Characterization of the antioxidant properties of citrus mutants induced by Gamma-rays

Min Young Kim [1,2,*], In-Jung Kim [1,2], Hyo Yeon Lee [1,2], Dong-Sun Lee [1,2], Soon Jae Im [1], Ji Hye Kim [1], Ji Hee Byun [1], Ji Young Kim [1], Youn Ji Lee [1], Seo Rin Jeong [1], Jung Hyun Kim [3], Seo Hyun Moon [4]

[1.] Laboratory of Pharmacology and Toxicology, Faculty of Biotechnology, College of Applied Life Science and [2.] Subtropical Horticulture Research Institute, Jeju National University, Jeju 690-756, Republic of Korea
[3.] Department of Tourism and Food Service Cuisine, Cheju Tourism College, Jeju 695-900, Republic of Korea
[4.] DNA Forensic Center, National Forensic Service, Seoul 158-707, Republic of Korea
jeffmkim@jejunu.ac.kr

Abstract: The antioxidant potential of the methanolic extracts obtained from fruits and leaves of gamma irradiated citrus was evaluated using 1,1-diphenyl-2-picrylhydrazyl (DPPH), superoxide anion, hydrogen peroxide and nitric oxide inhibition assays. Results showed that antioxidant activity of citrus was modulated by gamma irradiation. The DPPH, superoxide and hydrogen peroxide scavenging activities were significant higher ($p < 0.05$) for the pulp, leaf and peel extracts of citrus mutants, with IC_{50} value ranges of 1.02–1.10, 2.59–2.84, 0.82–0.91 mg/mL, respectively, but lower nitric oxide scavenging activities for the peel and pulp extracts of citrus mutants, as compared with the corresponding values of citrus wild-type.
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1. Introduction

Citrus is one of the most abundant and widely consumed crops globally because of their great economic and health value. Producer continuously seek varieties with morphological appearance, i.e. size, color and shape, taste characteristics, improved nutritional value, shelf life and other quality features to meet the consumers' expectations and concepts related to fruit traits and to promote international trade. However, it has been known that citrus is among the most difficult plants to improve through traditional breeding approaches.

Inducing mutations by gamma ray has been effectively used to improve more desirable and economically useful traits with several species of citrus. Irradiation of gamma rays on budstick can produce higher frequencies of mutation, leading to the creation of new variants compared to the control. Nowadays, the number of cultivars derived from mutation induction increases constantly (Bermejo et al., 2011; Chaudhuri, 2002; Deng, 2005; Ling et al., 2008; Raza et al., 2003). Although many of the effects of gamma irradiation on seedlessness, pollen germination, fruit quality and growth of citrus have received considerable attention, little experimental study that takes into account the antioxidant activity of citrus mutants obtained by gamma irradiation. Our objective was, therefore, to evaluate the potential antioxidant activities of these citrus mutants by employing the 1,1-diphenyl-2-picrylhydrazyl (DPPH), superoxide, hydrogen peroxide and nitric oxide scavenging assays. Leaves and different fruit parts (peel and pulp) were chosen in an attempt to make systematic comparisons among their potent bioactivities and to identify the part with high antioxidant activity for further studies.

2. Material and Methods

2.1. Irradiation and sample preparation

Budsticks obtained from one year-old citrus (*Citrus unshiu* Marc. cv. Miyagawa) were exposed to cobalt (^{60}CO) source with the dose of 120 Gy at the Institute for Nuclear Science and Technology, Jeju National University. The budsticks were then refrigerated until the following day when buds were grafted onto one year old sour orange (*C. aurantium* L.) seedling in the greenhouse and then selected mutant plants were moved to an open field. Initially, the leaf type, early and late fruit developments, thorn formation, size of petal leaves, adaptability to environmental conditions, fruit yield, and other plant characteristics were recorded. All cultivars shared the same environmental, cultural and soil conditions, thus the differences among cultivars were not influenced by climatic factors or crop technique. Representative samples studied in this paper were harvested in 2010. Based on preliminary observations of fruit characteristics, mutation had stable targeted fruit quality traits, with three mutants (Mut) selected for antioxidant analysis (Oh and Kim, 2011), test subjects were divided into four groups: (i) citrus wild-type derived from non-irradiated shoots

(WT); (ii) citrus mutants with comparatively high sugar/acid ratio (Mut I); (iii) citrus mutants with red color (Mut II); (iv) citrus mutants with rough shape (Mut III). The fruit peel and pulp, and leaves of citrus were dissected, lyophilized and then ground into a fine powder using a blender. Portions (25 g) of the powered samples were successively extracted with 250 mL of methanol in the 25 ºC shaking incubator for 24 h and subsequently purified by using a 0.45 μm membrane filter (Waters, Milford, MA, US), which were stored at 4 ºC until further use.

2.2. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) and superoxide radical scavenging assays

DPPH radical and superoxide scavenging activities of citrus WT and Mut methanolic extracts were determined as described in our recently published paper (Kim and Kim, 2011). A dose response curve was plotted to determine the IC_{50} values. IC_{50} is defined as the concentration sufficient to obtain 50 % of a maximum scavenging capacity. All tests were performed in triplicate.

2.3. Hydrogen peroxide scavenging assay

The hydrogen peroxide scavenging of the methanolic extracts was measured by using the method of (Senevirathne et al, 2010). Each experiment was performed at least in triplicate.

2.4. Nitric oxide radical scavenging assay

Nitric oxide scavenging activity was evaluated following the method of Green et al (1982) with slight modifications. The reaction mixture (100 μL) containing 10 mM sodium nitroprusside in phosphate-buffered saline (pH 7.0), with or without methanolic extracts at concentrations of 0.125, 0.25, 0.5, 1 and 2 mg/mL, was incubated at 25 °C for 3 h. Following incubation, reaction mixture was mixed with an equal amount of Greiss reagent (1% sulfanilamide and 0.1% *N*-1-naphthylethylene diamine dihydrochloride in 2.5% polyphosphoric acid), which was allowed to stand for 5 min, then absorbance of assay mixture was determined at 540 nm. Three replicates were made for each test sample to calculate IC_{50} values.

2.5. Statistical analysis

All values are expressed as the means ± standard deviation. Treatment effects were analyzed by one-way analysis of variance followed by a Duncan's multiple range tests using SPSS software (ver. 12.1, SPSS Inc., Chicago, IL, US). Differences were considered statistically significant at $p < 0.05$.

3. Results and Discussion

The prevention of the chain initiation step by scavenging various reactive species such as free radicals is considered to be an important antioxidant mode of action (Dastmalchi et al, 2007).

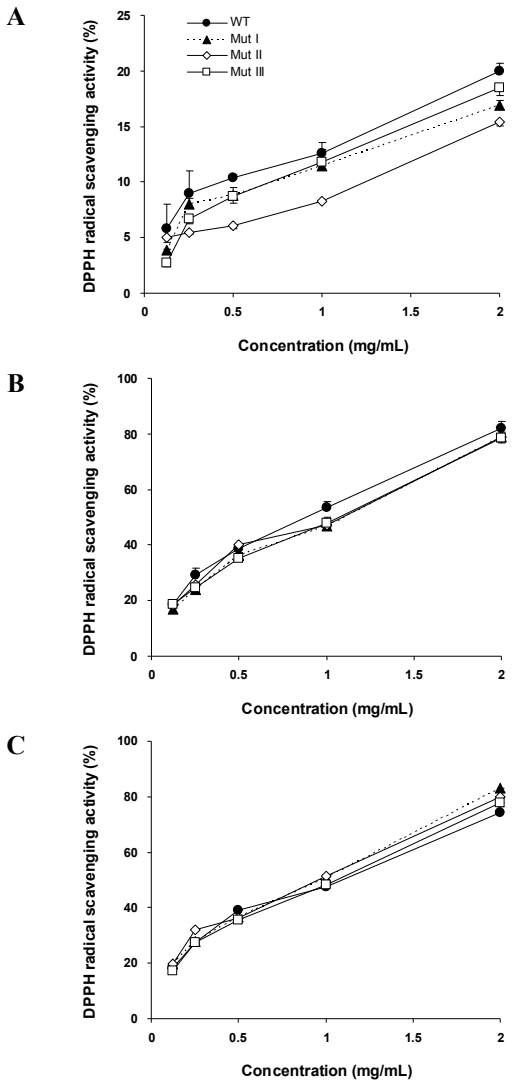

Fig. 1. Scavenging DPPH radical effects of leaf (A), peel (B) and pulp (C) extracts of citrus derived from non-irradiated and irradiated shoots. Data represent the mean ± standard deviation of three determinations.

Scavenging activity for free radicals of DPPH has been widely used to evaluate the antioxidant activity of natural products from plant sources (Huang et al, 2005; Zhu et al, 2004). The decrease in absorbance of the DPPH radical due to its reduction by different antioxidants is illustrated. Absorbance decreases as a result of a color change from purple to yellow as the radical is scavenged by

antioxidants through donation of hydrogen to form the stable DPPH free radical.

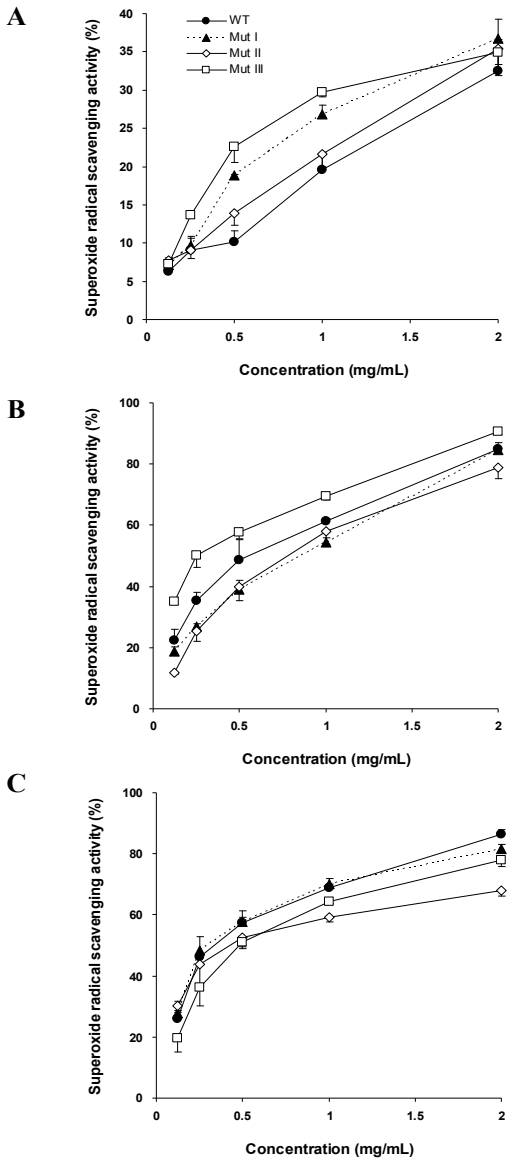

Fig. 2. Scavenging superoxide radical effects of leaves (A), peel (B) and pulp (C) extracts of citrus derived from non-irradiated and irradiated shoots. Data represent the mean ± standard deviation of three determinations.

Methanolic extracts of citrus WT and Mut plants were prepared for investigation of their antioxidant activities. As the data shown in Figure 1, scavenging effects of three parts of citrus extracts on DPPH radicals sharply increased from 0.125 to 2 mg/mL and were 15–20%, 79–82% and 74–83% for leaf, peel and pulp at 2 mg/mL, respectively. The top three most potent scavenging DPPH radical activity was observed in the pulp extracts of all Mut groups (1.02, 1.03 and 1.10 mg/mL IC_{50} for Mut I, II and III, respectively), which possess significantly higher activity on scavenging DPPH radicals than that of citrus WT (1.13 mg/mL IC_{50}) ($p < 0.05$) (Table 1). However, citrus leaf and peel extracts of Mut groups showed lower IC_{50} value than that of WT group extracts (Figure 1 and Table 1).

Superoxide anion is one of the most representative free radicals. In biochemical systems, superoxide radical can be converted into hydrogen peroxide by the action of superoxide dismutase and the hydrogen peroxide can subsequently generate extremely reactive hydroxyl radicals in the presence of certain transition metal ions or by UV photolysis (Halliwell and Gutteridge, 1999). In the present study, the superoxide anion scavenging activities of citrus WT and Mut plants were investigated and compared by using a NBT reduction method. With regard to scavenging effects of methanolic extracts on superoxide radicals, citrus peel and pulp more effective than leaves; the scavenging effects of citrus peel and pulp extracts increased from 12–35% and 20–30% at 0.125 mg/mL to from 79–90% and 68–87% at 2 mg/mL, respectively, much better that of leaf extracts (Figure 1 and Table 1). The citrus leaf extracts of all Mut groups (2.59–2.84 mg/mL IC_{50}) and peel extracts of Mut III (0.63 mg/mL IC_{50}) had relatively higher potential scavenging effects on superoxide as compared with the corresponding extracts of WT (3.15 and 0.86 mg/mL IC_{50}, respectively) ($p < 0.05$) (Table 1). However, IC_{50} values of peel extracts of Mut I and II, and pulp extract of Mut II and III in superoxide scavenging activities were above those of WT extracts, indicating they had relatively little scavenging properties (Table 1). No statistically significant differences were observed between the other Mut and the WT samples (Table 1).

Although hydrogen peroxide itself is not very reactive, it can sometimes cause cytotoxicity by giving rise to hydroxyl radicals in the cell. The results of hydrogen peroxide inhibition by the methanolic extracts of citrus WT and Mut plants are summarized in Figure 3 and Table 1. The IC_{50} values of peel and pulp extracts were 0.82–1.18 and 1.17–1.83 mg/mL, respectively, which were lower than that of leaf extracts (2.21–2.31 mg/mL) (Table 1). Obviously, the hydrogen peroxide scavenging activities were concentration-dependent for all parts of citrus extracts and showed significantly higher in peel extracts of Mut I and II, and in pulp extracts of Mut III than those of WT group extracts, while lower in the extracts of Mut III peels, and Mut II and III

pulps (Figure 3 and Table 1). Data represent the mean ± standard deviation of three determinations.

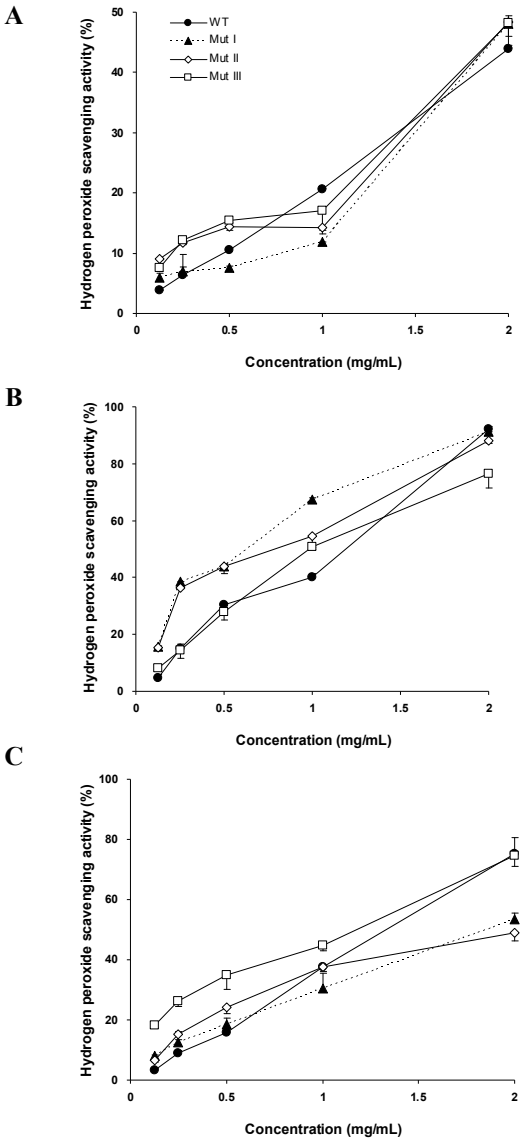

Fig. 3. Scavenging hydrogen peroxide effects of leaves (A), peel (B) and pulp (C) extracts of citrus derived from non-irradiated and irradiated shoots.

Nitric oxide is an essential bioregulatory molecule required for several physiological processes. The elevation of the nitric oxide results in inflammation, cancer and other pathological conditions. The nitric oxide scavenging abilities of the methanolic extracts of citrus WT and Mut plants are presented in Figure 4 and their IC_{50} in Table 1. The same trend was observed for the level of nitric oxide scavenging effects in tested citrus; three parts of citrus extracts scavenged nitric oxide in a dose-dependent manner, and citrus peel and pulp extracts had higher nitric oxide scavenging ability than citrus leaf extracts. However, these extracts of all Mut groups showed no better scavenging effects on nitric oxide compared with the corresponding extracts of WT (Table 1). Moreover, the capacities of scavenging effects on nitric oxide in the extracts of Mut I and II peels, and Mut II and III pulps were lower (higher IC_{50} values) than those of WT extracts (Table 1).

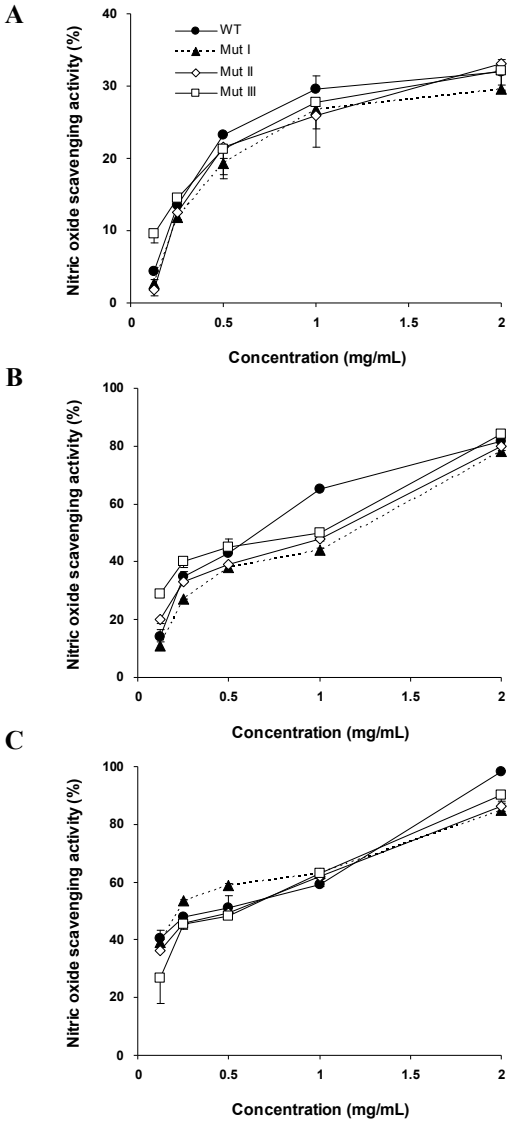

Fig. 4. Scavenging nitric oxide radical effects of leaves (A), peel (B) and pulp (C) extracts of citrus derived from non-irradiated and irradiated shoots. Data represent the mean ± standard deviation of three determinations.

Table 1. IC_{50} value in antioxidant capacities of leaves, peel and pulp extracts of citrus derived from non-irradiated and irradiated shoots

Group	IC_{50} value (mg/mL)*			
	DPPH radical	Superoxide radical	Hydrogen peroxide	Nitric oxide radical
Leaves				
WT	5.52 ± 0.358[a]	3.15 ± 0.450	2.31 ± 0.041	2.82 ± 0.160
Mut I	6.46 ± 0.333[b]	2.59 ± 0.194	2.31 ± 0.063	3.08 ± 0.057
Mut II	7.41 ± 0.253[c]	2.84 ± 0.239	2.27 ± 0.180	2.83 ± 0.374
Mut III	5.61 ± 0.261[a]	2.64 ± 0.121	2.21 ± 0.107	3.00 ± 0.338
Peel				
WT	1.00 ± 0.050[a]	0.86 ± 0.054[a]	1.09 ± 0.013[a]	0.92 ± 0.032[a]
Mut I	1.11 ± 0.051[b]	0.98 ± 0.017[b]	0.82 ± 0.005[b]	1.11 ± 0.012[b]
Mut II	1.08 ± 0.027[b]	1.03 ± 0.025[b]	0.91 ± 0.032[c]	1.02 ± 0.013[c]
Mut III	1.10 ± 0.011[b]	0.63 ± 0.026[c]	1.18 ± 0.058[d]	0.90 ± 0.018[a]
Pulp				
WT	1.13 ± 0.027[a]	0.72 ± 0.001[a]	1.35 ± 0.068[a]	0.66 ± 0.031[a]
Mut I	1.02 ± 0.004[b]	0.72 ± 0.056[a]	1.82 ± 0.137[b]	0.65 ± 0.013[a]
Mut II	1.03 ± 0.007[b]	0.95 ± 0.080[b]	1.83 ± 0.072[b]	0.75 ± 0.014[b]
Mut III	1.10 ± 0.004[c]	0.89 ± 0.067[b]	1.17 ± 0.066[c]	0.76 ± 0.062[b]

* IC_{50}, the concentration of citrus methanolic extracts that inhibited 50% of radicals. IC_{50} was obtained by interpolation from linear regression analysis. Each values is expressed as mean ± standard deviation ($n = 3$).
[a-d] Values with different superscripts in a column are significantly different ($p < 0.05$)

Reactive oxygen species such as superoxide, hydrogen peroxide and nitric oxide radicals, are very unstable and play an important role in oxidative stress related to the pathogenesis of various diseases through the direct reaction with other substances (Halliwell and Gutteridge, 1999; Finkel and Holbrook, 2000). Antioxidant properties of the various extracts from many plants are of great interest in both academia and the food industry, since their possible use as natural additives emerged from a growing tendency to replace synthetic antioxidant by natural ones. Previously, we observed great variations in the phenolic contents of citrus fruits and leaves obtained by gamma irradiation and some authors have shown the important role played by phenolics in the antioxidant capacity of citrus (Proteggente et al, 2003; Gorinstein et al, 2004; Anagnostopoulou et al, 2006). Considering above results obtained, it may anticipated that gamma irradiation modulates antioxidant activity achieved by the scavenging of DPPH, superoxide, hydrogen peroxide and nitric oxide, possibly because of the correlation between antioxidant activity and the contents of phenolics. A further research is recommended to verify the mechanism of antioxidative action of citrus extracts induced by gamma irradiation.

Corresponding Author:
Dr. Min Young Kim
Faculty of Biotechnology
College of Applied Life Sciences
Jeju National University
Jeju 690-756, Republic of Korea
E-mail: jeffmkim@jejunu.ac.kr

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