitory activity was carried out according to the procedure explained by Momtaz et al. [18] previously. The test concentrations (0.3125-2.5 mg/mL) were mixed with 80 μL phosphate buffer (50 mM) and 20 μL of mushroom tyrosinase solution (125 units/mL) in 50 mM phosphate buffer, pH 6.8. After pre-incubation at room temperature for 10 min, a reaction was performed by adding 40 μL L-tyrosine (2 mM). The amount of dopachrome in the reaction was measured at 475 nm using an ELISA reader. Kojic acid was used as the positive control.

**Determination of cytotoxicity (using MTT assay)**
Human melanoma cells (SKMEL-3) were obtained from the cell bank of Pasteur Institute of Iran (NCBI). For cytotoxicity study, SKMEL-3 cells were seeded at a density of 5 × 10³ cells/well in 96-well plates. After 24 h of incubation, the extracts (500 μg/mL) and kojic acid (100 μg/mL) were added to the wells. The cytotoxicity was measured using the MTT reduction method [19] after 72 h at 37 °C.

**Determination of melanin content**
Melanin content was measured as described previously by Chan et al. [20] with slight modifications. The human melanoma cells were seeded at 3×10⁵ cells/well in 6-well culture plates and incubated overnight to allow cells to adhere. The cells were exposed to the Z. jujuba fruit extracts (500 μg/mL) or kojic acid (100 μg/mL as the positive control) for 72 h. At the end of the treatment, the cells were washed with PBS and lysed with 800 μL of 1 N NaOH containing 10% DMSO for 1 h at 75 °C. The melanin content was
determined at 405 nm using a microplate reader.

Statistical analysis
The results were reported as mean ± SEM of three replicate determinations. Data were subjected to independent t-tests and the group means were compared using the ANOVA followed by Tukey post hoc test. p < 0.05 was regarded as statistically significant.

Results and discussion
The yield of percolation and soxhlet methods were 22.5% and 18.3% w/w, respectively. The Z. jujuba fruit extract obtained by percolation method (ZP) possessed a significantly (p < 0.05) higher total phenolic content (26.91 ± 0.55 mg of GA/g extract) than the extract obtained by soxhlet method (ZS) (21.66 ± 0.34 mg of GA/g extract). In addition, the total flavonoid content of ZP extract (15.49 ± 0.35 mg of quercetin/g extract) was significantly (p < 0.05) higher than the total flavonoid content of ZS extract (9.13 ± 0.32 mg of quercetin/g extract). The lower total phenolic and flavonoid contents of ZS extract in comparison with ZP extract is due to the thermal degradation of polyphenols during hot method. Considering the results, the temperature was one of the main factors in extraction procedures and could influence the pattern of active compounds in Z. jujuba fruits.

Free radicals could induce biological damage and aggravate the progression of cells to pathological conditions, such as inflammation, aging and diabetes. Antioxidant agents can improve the management of those diseases through their antioxidant activity. These compounds exert their antioxidant activity directly by scavenging free radicals, chelating trace metals and indirectly by inhibiting some enzymes involved in radical generation such as lipoxygenases, cyclooxygenase and xanthine oxidase [23,24]; therefore, the level of antioxidant activity is often correlated with the amount of total phenolic and flavonoid contents. Since tyrosinase plays a crucial role in melanogenesis, we evaluated the effects of the two extracts on the activity of mushroom tyrosinase. In the present study, the ZP extract displayed higher anti-tyrosinase activity (IC$_{50}$, 0.54 mg/mL) against mushroom tyrosinase than ZS extract (IC$_{50}$, 1.01 mg/mL) (table 1). Moreover, the anti-tyrosinase activity of ZP extract was comparable to kojic acid (IC$_{50}$, 0.49 mg/mL), a well-known tyrosinase inhibitor. Due to the strong linear correlation (R$^2$, 0.95) between antioxidant activity and mushroom tyrosinase inhibition in both Z. jujuba fruit extracts, the free radical scavenging pathway can be one of the

Table 1. Antioxidant and anti-tyrosinase activities of Ziziphus jujuba fruit extracts

<table>
<thead>
<tr>
<th>Sample extract</th>
<th>DPPH assay (SC$_{50}$ mg/mL)$^d$</th>
<th>Mushroom tyrosinase assay (IC$_{50}$ mg/mL)$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZP$^b$</td>
<td>1.40</td>
<td>0.54</td>
</tr>
<tr>
<td>ZS$^b$</td>
<td>1.79</td>
<td>1.01</td>
</tr>
<tr>
<td>Quercetin</td>
<td>0.06</td>
<td>0.54</td>
</tr>
<tr>
<td>Kojic acid</td>
<td></td>
<td>0.49</td>
</tr>
</tbody>
</table>

$^a$ ZP: Z. jujuba fruit extract obtained by cold method; $^b$ ZS: Z. jujuba fruit extract obtained by hot method. $^c$ SC$_{50}$ is the mean 50% free radical scavenging activity. $^d$ IC$_{50}$ is the mean 50% tyrosinase inhibition.

Previous studies have confirmed that some phenolic and flavonoid compounds have significant antioxidant activity. These compounds exert their antioxidant activity directly by scavenging free radicals, chelating trace metals and indirectly by inhibiting some enzymes involved in radical generation such as lipooxygenases, cyclooxygenase and xanthine oxidase [23,24]; therefore, the level of antioxidant activity is often correlated with the amount of total phenolic and flavonoid contents. Since tyrosinase plays a crucial role in melanogenesis, we evaluated the effects of the two extracts on the activity of mushroom tyrosinase. In the present study, the ZP extract displayed higher anti-tyrosinase activity (IC$_{50}$, 0.54 mg/mL) against mushroom tyrosinase than ZS extract (IC$_{50}$, 1.01 mg/mL) (table 1). Moreover, the anti-tyrosinase activity of ZP extract was comparable to kojic acid (IC$_{50}$, 0.49 mg/mL), a well-known tyrosinase inhibitor. Due to the strong linear correlation (R$^2$, 0.95) between antioxidant activity and mushroom tyrosinase inhibition in both Z. jujuba fruit extracts, the free radical scavenging pathway can be one of the
mechanisms of tyrosinase enzyme inhibition of Z. jujuba fruits, as suggested by earlier reports [25]. Polyphenols are considered as one of the main groups of tyrosinase inhibitors. The tyrosinase inhibition capacity of the extracts may be associated with the hydroxyl groups of the polyphenolics that bind to the active site on tyrosinase, leading to a lower enzymatic action. These results suggest that phenolic compounds can be defined as the main ingredients of Z. jujuba fruits for antioxidant activity and tyrosinase inhibition effect. However, these activities can also be affected by other non-phenolic antioxidants present in the samples, such as vitamin C.

Before evaluating the anti-melanogenic activity of Z. jujuba fruits on SKMEL-3 cell line, we considered its possible cytotoxic effects on Skmel-3 cells during 72 h incubation, to avoid misinterpretation of depigmenting results. In MTT assay, both extracts showed no significant cytotoxic activity on SKMEL-3 cells at the high dose (500 µg/mL) after 24 and 48 hours treatment, but reduced the viable cells slightly after 72 hours. The percentage viability of SKMEL-3 cells after exposure to 500 µg/mL of ZP and ZS extracts at 72 h was near 87% and 80%, respectively in comparison to the negative control group (100% cell viability). This result showed that both extracts from Z. jujuba fruits had low toxicity effects on melanoma cells at dose of 500 µg/mL.

After 72 hours, the melanin content assay indicated no reduction at doses of 100 and 250 µg/mL of the extracts, but at 500 µg/mL, the ZP extract showed about 30% reduction in melanin content of SKMEL-3 cells. At this concentration, the melanin secretion level in the treated cells with ZP extract was 69.37 ± 3.41% of control, while ZS extract (108 ± 2.50%) and kojic acid (91.96 ± 3.3%) showed no significant (p < 0.05) reduction in melanin content (figure 1). According to the results of this study, it can be suggested that the inhibition of melanin production is probably related to the reduction of free radicals and/or inhibition of tyrosinase activity in the melanoma cells. The current investigation was a preliminary study for evaluation of a new herbal source for hyperpigmentation treatment, but more in vivo studies are needed to describe the mechanism of anti-melanogenic activity of Z. jujuba fruits. Based on our findings, the Z. jujuba fruit extract obtained by cold extraction method could decrease tyrosinase activity and melanin production in vitro.

![Figure 1](image-url)  
Figure 1. Effect of ZP and ZS extracts on melanin formation in SKMEL-3 cells after 72h. Results were expressed as percentages relative to the control, ***p<0.001.

Thus, it could be recommended as a preventative or treatment for some dermatologic disorders resulting from excess melanin production/accumulation such as melasma, age spots and acne. In addition, this study showed that the temperature of extraction could influence the pattern of active compounds and level of antioxidant and anti-melanogenic activities in Z. jujuba fruits.

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**Declaration of interest**

The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.
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