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# Free Radical Scavenging Activity of Butanol Fraction from the Fruit of *Citrus junos*

**– Research Note –**

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## Abstract

**In this study, we investigated the free radical [1,1-diphenyl-2-picrylhydrazyl (DPPH), hydroxyl radical (·OH) and superoxide anion ($O_2^-$)] scavenging activity of MeOH extract and 3 fractions of *Citrus junos*. Of the tested fractions, the BuOH fraction showed the strongest DPPH scavenging activity, showing the $IC_{50}$ values of 63.4 mg/mL. Therefore, we continuously carried out DPPH, ·OH and $O_2^-$ scavenging activity tests of BuOH fraction of *Citrus junos*. The BuOH fraction of *Citrus junos* inhibited DPPH radical to 97.5% at a concentration of 1000 mg/mL and the scavenging activities were increased concentration-dependently. In addition, BuOH fraction from *Citrus junos* also scavenged ·OH in a concentration dependent manner from 5 to 1000 mg/mL. Furthermore, BuOH fraction showed about 56% $O_2^-$-scavengimg activity at 25 mg/mL concentration but, the scavenging activities were not enhanced in a dose dependent manner. The present results suggest that BuOH fraction of *Citrus junos* would have the protective potential from oxidative stress induced by free radicals.**

**Key words:** *Citrus junos*, free radical, hydroxyl radicals, superoxide anion

## INTRODUCTION

Free radicals and other reactive species are considered to be important causative factors in the development of various diseases and aging process (1-4). It is well known that an imbalance between free radical-generating and free radical-scavenging systems results in oxidative stress, a condition that has been associated with the cell injury seen in many pathologic conditions (5-7). This relationship has led to considerable interest in search for the antioxidant to scavenge free radicals and elevate defense system. Although several synthetic antioxidants have been suggested for the prevention and treatment of diseases, the various kinds of side effects and toxicities have become an issue. Therefore, the natural antioxidants from foods have attracted much attention and great effort has been made to search for safe and effective therapeutic agents for oxidative stress-related diseases. Compelling evidences indicate that increased consumption of dietary antioxidants or vegetables with antioxidant properties may contribute to the improvement of the quality of life by delaying onset and reducing the risk of degenerative diseases (8-10). Epidemiological studies have shown that increased consumption of fruits and vegetables containing high levels of phytochemicals has been recommended to prevent chronic diseases related to oxidative stress in the human body (11-14).

*Citrus junos* Sieb (*Rutaceae*), the hybrid of *C. inchangensis* and *C. reticulata* var. *austera* (15) has been cultivated mainly in Korea, China, and Japan (16). The fruit has been used as an aromatic bitter peptic, an expectorant, and a cough remedy in folk medicine (16). It is widely cultivated in southern seashore of Korea and mainly consumed as *Citrus junos* honey and *Citrus junos* juice in Korea. Shin et al. (17,18) reported the antioxidant activities of *Citrus junos* juice and hot-water extracts of *Citrus junos* peel. Kwon et al. (19) also showed that ethanol extract from *Citrus junos* seed has DPPH and hydroxyl radical scavenging activity. However, the antioxidative activities of MeOH extract and various solvent fractions from *Citrus junos* has not been demonstrated. Therefore, to investigate antioxidative capacity of *Citrus junos*, we tested 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity of MeOH extract and 3 fractions of *Citrus junos* and then examined DPPH, hydroxyl radical (·OH) and superoxide anion ($O_2^-$) scavenging activities of BuOH fraction, the most active fraction, from *Citrus junos*.

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## MATERIALS AND METHODS

### Materials

1,1-Diphenyl-2-picrylhydrazyl (DPPH), nitroblue tetrazolium (NBT), malondialdehyde (MDA), and 2-deoxyribose were purchased from Sigma (Sigma-Aldrich, Korea). All other chemicals used were of analytical grade and were obtained from Merck and Sigma (Sigma-Aldrich, Korea).

### Preparation of sample

Fruits of *Citrus junos* were purchased from Garak agricultural and marine products wholesale market in Seoul. The whole fruits with peel of *Citrus junos* (10 kg) was extracted with MeOH under reflux. Methanol was removed in reduced pressure and the extract (980 g) was partitioned between $CH_2Cl_2$ and water to yield $CH_2Cl_2$-soluble fraction (12.8 g). The aqueous layer was further extracted with *n*-BuOH to yield BuOH-soluble fraction (66.7 g).

### DPPH radical scavenging activity

DPPH radical scavenging activity was measured by the method of Hatano et al (20). The reaction mixture contains 100 mL of 60 mM DPPH and 100 mL of methanol extracts whose concentration was predetermined. Reaction mixture left stand at dark room for 30 min. Absorbance of reaction mixture was determined at 540 nm. Ethanol (95%) was used as a control. The scavenging activity of DPPH radical was expressed as $IC_{50}$ and an inhibition rate as follow.

DPPH-scavenging activity (%) = $(Abs_c - Ab_s)/Abs_c \times 100$
$Abs_c$: Absorbance of control, $Abs_s$: Absorbance of sample

### Hydroxyl radical scavenging activity

The oxidized 2-deoxyribose by hydroxyl radical produced from the Fenton reaction is degraded to malondialdehyde (MDA) (21). Reaction mixture was prepared with 0.2 mL of 10 mM $FeSO_4$-$7H_2O$ with 10 mM EDTA, 10 mM 2-deoxyribose solution (0.2 mL) and methanol extracts (1.4 mL)/or 0.2 M phosphate buffer (1.4 mL, pH 7.4). The reaction was initiated adding 1 mM $H_2O_2$ (0.2 mL) followed incubation at 37°C for 4 hr. After incubation, 1 mL of each 2.8% trichloroacetic acid (TCA) and 1 mL of 1.0% thiobarbituric acid (TBA) were added to incubation medium. It was boiled (95～100°C) for 10 min followed immediate cooling on the ice water. MDA produced during the reaction was measured at 520 nm. Phosphate buffered saline (pH 7.0) was used as a control. The ·OH scavenging activity was expressed as an inhibition rate as follow.

·OH-scavenging activity (%) = $(Abs_c - Ab_s)/Abs_c \times 100$
$Abs_c$: Absorbance of control, $Abs_s$: Absorbance of sample

### Superoxide radical scavenging activity

Superoxide radical generated in the xanthine-xanthine oxidase system was determined spectrophotometrically via monitoring the product of NBT as an end product (22). Reaction mixture was prepared with 400 mL of each methanol extracts (100～1000 mg/mL), 100 mM xanthine, 60 mM NBT, 0.05 U/mL xanthine oxidase and 0.1 M phosphate buffer (pH 7.4) to be the final volume of 2.0 mL. After incubation at 37°C for 10 min, the absorbance was measured at 560 nm, compared with the control samples run without xanthine oxidsase. Percent inhibition was calculated from the optical density of the BuOH fraction treated and control samples.

Inhibitory rate (%) = $[(C - CB) - (S - SB)]/(C - CB) \times 100$
C: control, CB: control blank, S: sample, SB: sample blank

### Statistical analysis

All statistical analyses performance was assessed by SAS software (SAS Institute, Cary, NC, USA). $p<0.05$ was determined as statistically significant. Measurement data were expressed as mean ± standard deviation.

## RESULTS AND DISCUSSION

Reactive oxygen species (ROS) currently known to be produced in the body include $O_2^-$, hydrogen peroxide ($H_2O_2$), ·OH, singlet oxygen ($^1O_2$) and lipid hydroperoxide (LOOH). These ROS induce oxidative tissue damage, react with cell membranes, and thus induce lipid peroxidation or cause inflammation, playing an important role as pathological mediators in many clinical disorders such as heart disease, diabetes, gout and cancer (23-25). Free radicals such as $O_2^-$, $H_2O_2$, ·OH and lipid radical cause DNA damage, protein denaturation and lipid peroxidation that can lead to cell death (26-28). Lipid peroxidation is initiated by radicals attacking unsaturated fatty acid, and propagated by a chain reaction cycle (29). Since unsaturated fatty acids are the most important components of biological membranes and impart desirable properties upon the fluidity of cellular membrane structure, the peroxidation of unsaturated fatty acids in biological membranes leads to disruption of membrane structure and function (30).

In this study, to investigate antioxidative capacity of *Citrus junos*, we tested 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity of MeOH extract and 3 fractions of *Citrus junos* and then examined DPPH, hydroxyl radical (·OH) and superoxide anion ($O_2^-$) scavenging activities of BuOH fraction, the most active fraction, from *Citrus junos*.

DPPH is a stable free radical and has been widely used to test the ability of compounds or plant extracts to act as free radical scavengers or hydrogen donors (20,31). The DPPH radical is stable in ethanolic solution for more than 60 min (20). Antioxidants react with the DPPH radical directly and restore it by transfer of electrons or hydrogen. Therefore, we used this system for assessing the radical scavenging activity of MeOH extract and 3 fractions of *Citrus junos*. In the DPPH scavenging activity tests of MeOH extract and 3 fractions of *Citrus junos* (Table 1), the BuOH fraction showed the strongest DPPH radical scavenging activity, showing the $IC_{50}$ values of 63.4 mg/mL. Therefore, based on this result for DPPH radical, we continuously investigated the radical scavenging activities with BuOH fraction of *Citrus junos*.

As shown in Table 2, the treatment of BuOH fraction from *Citrus junos* increased DPPH radical scavenging activity dose-dependently. At 1000 mg/mL concentration, 97.5% inhibition of DPPH radical was observed. These results suggest that BuOH fraction from *Citrus junos* is a promising against for scavenging of free radicals.

Moreover, $O_2^-$ and ·OH induce various injuries to the surrounding organs and play a vital role in some clinical disorders. Therefore, removal of $O_2^-$ and ·OH is the most effective defense of living body against disease (32). In particular, among various different radicals the ·OH is an extremely reactive and short-lived species that can attack biological molecules such as DNA, proteins, and lipids. The reactivity of ·OH has been related to several human diseases such as neurodegenerative disease and diabetes. Therefore, its scavenging activity has received much attention (33-35). Also, Kwon et al. (19) reported that ethanol extract from *Citrus junos* seed has ·OH scavenging activity. Table 3 shows the effect of BuOH fraction from *Citrus junos* against ·OH. Similar to the result on DPPH, the BuOH fraction also had the strongest protective effect against ·OH and increased ·OH scavenging activity as dose-dependant manner. At 1000 μg/mL concentration, the scavenging activity was elevated to 79.3%. On the other hand, the BuOH fraction from *Citrus junos* showed about 56% $O_2^-$-scavengimg activity at 25 mg/mL concentration but, the scavenging activities were not enhanced in a dose dependent manner, implying that BuOH fraction from *Citrus junos* scavenge $O_2^-$ effectively at low concentration and reached maximum activity at 25 mg/mL concentration (Table 4). From these results, we could confirm that BuOH fraction from *Citrus junos* may be an effective ·OH and $O_2^-$ scavenger and protect against radical-induced oxidative damage.

In summary, the BuOH fraction from *Citrus junos* effectively scavenged DPPH radical and ·OH with a dose-dependent manner. The present results demonstrated the protective effect on free radical-induced oxidative stress. The further study on active components from BuOH fraction from *Citrus junos* with antioxidative activity has to be supported.

**Table 1.** $IC_{50}$ values of methanol extract and each fractions of *Citrus junos* against DPPH radical

| Group | $IC_{50}$ (μg/mL) |
|---|---|
| MeOH extract | $164.5 \pm 21.0^{c}$ |
| $CH_2Cl_2$ fraction | $193.8 \pm 8.9^{b}$ |
| BuOH fraction | $63.47 \pm 2.7^{d}$ |
| $H_2O$ fraction | $217.5 \pm 7.6^{a}$ |

Values are mean ± SD. [a-d]Means with the different letters are significantly different ($p<0.05$) from treatment concentration by Duncan's multiple range test.

**Table 2.** DPPH radical scavenging activities of BuOH fraction from *Citrus junos*

| Concentration (mg/mL) | Scavenging activity (%) |
|---|---|
| 5 | $35.0 \pm 2.5^{d}$ |
| 25 | $37.5 \pm 0.0^{cd}$ |
| 50 | $37.5 \pm 0.0^{cd}$ |
| 100 | $45.0 \pm 3.0^{bc}$ |
| 200 | $51.5 \pm 1.5^{b}$ |
| 500 | $95.0 \pm 5.0^{a}$ |
| 1000 | $97.5 \pm 2.5^{a}$ |

Values are mean ± SD. [a-d]Means with the different letters are significantly different ($p<0.05$) from treatment concentration by Duncan's multiple range test.

**Table 3.** Hydroxyl radical scavenging activities of BuOH fraction from *Citrus junos*

| Concentration (mg/mL) | Scavenging activity (%) |
|---|---|
| 5 | $9.6 \pm 0.3^{g}$ |
| 25 | $12.5 \pm 0.2^{f}$ |
| 50 | $22.1 \pm 0.4^{e}$ |
| 100 | $44.2 \pm 0.5^{d}$ |
| 200 | $59.6 \pm 0.5^{c}$ |
| 500 | $72.3 \pm 0.2^{b}$ |
| 1000 | $79.3 \pm 0.2^{a}$ |

Values are mean ± SD. [a-g]Means with the different letters are significantly different ($p<0.05$) from treatment concentration by Duncan's multiple range test.

**Table 4.** Superoxide anion scavenging activities of BuOH fraction from *Citrus junos*

| Concentration (mg/mL) | Inhibition (%) |
|---|---|
| 5 | $34.8 \pm 2.6^{b}$ |
| 25 | $56.7 \pm 4.8^{a}$ |
| 50 | $56.7 \pm 1.0^{a}$ |
| 100 | $56.1 \pm 1.7^{a}$ |

Values are mean ± SD. [a,b]Means with the different letters are significantly different ($p<0.05$) from treatment concentration by Duncan's multiple range test.

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## REFERENCES

1. Yu BP, Chung HY. 2001. Oxidative stress and vascular aging. *Diabetes Res Clin Pract* 54: S73-S80.
2. Maxwell SR. 2000. Cornary artery disease-free radical damage, antioxidant protection and the role of homocystein. *Basic Res Cardiol* 95: I/65-I/71.
3. Ray G, Batra S, Shukla NK, Deo S, Raina V, Ashok S, Husain SA. 2000. Lipid peroxidation, free radical production and antioxidant status in breast cancer. *Breast Cancer Res Treat* 59: 163-170.
4. Butterfield DA, Lauderback CM. 2002. Lipid peroxidation and protein oxidation in Alzheimer's disease brain: potential causes and consequences involving amyloid b-peptide-associated free radical oxidative stress. *Free Radic Biol Med* 32: 1050-1060.
5. Cho KJ, Han SH, Kim BY, Hwang SG, Park KK, Yang KH, Chung AS. 2000. Chlorophyllin suppression of lipopolysaccharide-induced nitric oxide production in RAW 264.7 cells. *Toxicol Appl Pharmacol* 166: 120-127.
6. Surh YJ, Chun KS, Cha HH, Han SS, Keum YS, Park KK, Lee SS. 2001. Molecular mechanisms underlying chemopreventive activities of anti-inflammatory phytochemicals: down-regulation of COX-2 and iNOS through suppression of NF-kappa B activation. *Mutat Res* 480-481: 243-268.
7. Metsaars WP, Hauw JJ, van Welsem ME, Duyckaerts C. 2003. A grading system of Alzheimer disease lesions in neocortical areas. *Neurobiol Aging* 24: 563-572.
8. Meydani M, Lipman RD, Han SN, Wu D, Beharka A, Martin KR, Bronson R, Cao G, Smith D, Meydani SN. 1998. The effect of long-term dietary supplementation with antioxidants. *Ann NY Acad Sci* 854: 352-360.
9. Ames BN, Shigenaga MK, Hagen TM. 1993. Oxidants, antioxidants, and the degenerative disease of aging. *Proc Natl Acad Sci USA* 90: 7915-7922.
10. Steinmetz KA, Potter JD. 1996. Vegetables, fruits, and cancer prevention. *J Am Diet Assoc* 96: 1027-1039.
11. Chu YF, Sun J, Wu X, Liu RH. 2002. Antioxidant and antiproliferative activities of common vegetables. *J Agric Food Chem* 50: 6910-6916.
12. Liu RH. 2003. Health benefits of fruit and vegetables are from additive and synergistic combinations of phytochemicals. *Am J Clin Nutr* 78: 517S-520S.
13. Rimm EB, Ascherio A, Giovannucci E, Spiegelman D, Stampfer MJ, Willett WC. 1996. Vegetable, fruit, and cereal fiber intake and risk of coronary heart disease among men. *JAMA* 275: 447-451.
14. Gillman MW, Cupples LA, Gagnon D, Posner BM, Ellison RC, Castelli WP, Wolf PA. 1995. Protective effect of fruits and vegetables on development of stroke in men. *JAMA* 273: 1113-1117.
15. Herman Z, Hasegawa S, Fong Chi H, OU P. 1989. Limonoids in *Citrus ichangensis*. *J Agric Food Chem* 37: 850-851.
16. Han DR. 1981. *Hyundai Sang Yak Hak*. Hak Chang Sa, Seoul, Korea. p 80-81.
17. Shin JH, Lee JY, Ju JC, Lee SJ, Cho HS, Sung NJ. 2005. Chemical properties and nitrite scavenging ability of citron (*Citrus Junos*). *J Korean Soc Food Sci Nutr* 34: 496-502.
18. Shin JH, Lee SJ, Seo JK, Cheon EW, Sung NJ. 2008. Antioxidant activity of hot-water extract from Yuza (*Citrus junos* SIEB ex TANAKA) Peel. *J Life Sci* 18: 1745-1751.
19. Kwon OC, Shin JH, Kang MJ, Lee SJ, Choi SY, Sung NJ. 2006. Antioxidant activity of ethanol extracts from citron (*Citrus junos* SIEB ex TANAKA) seed. *J Korean Soc Food Sci Nutr* 35: 294-300.
20. Hatano T, Edamatsu R, Hiramatsu M, Mori A, Fujita Y, Yasuhara T, Yoshida T, Okuda T. 1989. Effects of the interaction of tannins with co-existing substances, Ⅵ. Effects of tannins and related polyphenols on superoxid anion radical, and on 1,1-diphenyl-2-picryhydrazyl radical. *Chem Pharm Bull* 37: 2016-2021.
21. Chung SK, Osawa T, Kawakishi S. 1997. Hydroxyl radical-scavenging effects of spices and scavengers from brown mustard (*Brassica nigra*). *Biosci Biotech Biochem* 61: 118-123.
22. Robak J, Gryglewski RJ. 1988. Flavonoids are scavengers of superoxide anions. *Biochem Pharmacol* 37: 837-841.
23. Slater TF. 1984. Free-radical mechanisms in tissue injury. *Biochem J* 222: 1-15.
24. Vuillaume M. 1987. Reduced oxygen species, mutation, induction and cancer initiation. *Mutat Res* 186: 43-72.
25. Meneghini R. 1988. Genotoxicity of active oxygen species in mammalian cells. *Mutat Res* 195: 215-230.
26. Fraga CG, Shigenaga MK, Park JW, Degan P, Ames BN. 1990. Oxidative damage to DNA during aging: 8-hydroxy-2'-deoxyguanosine in rat organ DNA and urine. *Proc Natl Acad Sci USA* 87: 4533-4537.
27. Stadtman ER. 1992. Protein oxidation and aging. *Science* 257: 1220-1224.
28. Halliwell B. Gutteridge JMC. 1999. Free radicals, other reactive species and disease. In *Free Radicals in Biology and Medicine*. 3rd ed. Halliwell B, Gutteridge JMC, eds. Oxford University Press, New York. p 246-350.
29. Shimazaki H. 1994. Antioxidants. In *Free Radicals and Biological Defense.* Niki E, Shimazaki H, Mino M, eds. Japanese Science Societies Press, Tokyo. p 45-57.
30. Machlin LJ, Bendich A. 1987. Free radical tissue damage: protective role of antioxidant nutrients. *FASEB J* 1: 441-445.
31. Zhu QY, Hackman RM, Ensunsa JL, Holt RR, Keen CL. 2002. Antioxidant activities of oolong tea. *J Agric Food Chem* 50: 6929-6934.
32. Lin JM, Lin CC, Chen MF, Ujiie T, Takada A. 1995. Scavenging effects of *Mallotus repandus* on active oxygen species. *J Ethnopharmacol* 46: 175-181.
33. Halliwell B, Gutteridge JMC. 1984. Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochem J* 219: 1-14.
34. Halliwell B, Gutteridge JMC, Cross CE. 1992. Free radicals, antioxidants, and human disease: where are we now? *J Lab Clin Med* 119: 598-620.
35. Zhang D, Yasuda T, Yu Y, Zheng P, Kawabata T, Ma Y, Okada S. 1996. Ginseng extract scavenges hydroxyl radical and protects unsaturated fatty acids from decomposition caused by iron-mediated lipid peroxidation. *Free Radic Biol Med* 20: 145-150.

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