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EVALUATION OF ANTIOXIDANT ATTRIBUTES OF ORANGE (CITRUS SINENSISL.)

Shivanki Aggarwal[*1] & Sheel Sharma[2]

[*1]Research Scholar, Department of Food Science and Nutrition, Banasthali University, Rajasthan, India

[2]Professor, Department of Food Science and Nutrition, Banasthali University, Rajasthan, India

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ABSTRACT

Phytochemicals are gaining unprecedented recognition as cardinal food constituentsto effectively mitigate rampant scenario of chronic, degenerative diseases. This study attempted to explore the antioxidant attributes of Orange (Citrus sinensisL.) in three categories: nutrient antioxidants estimation i.e. vitamin C and β-carotene, phytochemical quantification i.e. total phenols and flavonoids, and in vitro antioxidant assays expressed in terms of DPPH (2,2-Diphenyl-1-picrylhydracyl) radical scavenging assay, ABTS (2,2'-Azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) radical scavenging assay and FRAP (Ferric reducing antioxidant power) assay. To undertake latter two prospects, aqueous-organic extracts were prepared from fresh orange sample. Results revealed the fruit to be rich in nutrient- and phytochemical-associated antioxidant potential, mean values of vitamin C, β-carotene, total phenols, total flavonoids, DPPH assay, ABTS assay and FRAP assay being noted as 14.84±0.23 mg/100g, 182.93±5.84 µg/100g, 81.42±7.47 mg GAE/100g, 11.35±0.99 mg CE/100g, 61.01±2.61 mg TE/100g, 96.92±6.05 mg TE/100g and 62.77±0.94 mg FeSO4/100g respectively, and therefore validate it to be a novel dietary agent as an antidote to chronic diseases linked with oxidative stress.

KEYWORDS: Orange, phenolics, vitamins, antioxidants, antiradical efficacy.

ABBREVIATIONS

DPPH: 2,2-Diphenyl-1-picrylhydracyl, ABTS: 2,2'-Azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) , FRAP: Ferric reducing antioxidant power, TPC: Total phenolic content, TFC: Total flavonoid content, TE: Trolox equivalent, GAE: Gallic acid equivalent, CE: Catechin equivalent, TROLOX: 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid

I. INTRODUCTION

Due to grim burden of chronic diseases in India well-documented to emanate from oxidative stress and abortive results of high doses of isolated antioxidant supplements in human intervention trials, recent nutritional approaches is giving due attention to phytochemicals as mainstay of balanced diet. Their significance as 'Life Span Essentials' attributes to their antioxidant properties i.e. structure-derived free radical scavenging and metal chelating mechanisms; and origin-derived stress inducing bioactivity leading to cell signaling pathways associated with antioxidant upregulation), easy availability from plant foods, promising role in health promotion and disease prevention (Liu Rh, 2004).Fruits and vegetables, apart from being good sources of vitamins, minerals, and fiber, are also dubbed as rich sources of phytochemicals. Several epidemiological studies in citing an association between fruit and vegetable consumption and reduced risk of chronic diseases i.e. cancer, stroke, heart disease, obesity, diabetes and the like, have purported much of the disease prevention potential of fruits and vegetables in human health to be provided by phytochemicals (Palafox-Carlos et al, 2011). Orange, in particular, has been extolled to contain 170 phytonutrients and 60 flavonoids with anti-tumor, anti-inflammatory, blood-clot inhibiting and antioxidant properties (Etebu andNwauzoma, 2014).

II. MATERIALS AND METHODS

Sample procurement

Orange was randomly purchased in triplicate from different local food markets of Newai, Rajasthan and pooled together to get representative sample.

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Sample preparation

Oranges werepeeled, edible parts were obtained, then crushed with mortar-and-pestle using some water to obtain a paste-like consistency.Samples were immediately used for extract preparation.

Quantification of nutrient antioxidants

1) Vitamin C

Principle: Vitamin C (L-ascorbic acid), after extraction in a mixture of metaphosphoric acid and dilute acetic acid, is estimated by titrating it with 2,6Dichlorophenol indophenol dye solution. Oxidized form of this dye has a blue and red colour in alkaline and acidic medium respectively while reduced form has no colour (leuco form). Upon redox reaction with vitamin C, the dye generates its leuco form.

Procedure

Sample extraction- 10g fresh sample was homogenized with about 50ml of 5% metaphosphoric-10% acetic acid solution. Then it was transferred into a 100ml volumetric flask and was shaken gently until a homogeneous dispersion was obtained. Then it was diluted upto the mark by 5% metaphosphoric-10% acetic acid solution. Then the solution was filtered and the clear filtrate was collected for the determination of vitamin C in that sample.

Sample analysis

The burette was filled with dye solution (52mg of sodium salt of the dye + 42mg of sodium bicarbonate in water and made upto 500ml). 20ml of standard vitamin C solution (10mg/L in 5% metaphosphoric-10% acetic acid solution) was transferred into a titration flask and titrated against the dye solution to the appearance of a light pink color. Noted down the volume. Similarly, 20ml of test solution was titrated against the dye solution and value was noted (Raghuramulu et al 2003).

Calculations: Since the concentration of 20ml standard vitamin C solution is 200µg, vitamin C content in 100ml or 10g test sample is calculated as $\frac{Y}{X} \times 1mg$, where Y= amount (ml) of dye causing oxidation of test vitamin C solution (20ml) and X= amount (ml) of dye causing oxidation of standard vitamin C solution (20ml).

2) β-carotene

Procedure: 10g of macerated sample was placed into a conical flask containing 50ml of 95% ethanol and maintained at a temperature of 70-80°C in a water bath for 20 minutes with periodic shaking. The supernatant was decanted, allowed to cool and its volume was measured by means of a measuring cylinder and recorded as initial volume. The ethanol concentration of the mixture was brought to 85% by adding 15ml of distilled water and it was further cooled in a container of ice water for about 5 minutes. The mixture was transferred into a separating funnel and 25ml of petroleum ether was added and the cooled ethanol was poured over it. The funnel was swirled gently to obtain a homogeneous mixture and it was later allowed to stand until two separate layers were obtained. The bottom layer was run off into a beaker while the top layer was collected into a 250ml conical flask. The bottom layer was transferred into the funnel and re-extracted with 10ml petroleum ether for 5-6 times until the extract became fairly yellow. The entire petroleum ether was collected into 250ml conical flask and transferred into separating funnel for re-extraction with 50ml of 80% ethanol. The final extract was measured, its absorbance was measured using a spectrophotometer at a wavelength of 436nm (Mustafa and Babura, 2009).

Calculations

Equation of Bear-Lamberts law was applied i.e. A=ECL or C=A/EL; where C= concentration of carotene, A= absorbance, E= extinction coefficient, L= thickness of cuvettes (1 cm), E = 1.25×10^4µg/l

Phytochemical estimation and antioxidant activity assessment

1) Sample extraction

Macerated samples (1g) were placed in test tubes with 10ml of methanol/water (50:50, v/v). The pH was adjusted to 2 using 2M HCl. The tubes were thoroughly shaken, using orbital shaker, at room temperature for 1 hr, and then centrigued at 2500 g for 10 minutes. Supernatants were collected in clean dry test tubes. Then the residues were extracted again with 10 ml of acetone/water mixture (70:30, v/v). The methanol and acetone extracts were combined and subsequently used for various assays. Extracts produced in duplicate. In case of non-usage of extracts on the same day, they were stored at 4°C and used within a week for all analysis (Pérez-Jiménez et al 2008).

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2) Phytochemical analysis

a) Total phenolic content (TPC)

Principle:It is based on the single electron transfer (SET) in alkaline medium (7% $NaCO_3$) from phenolic compound to molybdenum, forming blue complex which is measured spectrophotometrically at 750-765nm.
Procedure:The mixture of sample solution (0.1 ml), deionized water (6 ml), FCR solution (0.5 ml), 7% $NaCO_3$ (1.5 ml) was vortexed for 1 min and incubated for 8 min at room temperature. Then a dose of 1.9 ml of deionized water was added. The mixture was allowed to stand for 2 h at room temperature. The absorbance was measured at 765 nm against blank (Xu and Chang, 2007).

Calculations: TPC was expressed as gallic acid equivalent (mg GAE/100g sample) through the calibration curve of gallic acid. Linearity range of the calibration curve was 100 to 1000 µg/ml.

b) Total flavonoid content (TFC)

*Principle:*Aluminum chloride forms acid complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonois. In addition, aluminum chloride forms acid labile complexes with orthodihydroxyl groups in the A or B ring of flavonoids.

Procedure: 0.5 ml of sample was mixed with 2.5 ml of deionized water in a test tube followed by adding 0.15 ml of 5% sodium nitrite ($NaNO_2$) solution. After 6 minutes, 0.3 ml of 10% aluminum chloride hexahydrate ($AlCl_3.6H_2O$) solution was added and allowed to stand for another 5 minutes before adding 0.1 ml of 1 M NaOH. The mixture was brought to 5 ml with the addition of 0.55 ml of deionized water and mixed well. The absorbance was measured immediately against the blank (the same mixture without the sample) at 510 nm using UV-spectrophotometer (Xu and Chang, 2007).

Calculations: TFC was expressed ascatechin equivalents (mg CE/100g sample). Linearity range of catechin calibration curve was 62.5-375 µg/ml.

3) Antioxidant activity analysis

a) DPPH radical scavenging activity

Principle: It is based on antioxidant-catalyzed reduction of purple-colored DPPH radical to its yellow-colored non-radical form which is measured spectrophotometrically at 517nm.

*Procedure:*A doseof 0.2 ml of tested legume extract was added to 7.6 ml ethanol solution of DPPH radical (final concentration was 0.1 mM). The mixture was shaken vigorously for 1 min by vortexing and left to stand at room temperature in the dark for 30 min. Thereafter, the absorbance was measured at 517 nm against ethanol blank (Xu and Chang, 2007).

Calculations:DPPH scavenging activity was expressed as trolox equivalents (mg TE/100g sample). Linearity range of trolox calibration curve was **0**.05 µM to 0.4 mMtrolox.

b) ABTS radical scavenging activity

Principle:It is based on antioxidant inhibition of the absorbance of blue-green coloured ABTS radical, generated via persulfate-induced ABTS oxidation, measured spectrophotometrically at 734nm.
Procedure:Thestock solutions included 7.4 mM ABTS solution and 2.6 mM potassium persulfate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in the dark. The solution was then diluted by mixing 1 ml $ABTS^{\bullet+}$ solution with 30 ml methanol to obtain an absorbance of 1.1 ±0.02 units at 734 nm using the spectrophotometer. In sample (0.3 ml), prepared $ABTS^{\bullet+}$ solution (5.7 ml) was added and kept it for 2 hrs in a dark condition. Then the absorbance was taken at 734 nm using spectrophotometer (Thaipong et al., 2006).
Calculations:ABTS scavenging activity was expressed as trolox equivalents (mg TE/100g sample). Linaerity range of trolox calibration curve was 0**.**1-0**.**7 mMtrolox.

c) FRAP assay

Principle:It is based on antioxidant-catalyzed reduction of ferric-TPTZ complex (colorless) to ferrous form (intensely blue coloured) which is measured spectrophotometrically at 593nm

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*Procedure:*The working FRAP reagent was prepared by mixing 10 volumes of 300 mM acetate buffer, pH 3.6, with 1 volume of 10mM TPTZ (2,4,6-Tripyridyl-s-triazine) in 40mM HCl and with 1 volume of 20mM FeCl3 ×6H2O (ferric chloride hexahydrate). Prepared working FRAP reagent was warmed to 37◦C. In 6 ml FRAP reagent, 0.2 ml of sample and 0.6 ml of deionized water were added and the absorbance was taken at 593 nm against reagent blank after 4min (Xu and Chang, 2007).

*Calculations:*FRAP value was expressed as Fe^{2+} (Iron (II) sulfate heptahydrate or ferrous sulfate) equivalent (mg FRAP/100g sample). Linearity range of the calibration curve was 0.1-0.9mM.

III. RESULTS AND DISCUSSION

Orange, besides being a good source of vitamins and minerals, has been stressed to be rich in phytochemicals too. This study attempted to quantify nutrient antioxidants, phenolics, flavonoids and antioxidant activity of orange expressed in terms of DPPH radical scavenging activity, ABTS radical scavenging activity and FRAP reducing assay. Results with respect to nutrient antioxidants, as shown in table 1, exhibit orange to be rich in vitamin C and β-carotene. These results are in line with Gopalan et al, 2002.Other carotenoids of antioxidant significance documented to be present in orange are β-cryptoxanthin, lutein (Ashcoff et al, 2015).

Table 1: Antioxidant vitamins (vitamin C and β-carotene) content of Orange

Vitamin C (mg/100g)	β-carotene (µg/100g)
14.84±0.23	182.93±5.84

Values are mean±standard deviation, n=3

In context of phytochemical profile, as presented in table 2, orange was found to be a good source of phenols and flavonoids and the results are consistent with Yan et al, 2006;Prakash et al, 2011; and Srivastava et al, 2013. The major phenolic class cited to predominate in oranges are flavonoids, particularly flavanones (hesperidin, narirutin, didymin, eriocitrin, naringin, naringenin, neohesperidin), flavones (6,8-di-C-glucosyl-apigenin, diosmin, poncirin, neoeriocitrin), polymethoxyflavones (sinensetin, nobiletin). They have been well-explained to exert cardioprotective, chemopreventive and neuroprotective effects and the purported mechanisms underlying such health effects are characterized as their antioxidant, anti-inflammatory bioactivities and signaling regulation, at molecular level, associated with upregulation of cellular antioxidant defense systems (Hwang et al, 2012; Gattuso et al, 2007; Peterson et al, 2006).Phenolic acids i.e. p-coumaric, ferulic, caffeic and sinapic acids have also been studied to locate in orange(Rapisarda et al, 2008).

Table 2: Phytochemical content of aqueous-organic extracts of Orange

Total phenols (mg GAE/100g)	Total flavonoids (mg CE/100g)
81.42±7.47	11.35±0.99

Values are mean±standard deviation, n=3

In vitro antioxidant activity assays (DPPH, ABTS, FRAP) in pursuit of exploring antioxidant potential of orangerevealed significant results, shown in table 3, which are in agreement with Ali et al, 2010; Murilio et al, 2012 and Yan et al, 2006.*In vivo* study by Franke et al, 2005 corroborated antioxidant bioactivity of orange by administering orange juice (236ml) to healthy subjects (n=13) for 3 weeks which resulted in significant increase in plasma concentrations of vitamin C, folate, carotenoid, flavonone and 16-29% decrease in 8-hydroxy deoxyguanosine (by-product of oxidative stress) in white blood cells.This suggests that orange holds the capacity to scavenge free radicals and to intervene in free radical-inflicted oxidative stress in body, which consequently paves a prudent way to health promotion and disease prevention.

Table 3: Antioxidant activity of aqueous-organic extracts of Orange

DPPH (mg TE/100g)	ABTS (mg TE/100g)	FRAP (mg $FeSO_4$/100g)
61.01±2.61	96.92±6.05	62.77±0.94

Values are mean±standard deviation, n=3

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IV. CONCLUSION

Orange(*Citrus sinensis*L.), besides being a rich source of vitamins, minerals, fiber, has been extolled to contain numerous phytochemicals characterized to confer cardioprotective, chemopreventive, neuroprotective effects through their conventional and cell signaling antioxidant mechanisms. Analysis of nutrient antioxidants, phytochemicals and *in vitro* antioxidant activity of the fruit in above study highlights its importance as a therapeutic food ingredient in health promotion and disease prevention.

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