Antioxidative capacity of Iranian *Citrus deliciosa* peels

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ABSTRACT

While there is growing attention towards the consumption of *citrus* fruits, because they appear to be associated with lower colorectal, esophageal, gastric cancers and stroke risks. This study was aimed at evaluating the *in vitro* antioxidant properties and phenolics content of Iranian *C. deliciosa*. Polyphenol's total content was 92.08 ± 2.50. Tannic acid equivalent mg/g and the flavonoid total content was 4.55 ± 0.13. Rutin equivalents mg/g, DPPH (IC_{50}: 0.358/ml), TEAC (TEAC value: 19.58), FRAP (EC1: 0.152 mg/ml), deoxyribose (IC_{50}: 76 µg/ml) and xanthine/xanthine oxides' methods (IC_{50}: 94 µg/ml) were highly influenced by *C. deliciosa*. Results indicated that the herb can be used for the treatment of diseases related to radicals or, due to good antioxidant activity, as food additives.

Keywords: *Citrus deliciosa*; Antioxidant; DPPH; FRAP; TEAC; Hydroxyl; Superoxide.

INTRODUCTION

Reactive oxygen species (ROS) play a critical role in the pathogenesis of various diseases such as aging, arthritis, cancer, inflammation, and heart diseases in human body.[1] Molecules derived from oxygen such as superoxide anion, hydroxyl radicals and hydrogen peroxide are chemically reactivated by ROS. They are generated in living organisms as by-products through many of metabolic pathways. ROS can easily act in response with oxidizes, most biomolecules—carbohydrates, proteins, lipids and DNA.[2] Living organisms, therefore, possess some protective mechanisms in resistance to the oxidative stress and toxic effects of ROS. Studies were initiated in order to identify their effectiveness and potential therapeutic capacities in a variety of diseases with oxidative stress pathogenesis as antioxidants may attenuate oxidative damage by free radical or metal chelation (Aruoma 1996).

Because of their utilization, there is growing interest in citrus fruits which appear to be related to lower colorectal, esophageal, stomach cancers and stroke risks. It, furthermore, seems that they are associated with improved blood lipid profiles and survival in the elderly.[3] Citrus plants are rich in naturally-occurring flavonoids which are primarily found in peels. Flavonoids have a wide range of biological activities: cell-proliferation-inhibiting, apoptosis-inducing, enzyme-inhibiting, antibacterial and antioxidant effects.[4] Flavonoids: flavanones, flavones and flavonols occur in *Citrus* fruit.[5] The main flavonoids found in citrus peels are hesperidine, narirutin, 6.8-di-*C*-Glu-Apigenin, and 6,8-di-*C*-Glu-Diosmetin.[6]

Jointly with the sweet orange (*Citrus sinensis* L. Osbeck) group, the mandarins are the most cultivated *Citrus* species in the world. The mandarins, according to Tanaka (1954), include 36 true species; Webber (1943) recognized four taxonomically related groups and Swingle (1943) has assumed the mandarin to be a single species. The most important commercial scion varieties of mandarins are Common (*C. reticulate* Blanco), Dancy (*C. tangerine* Hort. Ex Tanaka), Clementine (*C. clementine* Hort. Ex. Tanaka), Satsuma (*C. unshiu* Marc.) and Mediterranean mandarins (*C. deliciosa Tenore*).[7]

Several methods were developed recently for measuring the total antioxidant capacity of food and beverages. Because different antioxidant compounds may act *in vivo* through different mechanisms, these assays, according to

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the generation of different radicals and/or target molecules, differ in their chemistry and in the way end points are measured. No single method can, thus, fully evaluate the total antioxidant activity of foods.[7]

Many papers have reported the antioxidants in juice and edible parts of oranges of different origin and varieties.[4,5,8,9] As far as the peels are concerned, extracts from this part of the fruit were found to have a good total radical antioxidative potential. The rind of *Citrus* is a huge waste; therefore, it can be used as a valuable and low-priced source for natural antioxidant.

The objective of this study was to assess the total antioxidant activity of *Citrus deliciosa* peels that are grown in Iran by different antioxidant (DPPH, TEAC and FRAP), and antiradical (hydroxyl radical, superoxide) methods. The Folin–Ciocalteu and Colorimetric aluminum chloride methods were used to determine the total phenolic and flavonoids contents of each sample, respectively.

MATERIALS AND METHODS

Chemicals

The 6-hydroxy-2, 5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), and 2,4,6-tripyridyls-triazine (TPTZ), 1,1-Diphenyl-2-picrylhydrazyl (DPPH), 2-deoxy- D-ribose, xanthine, xanthine oxidase (XOD), thiobarbituric acid (TBA), ferric chloride and L-ascorbic acid, nitroblue tetrazolium (NBT) and Folin-Ciocalteu reagent were purchased from Sigma; $FeSO_4 \cdot 7H_2O$, $FeCl_3$ anhydrous were purchased from Fluka Co. All other chemicals used were of analytical grade supplied by Merck.

Plant material

Fresh and ripe fruits were used in the experiments. The *Citrus delicious* which is natively known as Narangi was procured from a region of Iran named Dezful. The herbal product extract was prepared according to the method of Min-Sheng et al. (2008). Each citrus herbal product powder (10 g) was extracted with 100 ml of methanol overnight in a shaker at room temperature. The extract was centrifuged at 3500 rpm for 20 min to obtain the supernatant and the rest was also re-extracted under the same conditions. The combined filtrate was filtered through 0.45 μm of filter membrane and evaporated with a rotary evaporator (4003, Heidolph, Germany) below 50°C. After the evaporation of the organic solvent, it was used for further analysis.[4]

Total flavonoid content determination

The flavonoid content was estimated by the $AlCl_3$ method: 1 ml of methanolic extract solution was added to 1 ml of 2% methanolic $AlCl_3$, $6H_2O$. The absorbance was measured 10 min later at 430 nm (X-ma 3000, Human, Korea). Results were expressed in mg rutin/100 g dry matter by a comparison with standard rutin—a calibration curve of Rutin ranging from 0.5 to 25 μg/ml[10] was treated in the same conditions.

Total phenolic content determination

Total phenolic compound amount in extracts was determined by the Folin–Ciocalteu method: 0.5 ml of each extract and 2.5 ml of a 1/10 aqueous dilutions of Folin-Ciocalteu reagent were mixed. 2 ml of Na_2CO_3 7.5% was, after 5 minutes, added and incubated at room temperature for 120 minutes. Absorption at 765 nm was measured by using a spectrophotometer. The total phenolic content was expressed as Tannic acid.[11] A calibration curve of Tannic acid (ranging from 0.02 to 0.1 mg/ml) was prepared and the results, determined from the regression equation of the calibration curve ($y = 11.34x$, R2 = 0.0.988), were expressed as mg Tannic acid equivalents per the sample's each gramme.

DPPH free radicals scavenging activity assay

DPPH assay was performed according to the method of Brand-Williams 1995 et al. To 3.9 ml of DPPH solution (0.025 g/l), 0.1 ml of sample solution was added and the absorbance was measured at 515 nm. The tubes were then incubated at room temperature for 30 minutes under dark conditions and the absorbance was measured at 517 nm.[12] Inhibition of DPPH radical was calculated using the equation I (%) = $100 \times (A_0 - A_s)/A_0$, where A_0 is the absorbance of the control (containing all reagents except the test compound), and A_s is the absorbance of the tested sample; The IC_{50} value represents the concentration of the sample that caused a 50% inhibition.

TEAC (Trolox equivalent antioxidant capacity) assay

The method used was, as described by Zulueta et al (2009), based on the capacity of a sample to inhibit the ABTS radical ($ABTS^{\cdot+}$) which compared with a reference antioxidant standard (Trolox®). The $ABTS^{\cdot+}$ radical was generated by chemical reaction with potassium persulfate ($K_2S_2O_8$). 25 ml of ABTS (7 mM) was, for this purpose, spiked with 440 μl of $K_2S_2O_8$ (140 mM) and permitted to position in darkness at the room temperature for 12–16 h that is the time required for

formation of the radical. Taking a volume of the previous solution and diluting it in ethanol, the working solution was prepared until its absorbance at λ = 734 nm was 0.70 ± 0.02. The reaction took place directly in the measuring cuvette. For this purpose, again, 2 ml of the $ABTS^{\cdot+}$ radical was added; the absorbance (A_0) was measured and 100 µl of the sample or the standard were added immediately at which the point of the antioxidants present in the sample began to inhibit the radical and then with a quantitative relationship between the reduction and the concentration of antioxidants present in the sample, decreased the absorbance rate. The absorbance was measured 2, 4, and 6 minutes after mixing the reagent. Radical cation at 734 nm as 1 mM Trolox was calculated in terms of the Trolox equivalent antioxidant activity at each specific time-point. The plot's gradient of the absorbance's inhibition percentage vs. the concentration plot for the antioxidant in question was, for calculating the TEAC, divided by the plot's gradient for Trolox.[13]

Ferric-reducing antioxidant power (FRAP) assay

The FRAP reagent contained 2.5 ml of a 10 mM tripydyltriazine (TPTZ) solution in 40 mM HCl plus 2.5 ml of 20 mM $FeCl_3 \cdot 6H_2O$ and 25 ml of 0.3 M acetate buffer at pH 3.6. Freshly prepared FRAP reagent (3.0 ml) were mixed with 30 µl of sample and 10 µl of distilled water; the reaction mixtures were later incubated at 37° C. Absorbance at 593 nm was read with reference to reagent blank containing distilled water, which was also incubated at 37°C. Aqueous solutions of known Fe(II) concentrations in the range of 100–2000 µM ($FeSO_4 \cdot 7H_2O$) were used for calibration.[14] All tests were run in triplicate and mean values were used to calculate EC_1 and EC_{50} values. EC_1 is defined as the concentration of an antioxidant having a ferric reducing ability equivalent to that of a 1 mM ferrous salt.

Hydroxyl radicals scavenging activity assay

The reaction mixture contained 100 µl of extract, 500 µl of 5.6 mM 2-deoxy-d-ribose in KH_2PO_4–NaOH buffer (50 mM, pH 7.4), 200 µl of premixed 100 µM $FeCl_3$, 104 mM EDTA (1:1 v/v) solution, 100 µl of 1.0 mM H_2O_2, and 100 µl of 1.0 mM aqueous ascorbic acid were dissolved in water. Tubes were incubated at 50°C for 30 minutes. Thereafter, 1 ml of 2.8% TCA and 1 ml of 1.0% TBA were added to each tube. The samples were heated in a water bath at 50°C for 30 minutes. The extent of oxidation was estimated from the absorbance rate of the solution at 532 nm. The inhibition percentage values were calculated from the absorbance of the control (Ac) and of the sample (As) using the following equation: Inhibition (%) = ((Ac–As)/Ac) * 100, where the controls contained all the reaction reagents except the extract or positive control substance.[15]

Superoxide anion scavenging activity assay

Superoxide anion scavenging activity of *C. deliciosa* was measured by using the xanthine/xanthine oxidase method. A 0.5 ml of sample was added to a 1.0 ml mixture of 0.4 mM xanthine and 0.24 mM nitroblue tetrazolium chloride (NBT) in a 0.1 M phosphate buffer (pH 8.0). A 1.0 ml solution of xanthine oxidase (0.049 units/ml), diluted in 0.1 M phosphate buffer (pH 8.0), was added and the resulting mixture incubated in a water bath at 37°C for 40 minutes. The reaction was terminated by adding 2.0 ml of 69 mM sodium dodecylsulphate (SDS) and the absorbance of NBT was measured at 560 nm.[16]

Statistical analysis

The data determined was expressed as the mean of three replicate determinations and presented as mean ± SD (standard deviation). The IC_{50} values were estimated by linear/non-linear regression.

RESULTS

Total phenolic and flavonoid content

The extract yield was found to be 22.81 mg/g of peel. The total phenolic content of *C. deliciosa* peel extract was, in terms of tannic acid equivalent/g of the extract's dried weight, found to be 92.08 ± 2.50 mg and the total flavonoid content was 4.55 ± 0.13 mg in terms of Rutin equivalents/g of the extract's dried weight.

Antioxidant assay

The results of antioxidant assays are shown in Table 1.

DISCUSSION

Free radicals are harmful by-products generated during normal cellular metabolism which could initiate oxidative damage to the body. Antioxidants are believed to play a significant role in the body's defense system against free radicals. Recently, numerous reports have described antioxidants and compounds with radical-scavenging activity present in fruits, vegetables, herbs and cereals extracts.[17]

Table 1

Antioxidant Methods	Parameters	
DPPH assay	IC_{50}:0.358 mg/ml	Fig. 1
ABTS assay	TEAC value: mmol Trolox equvalents /100 g DW	Fig. 2
	2 min: 17.9	
	4 min: 18.86	
	6 min: 19.58	
FRAP assay	EC_1: 0.152 mg/ml	Fig. 3
	EC_{50}: 0.147 mg/ml	
Hydroxyl radical scavenging activity	IC_{50}: 76 µg/ml	Fig. 4
Superoxide radicals scavenging activity	IC_{50}: 94 µg/ml	Fig. 5

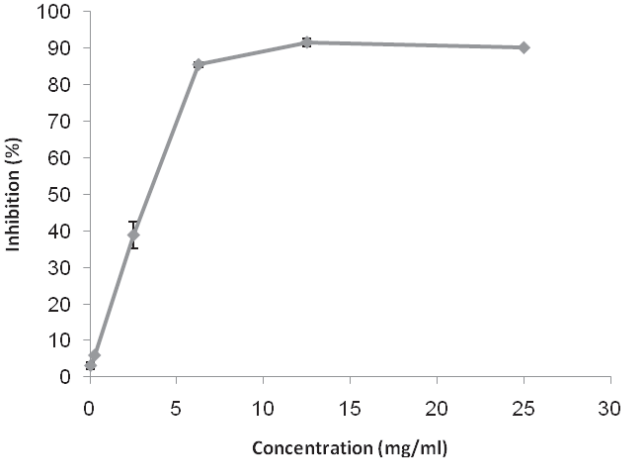

Figure 1. DPPH free radical scavenging activity of *C. deliciosa* extract. Values are expressed as mean ± standard deviation (n = 3).

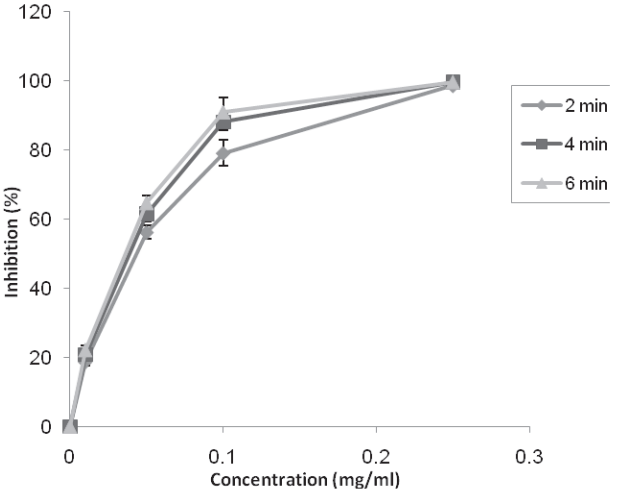

Figure 2. ABTS free radical scavenging activity of *C. deliciosa* extract. Values are expressed as mean ± standard deviation (n = 3).

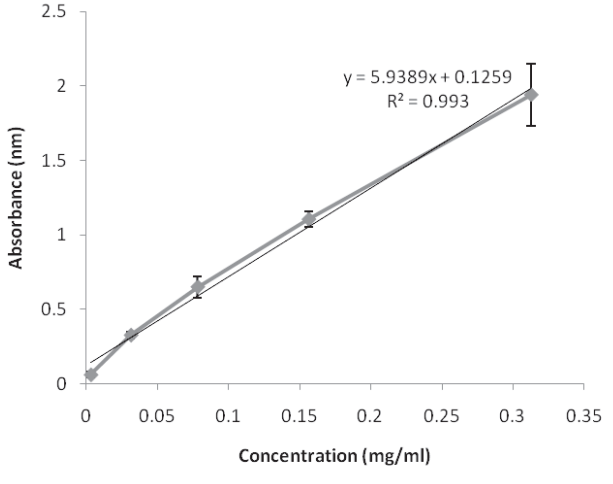

Figure 3. FRAP scavenging activity of *C. deliciosa* extract. Values are expressed as mean ± standard deviation (n = 3).

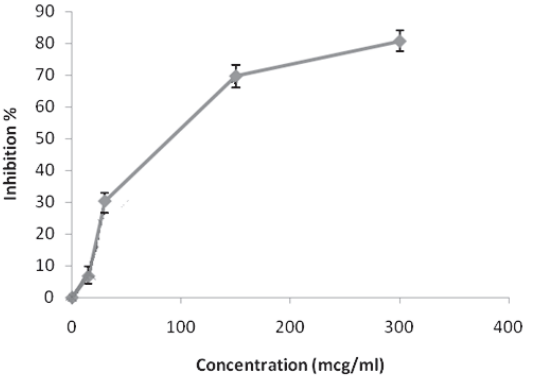

Figure 4. Hydroxyl radical scavenging activity of *C. deliciosa* extract. Values are expressed as mean ± standard deviation (n = 3).

Phenolics and polyphenolic compounds constitute the main class of natural antioxidants present in plants. The most widespread and diverse phenolics are the flavonoids, which are secondary metabolites, and widely dispersed throughout the plant kingdom. As antioxidants, there is a wide degree of effect variation between the different phenolic compounds. The number of hydroxyl groups and substitution with electron-donating alkyl or methoxy groups of flavonoid increase the antioxidant potential.[18] The total phenolic and flavonoid contents in *Citrus* species

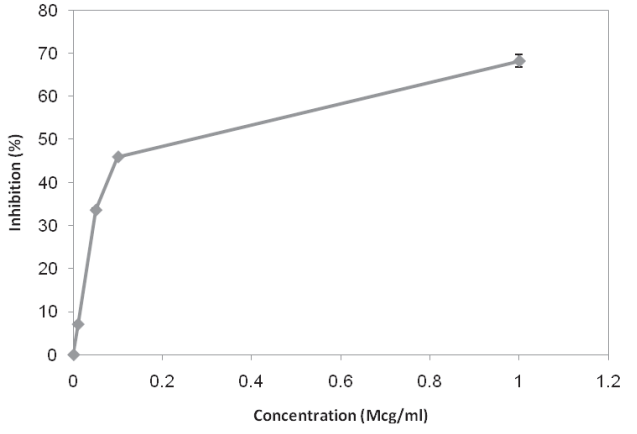

Figure 5. Superoxide anion radical scavenging activity of *C. deliciosa* extract. Values are expressed as mean ± standard deviation (n = 3).

were usually higher in peels respect to tissues[5] and the antioxidant activity of fruits and vegetables significantly increases with the increase of total polyphenol content concentration.

The activity of antioxidants in foods and biological systems depends on several factors such as the colloidal properties of the substrates, the conditions, the stages of oxidation and the localization of antioxidants in different phases. When testing natural antioxidants *in vitro*, it is therefore important to consider the system composition, the type of oxidisable substrate, the mode of accelerating oxidation, the methods to assess oxidation, and how to quantify antioxidant activity. For this reason, there cannot be a short-cut approach to determining antioxidant activity. Each evaluation, by using several methods to measure different products of oxidation, should be carried out under various conditions of oxidation.[19] We, for these reasons, used five antioxidant methods.

DPPH is a stable organic free radical with adsorption band at 515–528 nm which usually used as a reagent to evaluate free radical scavenging activity of antioxidants. Because it can accumulate many samples in a short period and is sensitive enough to detect active ingredients at low concentrations, it has been extensively used for screening antiradical activities of fruit and vegetable juices or extracts.[3]

The results suggest that *C. deliciosa* extract is capable of scavenging free radicals and, thus, it is able to prevent the initiation of free radical-mediated chain reactions by stabilizing reactive species before they can participate in deleterious reactions. The IC_{50} was 0.358 mg/ml. According to the studies carried out by Min-Sheng et al, IC_{50} value of *Citrus reticulata* Blanco peels and *C. reticulata* Blanco immature peels were 0.78 and 0.46 mg/ml;[4] Ghasemi et al have recorded IC_{50}, 1.1, 0.6, 1.9, 1.4, 2.1 mg/ml for experiments conducted with *C. sinensis* var, Washington Navel *C. reticulata* var. Ponkan, *C. unshiu* var. Mahalli, *C. limon* and *C. paradis* peels.[5]

The ABTS method, compared with a standard amount of Trolox, measures the relative antioxidant ability of the sample to scavenge the radical $ABTS^{\cdot+}$ in the aqueous phase. The $ABTS^{\cdot+}$, generated by potassium persulfate, is presented as an excellent tool for determining the antioxidant activity of hydrogen-donating (scavengers of aqueous phase radicals) and the chain breaking antioxidants (scavengers of lipid peroxyl radicals). Rice-Evans and co-workers have demonstrated that $ABTS^{\cdot+}$ assay can be used to measure a broad diversity of substances' antioxidant activity.[20] The TEAC value of the extracts at a 6 minutes reaction was 0.2 mmol Trolox equivalent/100 g DW. TEAC values of sweeties and grapefruits peels were 8.47 and 7.31 μmol Trolox/g FW.[21] Asghar et al have also reported TEAC value 16.19, 19.40, 4.56, 7.21, 1.28 mmol Trolox equivalent/liter of *Citrus* fruit extracts for *Citrus aurantium*, *Citrus medica*, *Citrus paradise*, *Citrus sinensis* and *Citrus aurantium* (22)

FRAP assay, with a ferric tripyridyltriazine (Fe^{3+}– TPTZ) complex and producing a colored ferrous tripyridyltriazine (Fe^{2+}–TPTZ), measures the reducing potential of an antioxidant reaction. Generally, the reducing properties are associated with the presence of compounds, which exert their action by breaking the free radical chain through donating a hydrogen atom (23). The EC_1 and EC_{50} values of *C. deliciosa* were 0.152 and 0.147 mg/ml. Min-Sheng *et al* have reported EC_{50} value 0.12, 0.25, 0.24, 0.24 mg/ml for *Aurantii* Fructus, *Citri Reticulatae* Pericarpium, *Citri Reticulatae* Viride Pericarpium, and *Aurantii* Immaturus Fructus (4).

Hydroxyl radicals are high reactive-oxygen species capable to attack most biological substrates, e.g. carbohydrates, DNA, polyunsaturated fatty acids, and proteins. The prevention of such deleterious reactions is highly significant in terms of both human health and the shelf-life of foodstuffs, cosmetics, and pharmaceuticals. Therefore, it is important to assess the protective ability of the extract against the hydroxyl radicals. When hydroxyl radical generated by the Fenton reaction attacks the deoxyribose, deoxyribose degrades into fragments that react with TBA on heating at low pH to form a pink color, which can be quantified spectrophotometrically at 532 nm So, the inhibition effect from the changes of absorption can

be calculated (3). The IC_{50} for *C. deliciosa* was 76 μg/ml. IC_{50} of Pericarpium *Citri Reticulatae* was 76.22 μg/ml (3)

Superoxide anion radical, as the precursor of the more reactive oxygen species including hydroxyl and peroxynitrite radicals, is very harmful to the cellular components in a biological system. The superoxide anion radical scavenging activities of the extracts from *C. deliciosa* assayed by the xanthine/xanthine oxidase method and IC_{50} was 94 μg/ml. Yi et al have reported IC_{50} 78.4 μg/ml for Pericarpium *Citri Reticulatae* in the PMS-NADH-NBT system (3).

The results suggest that *C. deliciosa* methanoic extract is capable for scavenging free radicals so, it is able to prevent the initiation of free radical-mediated chain reactions by stabilizing reactive species before they can participate in deleterious reactions.

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