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Comparative Study on Antioxidant Activity of Vegetable Oils under *in vitro* and Cellular System

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Abstract

Overproduction of free radical and oxidative stress are involved in the progression of degenerative disease. Therefore, to attenuate oxidative stress induced by free radical, natural antioxidants including vegetable oils with ω-3 or ω-6 fatty acids have been much concern. This study was to investigate the inhibitory effect against free radical and protective activity from oxidative stress of different vegetable oils as potential sources of antioxidants and linolenic acid. Olive, corn, and *Perilla* oil have free radical scavenging activity and protective effect from superoxide anion (O_2^-), and peroxynitrite ($ONOO^-$)-induced cellular damage. In addition, *Perilla* oil exert the relatively high antioxidant effect at low concentration. Based on these results, we further studied radical scavenging effect of linolenic acid, which highly contained in *Perilla* oil. Our results revealed that linolenic acid increased 1,1-diphenyl-2-picrylhydrazyl and hydroxyl radical scavenging activity in a dose-dependent manner. Furthermore, linolenic acid showed noticeable protective effect against oxidative stress in a dose-dependent manner under LLC-PK_1 cells. Thus, *Perilla* oil and linolenic acid as a major fatty acid from *Perilla* oil suppressed free radical production and protected from oxidative stress in O_2^- and $ONOO^-$-induced LLC-PK_1 cells. The present study clearly demonstrated that linolenic acid is responsible for the radical scavenging effect against oxidative stress. Therefore, this research suggests the protective role of olive, corn, *Perilla* oil, and linolenic acid against free radical production and oxidative stress-related degenerative diseases.

Keywords: linolenic acid, *Perilla* oil, oxidative stress, free radical, antioxidant

1. Introduction

Oxidative stress caused by production of reactive oxygen species (ROS) is associated with a number of pathological conditions (Valko et al., 2007). Free radical has known to implicate pathogenesis of a various disease including diabetes, hypertension, inflammation and cancer (Halliwell, 1994). On the other hand, natural antioxidant substances play a key role in interfering with the oxidation process by inhibiting free radicals. Therefore, consumers prefer natural antioxidant for the stabilization of fats and oils against oxidative rancidity.

Many different plant materials have used for source of oil with antioxidant activity. Oils also contain different substances with antioxidant activity (Velioglu et al., 1998). Antioxidants in oils are important in the stabilization of fatty acids (Baldioli et al., 1996). The antioxidant effect of phenol compounds or other compositions in oils has been well studied. Olive oil is abundance of oleic acid, a monounsaturated fatty acid (C18:1n-9), which ranges from 56~84% of total fatty acids (Waterman & Lockwood, 2007). Owen et al. (2004) reported that oleic acid has shown effect for cancer prevention. In addition, olive oil has phenolic constituents such as protocatechuic acid or tyrosol with antioxidant activity (Papadopoulos & Boskou, 1991). Corn oil is composed of 59% polyunsaturated fatty acids and linoleic acid (C18:2n-9). In previous study, it is highly effective food oil for lowering serum cholesterol (Dupont et al., 1990). *Perilla* oil consists of a high amount of linolenic acid (C18:3n-3) which has recognized as an essential fatty acid in nutrition (Burdge, 2006). It has been reported a variety of medicinal properties including antitumor, antimicrobial, antiinflammatory activities (Watanabe et al.,

1994; Inouye, Takizawa, & Yamaguchi, 2001). In addition, *Perilla* oil rich in linolenic acid was effective in reducing postprandial lipid level and related to the increase of eicosapentaenoic acid and docosahexaenoic acid contents in hepatic membrane fractions (Kim & Choi, 2001). However, the study on comparison of antioxidative property among vegetable oils and purified fatty acids from oils has not been carried out yet.

This study was to investigate the inhibitory effect against free radical and protective activity from oxidative stress of different vegetable oils as potential sources of antioxidants and linolenic acid purified from *Perilla* oil.

2. Materials and Methods

2.1 Reagents and Instruments

Olive, corn, and *Perilla* oils were purchased in local supermarkets in Korea. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) and 2-deoxyribose used to investigate radical-scavenging activity were obtained from Sigma Chemical Co. (St Louis, MO, USA) and hydrogen peroxide (H_2O_2) was purchased from Junsei Chemical Co. (Tokyo, Japan). Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), and penicillin/streptomycin were obtained from Welgene (Daegu, Korea). 3-(4,5-dimethylthiazol-2-yl)-2,3-diphenyl tetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were obtained from Sigma Chemical Co. (St Louis, MO, USA).

2.2 Isolation of Linolenic Acid

Linolenic acid was isolated by the method of Jeong et al. (1997) with slight modification. We could purify the linolenic acid from *Perilla* oil and the yield of linolenic acid was 79.89%.

2.3 DPPH Radical Scavenging Activity

In a microplate, 100 µl of sample was added to an ethanolic solution of DPPH (60 µM) according to the method described by Hatano et al. (1989). After being mixed gently and incubated for 30 min at room temperature, the DPPH radical was measured at 540 nm using a microplate reader.

$$\text{DPPH radical scavenging effect (\%)} = [(A_0 - A_1)/A_0] \times 100 \quad (1)$$

Where, A_0 was absorbance of the control and A_1 was absorbance of the sample/standard.

2.4 Hydroxyl Radical (·OH) Scavenging Activity

Scavenging of ·OH radicals was measured according to the method given by Chung et al. (1997). The reaction mixture contained 200 µl of 10 mM $FeSO_4 \cdot 7H_2O_2$-EDTA, 200 µl of 10 mM 2-deoxyribose, and 1400 µl of sample solutions. After incubation at 37 °C for 4 h, the reaction was stopped by adding 1 ml of 2.8% trichloroacetic acid and 1.0% thiobarbituric acid solution. The solution was boiled for 20 min and then cooled in a water bath. ·OH scavenging activity was measured at 490 nm.

$$\text{·OH radical scavenging effect (\%)} = [(A_0 - A_1)/A_0] \times 100 \quad (2)$$

Where, A_0 was absorbance of the control and A_1 was absorbance of the sample/standard.

2.5 Cell Culture

$LLC\text{-}PK_1$ porcine renal epithelial cells were provided by ATCC (Manassas, VA, USA). The cells were maintained in T-75 culture flask containing 5% FBS-supplemented DMEM medium at 37 °C in a 5% CO_2 incubator. Cells were sub-cultured weekly with 0.05% trypsin-EDTA in phosphate buffered saline.

2.6 Cell Viability

Cell viability was measured by MTT assay. $LLC\text{-}PK_1$ cells were seeded at a density of 5×10^4 cells per well in 96-well plate and incubated for 2 h. Next, 100 µl of 0.25 mM of pyrogallol and 1.0 mM of 3-morpholinosydnonimine (SIN-1) were treated. After 24 h, the 100 µl of oils and linolenic acid was added to each well and incubation was continued for 24 h. we dissolved the oils into DMSO and diluted with medium to treat $LLC\text{-}PK_1$ cells. And then, MTT solution (1 mg/mL) was added to each 96-well plate and 5% CO_2 incubated for 4 h at 37 °C. Then, the medium containing MTT was removed. The formazan crystals incorporated in the viable cells were solubilized with DMSO and the absorbance of each well was read at 540 nm using microplate reader (Mosmann, 1983).

2.7 Statistical Analysis

Significance was verified by performing Duncan's multiple range tests using SAS software (version 6.0, SAS Institute, Cary, NC, USA).

3. Results

We evaluated the antioxidant activity of olive, corn, and *Perilla* oils under *in vitro* and cellular system. The

DPPH radical scavenging activity was shown in Table 1. Oilve, corn, and *Perilla* oils increased scavenging activity dose-dependently. Among oils, *Perilla* oil has highest antiradical capacity, showing 12.53% scavenging effect against DPPH at the concentration of 500 μg/ml. Also, the result of ·OH radical scavenging activity was shown in Table 2. These three oils exerted strong scavenging activity against ·OH. At 25 μg/ml, all oils including *Perilla* oil scavenged ·OH radical over 80%.

Table 1. DPPH radical scavenging activity of vegetable oils

Treatment (μg/ml)	Scavenging activity (%)		
	Olive oil	Corn oil	*Perilla* oil
25	0.70 ± 2.25^{de}	-3.13 ± 2.41^{f}	0.02 ± 3.70^{ef}
125	1.30 ± 2.78^{cde}	-2.97 ± 2.63^{f}	2.63 ± 2.10^{cde}
250	3.90 ± 2.52^{cd}	0.36 ± 2.87^{de}	8.96 ± 2.38^{b}
500	4.41 ± 2.65^{c}	2.97 ± 2.09^{cde}	12.53 ± 3.75^{a}
L-Ascorbic acid [1] (IC_{50})		2.77 ± 0.25	

Note. Values are mean ± SD; [1] L-Ascorbic acid was used as a positive control; [a~f] Means with different letters are significantly different ($P < 0.05$) by Duncan's multiple range test.

Table 2. Hydroxyl radical scavenging activity of vegetable oils

Treatment (μg/ml)	Scavenging activity (%)		
	Olive oil	Corn oil	*Perilla* oil
25	89.58 ± 0.20^{d}	88.28 ± 0.37^{e}	82.88 ± 0.52^{f}
125	92.66 ± 0.05^{b}	91.79 ± 0.21^{c}	76.72 ± 0.36^{g}
250	93.60 ± 0.14^{a}	92.85 ± 0.44^{b}	67.40 ± 0.14^{h}
500	93.52 ± 0.07^{a}	93.54 ± 0.28^{a}	42.16 ± 1.19^{i}
L-Ascorbic acid [1] (IC_{50})		0.04 ± 0.00	

Note. Values are mean ± SD; [1] L-Ascorbic acid was used as a positive control; [a~i] Means with different letters are significantly different ($P < 0.05$) by Duncan's multiple range test.

As shown in Table 3, pyrogallol declined cell viability to 66.84% compared to 100% non-treated cells. When three kinds of oils were treated in pyrogallol-induced LLC-PK_1 cells, the cell viability were increased by 69.25%, 68.21% and 74.34% at the concentration 10 μg/ml of olive, corn, and *Perilla* oils, respectively. In addition, SIN-1 led to the significant decrease in cell viability to 55.63%. Otherwise, the treatment of oils increased cell viability compared to SIN-1-treated control group. In particular, at the concentration of 10 μg/ml, *Perilla* oil elevated cell viability to 66.69% (Table 4).

Table 3. The protective effect of vegetable oils on viability of LLC-PK_1 cells treated with pyrogallol

Treatment (μg/ml)	Cell viability (%)		
	Olive oil	Corn oil	*Perilla* oil
10	69.25 ± 3.34^{bc}	68.21 ± 3.98^{bc}	74.34 ± 2.87^{b}
50	73.69 ± 5.01^{b}	68.52 ± 7.17^{bc}	69.94 ± 0.57^{bc}
100	74.00 ± 2.62^{b}	70.12 ± 6.04^{bc}	68.19 ± 4.97^{bc}
Control		100.00 ± 1.91^{a}	
Pyrogallol-treated Control		66.84 ± 0.60^{c}	
L-Ascorbic acid [1]		65.44 ± 2.24	

Note. Values are mean ± SD; [1] L-Ascorbic acid (10 μg/ml) was used as positive control; [a~c] Means with different letters are significantly different ($P < 0.05$) by Duncan's multiple range test.

Table 4. The protective effect of vegetable oils on viability of LLC-PK_1 cells treated with SIN-1

Treatment (μg/ml)	Cell viability (%)		
	Olive oil	Corn oil	*Perilla* oil
10	56.06 ± 2.54^{d}	65.85 ± 6.85^{bc}	66.69 ± 5.93^{b}
50	59.69 ± 3.81^{cd}	58.76 ± 2.48^{d}	65.93 ± 1.73^{bc}
100	67.80 ± 4.53^{b}	69.94 ± 4.83^{b}	55.93 ± 4.08^{d}
Control		100.00 ± 5.10^{a}	
SIN-1-treated Control		55.63 ± 2.65^{d}	
L-Ascorbic acid [1)]		64.11 ± 2.79	

Note. Values are mean ± SD; [1)] L-Ascorbic acid (10 μg/ml) was used as a positive control; [a~d] Means with different letters are significantly different ($P < 0.05$) by Duncan's multiple range test.

From these data, the three kinds of vegetable oils have free radical scavenging activity and protective effect from superoxide anion (O_2^-), and peroxynitrite ($ONOO^-$)-induced cellular damage. In addition, *Perilla* oil exerts the strong antioxidant effect. Based on these results, we further studied radical scavenging effect of linolenic acid, which highly contained in *Perilla* oil.

The result of DPPH radial scavenging activity of linolenic acid was shown in Table 5. Linolenic acid increased DPPH scavenging ability as concentration-dependent manner. At concentration of 500 μg/ml linolenic acid showed the strongest DPPH radical scavenging activity of 21.87%. In addition, ·OH radical scavenging capacity increased as dose-dependent manner (Table 6). The highest inhibition of ·OH radical was found in the concentration of 25 μg/ml, showing 80.7%. The ·OH radical scavenging activity of linolenic acid from *Perilla* oil was higher than DPPH radical scavenging activity.

Table 5. DPPH radical scavenging activity of linolenic acid from *Perilla* oil

Concentration (μg/ml)	Scavenging activity (%)
125	9.57 ± 2.74^{c}
250	15.72 ± 2.79^{b}
500	21.87 ± 3.74^{a}
L-Ascorbic acid [1)] (IC_{50})	0.06 ± 0.05

Note. Values are mean ± SD; [1)] L-Ascorbic acid was used as a positive control; [a~c] Means with different letters are significantly different ($P < 0.05$) by Duncan's multiple range test.

Table 6. Hydroxyl radical scavenging activity of linolenic acid from *Perilla* oil

Concentration (μg/ml)	Scavenging activity (%)
5	51.45 ± 0.19^{c}
10	73.73 ± 0.09^{b}
25	80.70 ± 0.03^{a}
L-Ascorbic acid [1)] (IC_{50})	0.49 ± 0.00

Note. Values are mean ± SD; [1)] L-Ascorbic acid was used as a positive control; [a~c] Means with different letters are significantly different ($P < 0.05$) by Duncan's multiple range test.

To evaluate the protective activity of the linolenic acid from *Perilla* oil against oxidative stress, pyrogallol and SIN-1 were used. As shown in Table 7, O_2^- generated by pyrogallol decreased cell viability to 51.55% compared to 100% in control group. However, treatment of linolenic acid at concentration 5 μg/ml, the viability was increased to 58.49%. SIN-1 generated both NO and O_2^-, which formed $ONOO^-$, and declined cell viability (79.89%). Meanwhile, cell viability was increased in dose-dependently manner treated with linolenic acid. In particular, at the concentration of 100 μg/ml, cell viability increased to 81.02% (Table 8). From these results, the

linolenic acid from *Perilla* oil has protective effect against O_2^- and $ONOO^-$-induced oxidative stress in LLC-PK_1 cells.

Table 7. The protective effect of linolenic acid on viability of LLC-PK_1 cells treated with pyrogallol

Treatment (μg/ml)	Cell viability (%)
5	58.49 ± 5.02^{b}
25	53.17 ± 4.23^{bc}
50	54.63 ± 4.09^{bc}
100	48.42 ± 11.48^{c}
Control	100.00 ± 0.60^{a}
Pyrogallol-treated Control	51.55 ± 1.76^{bc}
L-Ascorbic acid [1)]	50.47 ± 2.24

Note. Values are mean ± SD; [1)] L-Ascorbic acid (10 μg/ml) was used as a positive control; [a~c] Means with different letters are significantly different ($P < 0.05$) by Duncan's multiple range test.

Table 8. The protective effect of linolenic acid on viability of LLC-PK_1 cells treated with SIN-1

Treatment (μg/ml)	Cell viability (%)
5	63.53 ± 9.66^{bc}
25	69.12 ± 4.75^{c}
50	70.24 ± 6.90^{c}
100	81.02 ± 9.18^{b}
Control	100.00 ± 1.72^{a}
SIN-1-treated Control	79.89 ± 3.35^{b}
L-Ascorbic acid [1)]	92.06 ± 2.79

Note. Values are mean ± SD; [1)] L-Ascorbic acid (10 μg/ml) was used as a positive control; [a~c] Means with different letters are significantly different ($P < 0.05$) by Duncan's multiple range test.

4. Discussion

Overproduction of free radical and oxidative stress are involved in the progression of degenerative diseases (Ames, Shigenaga, & Hagen, 1993). Therefore, to attenuate oxidative stress induced by free radical, natural antioxidants including vegetable oils with ω-3 or ω-6 fatty acids have been much concern (Sherwin, 1976). Fatty acid composition is related to the antioxidant activity. Corn oil is rich in linoleic acid (53.4 g/100 g fatty acids) and olive oil is rich in oleic acid (80.6 g/100 g fatty acids). Meanwhile, corn oil and olive oil typically contains linolenic acid at 1.0 g and 0.6 g/100 g fatty acids, respectively. *Perilla* oil contains high level of linolenic acid, 60.9 g/100 g fatty acids (Hunter, 1990; Okuno et al., 1997). The purpose of this work is to compare the antioxidant ability of high- and low linolenic acid in three kinds of vegetable oils and investigate the relationship between the antioxidant effects of three vegetable oils and main fatty acid composition

DPPH radical scavenging assay is the most common assay to determine the antioxidant activity. *Perilla* oil scavenged DPPH significantly greater than the other oils. ·OH radical is the most reactive species that can damage and implicate DNA, proteins and lipids. Therefore, removal of ·OH is important to defense from various disorders (Lin et al., 1995). It was obvious that *Perilla* oil had the greatest antioxidant activity against DPPH and showed protective capacity from ·OH radical. These results indicated that fatty acids in the vegetable oils would play the protective role from free radical-induced damage.

We further investigated antioxidant activity of the vegetable oils in a cellular system using LLC-PK_1 renal epithelial cells. LLC-PK_1 cells are used for measuring antioxidant ability because of its susceptible to oxidative stress (Schena et al., 2001). Therefore, experimental model of oxidative damage on LLC-PK_1 cells exposed to free radicals would be useful for determining effective protection ability from free radicals. To evaluate the protective effect of the vegetable oils against free radical, pyrogallol and SIN-1 were used. NO and O_2^- are demonstrated to rapidly react with $ONOO^-$, which is powerful oxidant exhibiting a wide range of tissue damage

(Hanaue et al., 2007). SIN-1, which produces $ONOO^-$ via generation of NO and O_2^-, also led to lose of viability. On the other hand, vegetable oils exerted a protective effect against oxidative damage by SIN-1. In addition, vegetable oils rescued LLC-PK_1 cells from pyrogallol-induced cell death. Among the three kinds of vegetable oils, we confirmed that *Perilla* oil showed the strongest protective effect against oxidative stress at the low concentration.

Taken together with these results, *Perilla* oil has the strongest radical scavenging activity and showed the highest cell viability at the low concentration in oxidative stress-induced LLC-PK_1 cells. Therefore, we examined antioxidant effect of linolenic acid. The unique composition of fatty acids of *Perilla* oil differentiates it from other vegetable oils. *Perilla* oil has amount of linolenic acid (> 60%) compared to other vegetable oils. Considering that linolenic acid can be a precursor of long chain fatty acids such as EPA and DHA, it has been reported that dietary fats rich in linolenic acid have the health beneficial roles. Our results revealed that linolnic acid increased DPPH radical scavenging activity in a dose-dependently. Furthermore, linolenic acid showed noticeable protective effect against ·OH radical in a dose-dependent manner. In cellular system, the linolenic acid from *Perilla* oil attenuated the oxidative stress induced by O_2^- and $ONOO^-$ through elevation of cell viability. Thus, *Perilla* oil and linolenic acid as major fatty acid from *Perilla* oil suppressed free radical production and markedly protected from oxidative stress. The present study clearly demonstrated that linolenic acid is responsible for the radical scavenging effect against oxidative stress.

From the results of present investigations, *Perilla* oil and linolenic acid, its active constituent, exhibited antioxidant activities through *in vitro* and cellular system. Several studies demonstrated that linolenic acid is effective anti-inflammatory through NF-κB signal transduction and ant-diabetes by regulating insulin secretion in spite of low concentration (Erdinest et al., 2012; Itoh et al., 2003). Linolenic acid exerts antioxidative effect even at low concentration. Although the further study has to be supported, *Perilla* oil also shows strong antioxidative activity even at low concentration because of high contents of linolenic acid in *Perilla* oil. Therefore, *Perilla* oil seems to be more attributable to its cell viability at low concentration rather than high concentration. Further study has to be supported on the biotechnical method to increase linolenic acid from *Perilla* oil together with the stabilization from oxidative rancidity.

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