

The Antioxidative Capacity of Iranian *Citrus sinensis* Var. *Valencia* Peels from Iran

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Available Online: 15th December, 2016

ABSTRACT

Orange peel, as a widely consumed fruit, is inedible and considered as a countless source of leftover. It contains several effective compounds such as flavonoids. Nowadays, as both the effectiveness of antioxidants and deadliness of free radicals are proved, many focus on plants and natural resources as an important source of antioxidants. This study was aimed at evaluating the antioxidative and anti-radical effect of orange peel using different tests. A methanolic extract was taken from peel and the amount of polyphenolic and flavonoid compounds was determined using spectrophotometric methods. The antioxidant activity was evaluated with FRAP and DPPH, ABTS, hydroxyl and superoxide radical scavenging activity assays. IC₅₀ for DPPH, ABTS (at the 6 minute), FRAP, hydroxyl radical scavenging assay, and superoxide radical scavenging assay was reported to be 491.52, 52.89, 125.23, 99.36 mg/ml, and 167.59 mg/ml, respectively. Polyphenolic and flavonoid compounds were obtained to be 58.62 and 3.25 mg/ml, respectively. Results indicate that orange peel has appropriate antioxidative, anti-hydroxyl radical, and anti-superoxide effect

Keywords: *Citrus sinensis*, *Valencia*, Antioxidant, polyphenol, superoxide radical scavenging assay

INTRODUCTION

The Genus *Citrus* is a shrub, from Rutaceae family; the peel residue of *Citrus sinensis* is the primary waste fraction and is an interesting source of phenolic compounds, which include phenolic acids and flavonoids. The main flavonoids of *C. sinensis* are polymethoxyflavanone, polymethoxychalcones¹, polymethoxy flavones (ie. sinensetin, nobiletin, tangeretin, quercetogetin)², flavanone glycosides (i.e., hesperidin and naringin)³, The citrus flavonoids have been found to have benefits effect, such as anticancer, antiviral, anti-inflammatory, analgesic and Neuroprotective activities, effects on capillary fragility, and an ability to inhibit human platelet aggregation⁴⁻⁶. Some of flavonoids effect are based in their antioxidant activity⁴. Antioxidants being defined as a molecule that can delay or inhibit the oxidation of other molecules cause by reactive oxygen species (ROS). They are generated in living organisms as by-products through many metabolic pathways, can readily react, oxidize and damage most biomolecules such as DNA, RNA, polysaccharide, cholesterol, protein and cause various

disease such as Alzheimer and neurodegenerative disorders, atherosclerosis and rheumatoid arthritis⁷⁻¹⁰. Living organisms possess a number of protective mechanisms against the oxidative stress and toxic effects of ROS. As antioxidants may attenuate oxidative damage by free radical or metal chelation, studies have been conducted in order to identify their potency, and their potential therapeutic capacities in various diseases with oxidative stress pathogenesis¹⁰.

Orange production in 2010 is 66.4 million tonnes (36.3 million tonnes fresh and 30.1 million tonnes processed). World consumption of oranges grew at a compound rate of 3.5 percent over the period 1987-89 to 1997-99. Consumption of fresh oranges grew at an annual rate of 2.8 percent¹¹. Peels are abundant byproducts of citrus and it can be used as inexpensive source for different medicinal purposes. Various studies investigated antioxidant effect of *C. sinensis* peels but most of them used limited test for assaying antioxidant capacity of peels. Different antioxidant compounds may act in vivo through different mechanisms, no single method can fully evaluate the TAC of foods, and therefore we used

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a panel of tests to evaluate the total antioxidant capacity of *Citrus sinensis* var. *Valencia* peels that grows in Iran.

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-tripyridyl-s-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), Folin-Ciocalteu reagent, were purchased from Sigma. $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, FeCl_3 anhydrous were purchased from Fluka Co. All other chemicals used were of analytical grade supplied by Merck.

Plant material

The *Citrus sinensis* var. *Valencia* locally known as porteghal, fresh and ripe fruits were prepared from region of Iran namely Dezful. The herbal product extract was prepared according to previous works¹⁰. Peels of *C. sinensis* were prepared manually and dried at room temperature.

Powdered peels (10 g) were extracted with 100 ml of methanol overnight at room temperature for 48 h. The extract was centrifuged at 3500 rpm for 20 min to obtain the supernatant and the residue was 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 evaporation of organic solvent, the extract was used for further analysis.

Determination total flavonoid contents

For determined of flavonoids content, 1 ml of methanolic extract was added to 1 ml of 2% methanolic $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$. The absorbance was measured 10 min later at 430 nm (X-ma 3000, Human, Korea). The results were expressed as mg rutin/ g dry extract^{12,13}.

Determination total phenolic content

For determined of phenolic compound, 0.5 ml of extract and 2.5 ml of a 1/10 aqueous dilution of folin-ciocalteu reagent were mixed. After 5 min 2 ml of Na_2CO_3 7.5% were added and incubated at room temperature for 120 min, Absorption at 765 nm was measured using a spectrophotometer. The total phenolic content was expressed as Tannic acid. The results were expressed as mg Tannic acid /g dry extract^{14,15}.

DPPH free radicals scavenging activity assay

DPPH assay was performed according to the method 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 absorbance at 515 nm was measured. The tubes were then incubated at room temperature for 30 min under dark conditions and the absorbance was measured at 517 nm¹⁶. 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 represented the concentration of sample that caused 50% inhibition^{17,18}.

ABTS free-radical scavenging activity

The $\text{ABTS}^{\cdot+}$ radical was generated by chemical reaction with potassium persulfate ($\text{K}_2\text{S}_2\text{O}_8$). For this purpose, 25 ml of ABTS (7 mM) was spiked with 440 μl of $\text{K}_2\text{S}_2\text{O}_8$ (140 mM) and allowed to stand in darkness at room temperature for 12–16 h. The working solution was prepared by taking a volume of the previous solution and diluting it in methanol until its absorbance at $\lambda = 734 \text{ nm}$ was 0.70 ± 0.02 . The reaction took place directly in the measuring cuvette. For this purpose, 2 ml of the $\text{ABTS}^{\cdot+}$ radical were added; the absorbance (A_0) was measured, and 100 μl of sample or standard were added immediately. The absorbance was measured 2, 4 and 6 min after mixing reagent at 734 nm¹⁷.

Ferric-reducing antioxidant power (FRAP) assay

The FRAP reagent contained 2.5 ml of a 10 mM tripyridyltriazine (TPTZ) solution in 40 mM HCl plus 2.5 ml of 20 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ 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 ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) were used for calibration. All tests were run in triplicate and mean values were used to calculate EC_1 values. EC_1 is defined as concentration of an antioxidant having a ferric reducing ability equivalent to that of 1 mM ferrous salt¹⁹.

Hydroxyl radicals scavenging activity assay

The reaction mixture contained 100 μl of extract dissolved in water, 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 and 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. Tubes were incubated at 50°C for 30 min. 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 min. The extent of oxidation was estimated from the absorbance of the solution at 532 nm. The percentage inhibition values were calculated from the absorbance of the control (A_c) and of the sample (A_s) using the equation: Inhibition (%) = $((A_c - A_s) / A_c) \times 100$, where the controls contained all the reaction reagents except the extract or positive control substance²⁰

Superoxide anion scavenging activity assay

Superoxide anion scavenging activity of *C. deliciosa* was measured using the xanthine/xanthine oxidase method. A 0.5 ml of samples was added to a 1.0 ml mixture of 0.4 mM xanthine and 0.24 mM nitroblue

tetrazolium chloride (NBT) in 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 min. The reaction was terminated by adding 2.0 ml of 69 mM sodium dodecylsulphate (SDS) and the absorbance of NBT was measured at 560 nm¹⁰.

Statistical analysis

The data determined were expressed as the mean of three replicate determinations and presented as mean ± SD (standard deviation). The IC₅₀ values were estimated by linear/non-linear regression.

RESULTS

Polyphenolic and flavonoid compounds are important plant compounds having antioxidative effects. In orange peel, there are a considerable amount of these compounds. Amount of polyphenolic compounds was obtained using Folin-Ciocalteu test (58.62 ± 0.22 mg Tannic acid /g dry extract) and amount of flavonoid compounds from flavonol and flavones was obtained using ALCL₃ test (3.25 ± 0.12 mg rutin/ g dry extract). The final methanolic extract was calculated using maceration method (27.73 ± 1.23 g/ 100 g herb dry powder). In the present study different test were carried out in order to have a better evaluation on the antioxidative capacity. Results of these tests are indicated in table 1 and Fig 1-5. Antioxidative activity of *C.deliciosa* peels has been studied in another study¹⁰. Results of this study are also indicated in table 1 to have a better comparison (parameters of both studies become the same).

DISCUSSION

Nowadays, Medicinal Plants play an important role in treating different diseases. Raw extracts and compounds purified from plants are extensively used for different purposes. Some of these therapeutic effects are antioxidative and anti-radical effects of plants, which

given the side effects and limitations of using synthetic antioxidants, use of plants as natural antioxidants is emphasized²¹. Different botanicals compounds such as carotenoids, Sesquiterpenes, polysaccharides and etc. have appropriate antioxidative effects. Among compounds, polyphenolic ones are important due to their powerful antioxidative effect, which have been studied in several studies²². Polyphenolic compounds have too important groups such as flavonoids, *Coumarin*, *stilbenes* and etc. Several studies have been carried out on flavonoids and their properties indicating a direct relation between the amount of flavonoid compounds and plants` antioxidative capacity²³. As indicated in table 1, amount of polyphenolic and flavonoid compounds in *C. deliciosa* is more than *C. sinensis*.

The DPPH radical-scavenging assay has been widely used to investigate the scavenging capacity of antioxidant compounds. DPPH is a stable organic free radical with adsorption band at 515–528 nm. The main disadvantage of DPPH assay is that DPPH is a long-lived nitrogen radical, which bears no similarity to the highly reactive and transient peroxy radicals involved in lipid peroxidation²⁴. Results of this test indicate that *C.sinensis* peel has proper effect in DPPH radical-scavenging but its effect is weaker compared with *C.deliciosa*. According to the studies carried out by Su *et al*, IC₅₀ value of *Citrus reticulata* Blanco peels and *C. reticulata* Blanco immature peels in DPPH assay were 0.78 and 0.46 mg/ml²⁵, Ghasemi *et al* have recorded IC₅₀ , 1.1, 0.6, 1.9, 1.4, 2.1 mg/ml for experiments conducted with *C. sinensis* var, *Washington*, *C. reticulata* var. *Ponkan*, *C. unshiu* var. *Mahalli*, *C. limonand* and *C. paradisi* peels²⁶.

ABTS^{•+}, the oxidant, was generated by persulfate oxidation of 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS²⁻). The ABTS^{•+} is presented as an excellent tool for determining the antioxidant activity of electron-donating and the chain breaking antioxidants (scavengers of lipid peroxy radicals)²⁴.

Table 1: IC₅₀ and EC₁ values of various assays for *C. deliciosa* and *C. sinensis* peels

Antioxidant Methods	Parameters (µg/ml) (<i>Citrus deliciosa</i> peels)	Parameters (µg/ml) (<i>Citrus sinensis</i> peels)	
DPPH assay	IC50: 353.3	IC50: 491.52	Fig. 1
ABTS assay	IC50: 2 min: 51.32 4 min: 44.12 6 min: 40.98	IC50: 2 min: 64.31 4 min: 56.82 6 min: 52.89	Fig. 2
FRAP assay	EC1: 77.96	EC1: 125.23	Fig. 3
Hydroxyl radical scavenging activity	IC50: 76.23	IC50: 99.36	Fig. 4
Superoxide radicals scavenging activity	IC50: 94.02	IC50: 167.59	Fig. 5

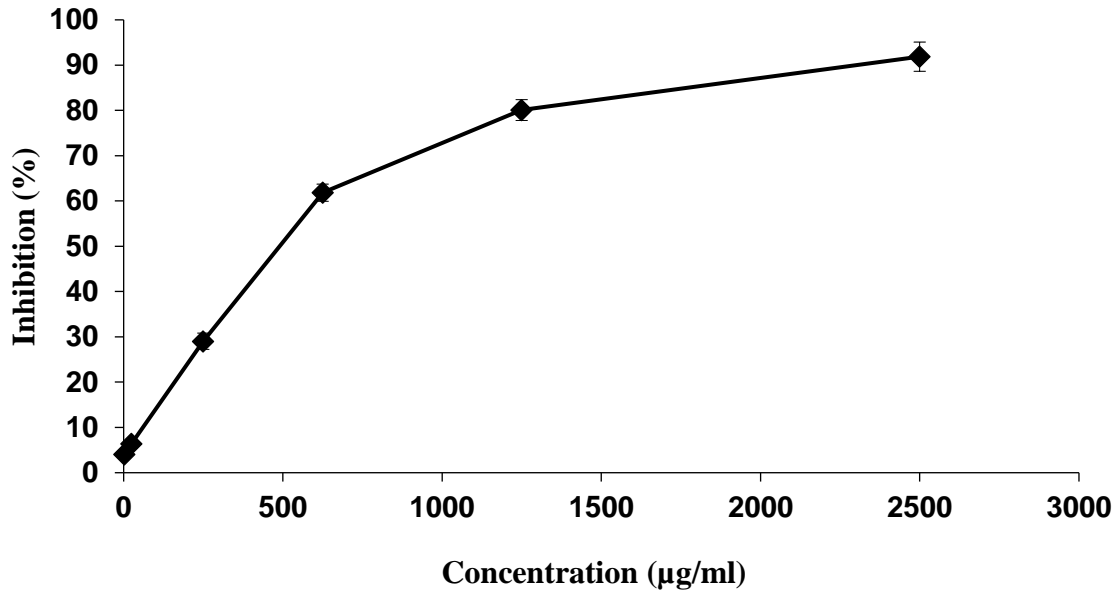


Figure 1

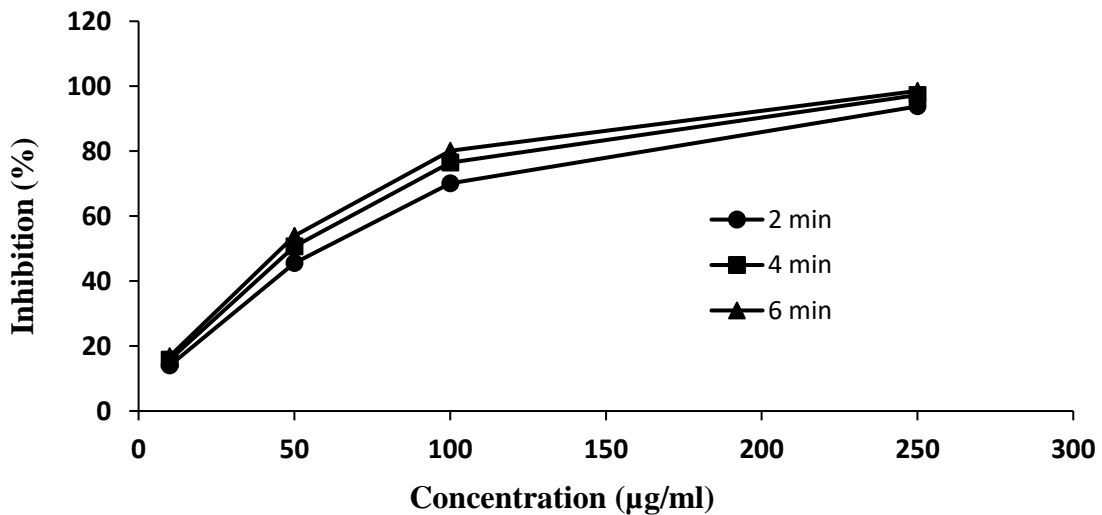


Figure 2

As seen in table 1, at minutes 2, 4, and 6, *C. deliciosa* peel has a lower IC_{50} compared by *C. sinensis*, which indicates that *C. deliciosa* shows a faster and more powerful effect than *C. sinensis*.

Antioxidant potential of *Citrus sinensis* extract was estimated from their ability to reduce TPTZ–Fe (III) complex to TPTZ–Fe (II) complex. The ferric reducing antioxidant power, generally measures the antioxidant effect of any substance in the reaction medium as its reducing ability²⁴. Results show that in FRAP test, reducing power of *C. deliciosa* is more powerful than *C. sinensis*. Comparing the results of this study with *Phoenix dactylifera* leaf (EC_{10} : 0.28 mg/mL), *P. dactylifera* var *Deyri* (EC_{10} : 5.35 mg/mL) have weaker effects than *C. sinensis* (EC_{10} : 125.23 µg/ml)^{12,17}. EC_{10} of

methanolic and aqueous extracts of *Allium hirtifolium* were 3.99 and 50.23 mg/ml¹⁹

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 pharmaceuticals²⁷. Hydroxyl radicals produced through complex reaction of Iron-EDTA with H_2O_2 attacks deoxyribose in the presence of ascorbic acid to form products that after being heated by the thiobarbituric acid in a low pH, produce pink pigments²⁸. A rate constant for the reaction of scavenger with hydroxyl radical can be inferred from inhibiting the formation of

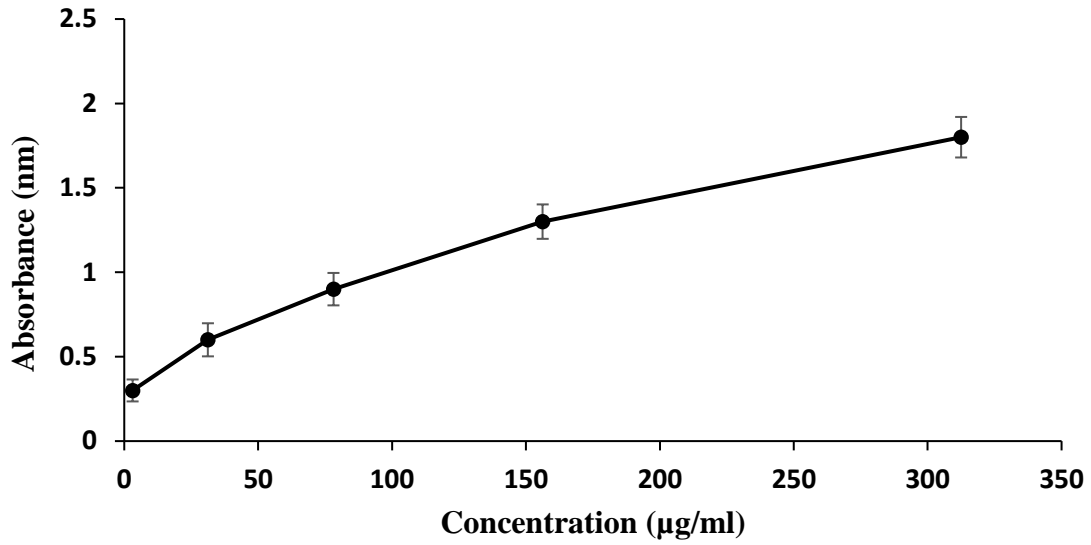


Figure 3

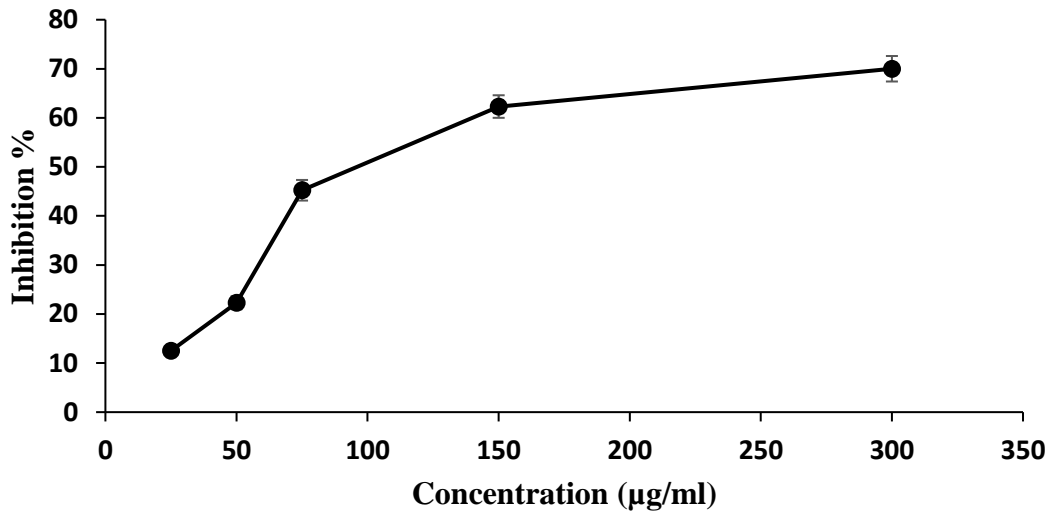


Figure 4

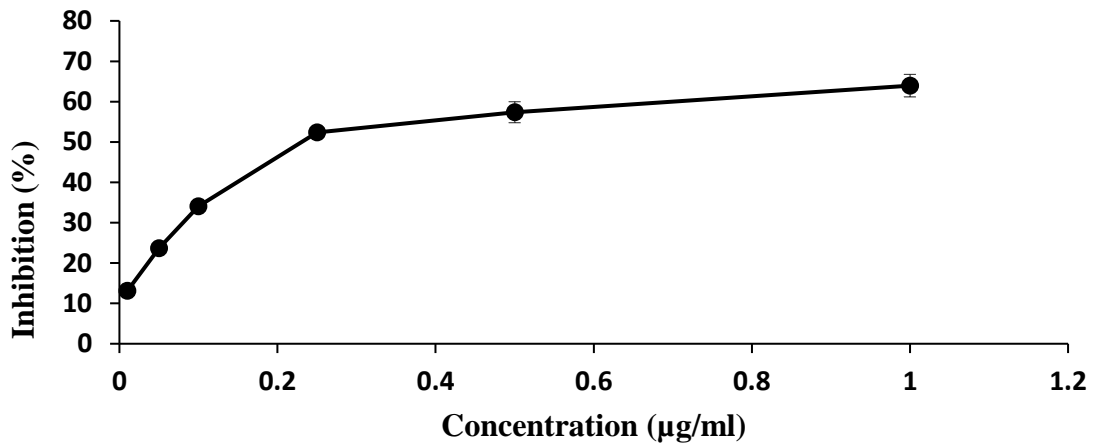


Figure 5

color. The IC₅₀ for *C. deliciosa* peels and Pericarpium *Citri reticulatae* were 76 and 76.22 µg/ml¹⁰.

The superoxide radical is generated within aerobic biological systems during both enzymatic and nonenzymatic oxidations. Superoxide anion radical, as the precursor of the more reactive oxygen species including hydroxyl and peroxy nitrite radicals, is very harmful to the cellular components in a biological system²⁹. IC₅₀ for *C. sinensis* using the method above is 167.59 µg/ml so *C. deliciosa* with IC₅₀ of 94.02 µg/ml has more powerful effects.

In superoxide anion scavenging activity assay the IC₅₀ value of the methanolic and aqueous extracts of *Allium hirtifolium* were found to be 3.59 and 34.88 µg/ml respectively. Yi et al have reported IC₅₀ 78.4 µg/ml for Pericarpium *Citri Reticulatae* in the PMS-NADH-NBT system³⁰.

CONCLUSION

Results of the present study indicate that antioxidative and anti-radical effects of *C. sinensis* peel is appropriate but its effect is lower than *C. deliciosa* peel.

ACKNOWLEDGMENT

This paper is issued from thesis of Fatemeh Javedani and financial support was provided by physiology research center, Ahvaz Jundishapur university of medical sciences, Ahvaz, Iran (PRC-46).

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