Research Journal of Pharmacognosy (RJP) 3(2), 2016: 1-7
Received: Nov 2015
Accepted: Jan 2016

Original article

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

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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 (SC_{50} =1.40 mg/mL) and anti-tyrosinase activities (IC_{50} = 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,

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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, *ρ*-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).

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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 flavonoids 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 $NaNO_2$ (15%, 6 µL). After shaking the mixture for 5 min, 6 µL of $AlCl_3$ (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 5×10^3 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 3×10^5 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 scavenging power. In the present study, electron donation ability of the extracts was measured from the bleaching of the purple colored DPPH. The extracts exhibited scavenging activity against the DPPH radicals in a concentration-dependent manner, but the ZP extract displayed significantly higher antioxidant activity in all concentrations. As shown in table 1, the ZP and ZS extracts indicated DPPH radical scavenging activity with SC_{50}, 1.40 and 1.79 mg/mL, respectively that were significantly lower than quercetin (SC_{50}, 0.06 mg/mL).

These results confirmed that the free radical scavenging activity of ZP extract was higher than ZS extract which may possibly be due to the higher level of phenolic or non-phenolic (vitamin C) compounds in this extract. Some studies have been shown that degradation of vitamin C and polyphenol compounds such as anthocyanins improved by increasing extraction temperature [21,22].

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

Sample extract	DPPH assay (SC_{50} mg/mL)[c]	Mushroom tyrosinase assay (IC_{50} mg/ml)[d]
ZP[a]	1.40	0.54
ZS[b]	1.79	1.01
Quercetin	0.06	-
Kojic acid	-	0.49

[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 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

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*.

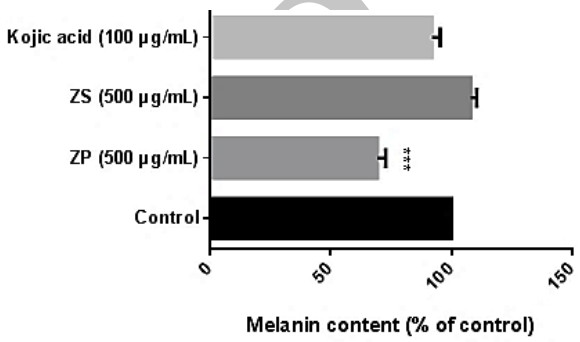

Figure 1. Effect of ZP and ZS extracts on melanin formation in SKMEL-3 cells after 72h. Results were expressed as percentages relative to the control, *** $p<0.001$.

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.

Acknowledgements

This work was financially supported by the Pharmaceutical Sciences Research Center, Tehran University of Medical Sciences (grant NO. 90/D/425/237), Tehran, Iran.

Declaration of interest

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

References

[1] Li JW, Ding SD, Ding XL. Optimization of the ultrasonically assisted extraction of polysaccharides from *Zizyphus jujuba* cv. *Jinsixiaozao*. *J Food Eng*. 2007; 80(1): 176-183.

[2] Gao QH, Wu CS, Wang M. The jujube (*Ziziphus jujuba* Mill.) fruit: a review of current knowledge of fruit composition and health benefits. *J Agric Food Chem*. 2013; 61(14): 3351-3363.

[3] Al-Reza SM, Yoon JI, Kim HJ, Kim JS, Kang SC. Anti-inflammatory activity of seed essential oil from *Zizyphus jujuba*. *Food Chem Toxicol*. 2010; 48(2): 639-643.

[4] Jiang JG, Huang XJ, Chen J. Separation and purification of saponins from Semen *Ziziphus jujuba* and their sedative and hypnotic effects. *J Pharm Pharmacol*. 2007; 59(8): 1175-1180.

[5] Ganachari MS, Shiv K, Bhat KG. Effect of *Ziziphus jujuba* leaves extract on phagocytosis by human neutrophils. *J Nat Rem*. 2004; 4(1): 47-51.

[6] Huang X, Kojima-Yuasa A, Norikur T, Kennedy DO, Hasuma T, Matsui-Yuasa I. Mechanism of the anti-cancer activity of *Zizyphus jujuba* in HepG2 cells. *Am J Chin Med*. 2007; 35(3): 517-532.

[7] Mahajan RT, Chopda MZ. Phyto-Pharmacology of *Ziziphus jujuba* Mill- A plant review. *Phcog Rev*. 2009; 3(6): 320-329.

[8] Naftali T, Feingelernt H, Lesin Y, Rauchwarger A, Konikoff FM. *Ziziphus jujuba* extract for the treatment of chronic idiopathic constipation: a controlled clinical trial. *Digestion*. 2008; 78(4): 224-228.

[9] Sabzghabaee AM, Khayam I, Kelishadi R, Ghannadi A, Soltani R, Badri S, Shirani S. Effect of *Zizyphus jujuba* fruits on dyslipidemia in obese adolescents: a triple-masked randomized controlled clinical trial. *Med Arch*. 2013; 67(3): 156-159.

[10] Zhao Z, Li J, Wu X, Dai H, Gao X, Liu M, Tu P. Structure and immunological activities of two pectic polysaccharides from the fruits of *Zizyphus jujuba* Mill. cv. *jinsixiaozao* Hort. *Food Res Int*. 2006; 39(8): 917-923.

[11] Hung CF, Hsu BY, Chang SC, Chen BH. Antiproliferation of melanoma cells by polysaccharide isolated from *Zizyphus jujuba*. *Nutrition*. 2012; 28(1): 98-105.

[12] Cheng G, Bai Y, Zhao Y, Tao J, Liu Y, Tu G, Ma L, Liao N, Xu X. Flavonoids from *Zizyphus jujuba* Mill. var. *spinosa*. *Tetrahedron*. 2000; 56(45): 8915-8920.

[13] Kim YJ, Uyama H. Tyrosinase inhibitors from natural and synthetic sources: structure, inhibition mechanism and perspective for the future. *Cell Mol Life Sci*. 2005; 62(15): 1707-1723.

[14] Solano F, Briganti S, Picardo M, Ghanem GH. Hypopigmenting agents: an updated review on biological, chemical and clinical aspects. *Pigment Cell Res*. 2006; 19(6): 550-571.

[15] Hajimehdipoor H, Gohari AR, Ajani Y, Saeidnia S. Comparative study of the total phenol content and antioxidant activity of some medicinal herbal extracts. *Res J Pharmacogn*. 2014; 1(3): 21-25.

[16] Chang CC, Yang MH, Wen HM, Chern JC. Estimation of total flavonoid content in propolis by two complementary colorimetric methods. *J Food Drug Anal*. 2002; 10(3): 178-182.

[17] Bahadori MB, Mirzaei M. Cytotoxicity, antioxidant activity, total flavonoid and phenolic contents of *Salvia urmiensis* Bunge and *Salvia hydrangea* DC. ex Benth. *Res J Pharmacogn*. 2015; 2(2): 27-32.

[18] Momtaz S, Mapunya BM, Houghton PJ, Edgerly C, Hussein A, Naidoo S, Lall N. Tyrosinase inhibition by extracts and constituents of *Sideroxylon inerme* L. stem

bark, used in South Africa for skin lightening. *J Ethnopharmacol*. 2008; 119(3): 507-512.

[19] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods*. 1983; 65(1-2): 55-63.

[20] Chan YY, Kim KH, Cheah SH. Inhibitory effects of *Sargassum polycystum* on tyrosinase activity and melanin formation in B16F10 murine melanoma cells. *J Ethnopharmacol*. 2011; 137(3): 1183-1188.

[21] Al-Farsi MA, Lee CY. Optimization of phenolics and dietary fibre extraction from date seeds. *Food Chem*. 2008; 108(3): 977-985.

[22] Jenshi Roobha J, Saravanakumar M, Aravindhan KM, Suganya Devi P. The effect of light, temperature, pH on stability of anthocyanin pigments in *Musa acuminata* bract. *Res Plant Biol*. 2011; 1(5): 5-12.

[23] Sarkhail P, Sarkheil P, Khalighi-Sigaroodi F, Shafiee A, Ostad N. Tyrosinase inhibitor and radical scavenger fractions and isolated compounds from aerial parts of *Peucedanum knappii* Bornm. *Nat Prod Res*. 2013; 27(10): 896-899.

[24] Rahman K. Studies on free radicals, antioxidants, and co-factors. *Clin Interv Aging*. 2007; 2(2): 219-236.

[25] Sanchez-Ferrer A, Rodrıgez-Lopez JN, Garcıa-Carmona F. Tyrosinase: A comprehensive review of its mechanism. *Biochim Biophys Acta*. 1995; 1247(1): 1-11.