

# Comparison of Antioxidant Properties of Evening Primrose Seeds by Different Processing Methods, and Physiological Properties of Evening Primrose Seed Powder

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**ABSTRACT:** This study proposes the processing method that could maximize the functional properties of evening primrose seeds (EPS) and its various nutritional components. EPS can be prepared by different methods, such as being left raw, roasting, steaming, and powdering. Processing of EPS to create EPS powder (EPSP) resulted in higher levels of vitamin E, fatty acids, total phenolic contents, and antioxidant activity, compared with the other processing methods. Also, EPSP maintained lipid peroxidation inhibitory activity for 49 days. In particular, the EPSP ethyl acetate (EtOAc) fraction exhibited high antioxidant, antidiabetic, and angiotensin I-converting enzyme inhibitory activities. The EPSP ethyl acetate fraction showed a high cytoprotective effect against H<sub>2</sub>O<sub>2</sub>-induced cell damage in both RAW264.7 and EA.hy926 cells. In addition, the EtOAc fraction showed anti-inflammatory activity by the inhibitory activity of nitric oxide (NO) in RAW264.7 cells, and antihypertensive activity by the activity of NO in EA.hy926 cells. These results suggest that EPSP could be useful as a food ingredient that assists the prevention of various diseases caused by oxidative stress.

**Keywords:** antihypertension, anti-inflammation, cytoprotective effect, evening primrose seed powder, processing methods

## INTRODUCTION

The increased incidence of chronic diseases, such as stroke, arteriosclerosis, hypertension, cancer, and diabetes, has increased the general interest in plant-derived and natural foods that can reduce the effects of these diseases. A potential cause of these diseases could be generation of reactive oxygen species (ROS). In healthy physiological conditions, harmful ROS are removed by a variety of antioxidant defense mechanisms. However, when ROS generation exceeds the intracellular antioxidative capacity, the resulting oxidative stress causes cell damage and, ultimately, cell death (Farooq et al., 2019). Biosynthetic antioxidant substances that remove ROS include antioxidant enzymes, such as superoxide dismutase (SOD) and peroxidase, and low-molecular weight antioxidant compounds, such as tocopherol, ascorbate, carotenoids, and flavonoids (Mittler et al., 2004; Zhu et al., 2019). These antioxidant substances are widely present in plants and animals, and numerous studies are being conducted to explore their properties.

Evening primrose (*Oenothera erythrosepala* Borbas) is a plant from the Onagraceae family. Since evening primrose seed (EPS) oil contains  $\gamma$ -linolenic acid (GLA), a prostaglandin hormone precursor, the plant has been used in various antioxidation-related studies. GLA in EPS has potential to improve blood cholesterol levels and circulation, the discomfort caused by premenstrual physiological changes, and immune hypersensitivity-related skin conditions (Kawamura et al., 2011). However, the EPS fat content is 5.3~5.8%, which is very low in comparison to that of sesame seeds (50.5~51.6%), therefore EPS yields far less oil. In this study, we explored the conditions of juice extraction and processing methods that could improve the low EPS yield while maximizing antioxidant activity and the vitamin E and fatty acid contents. Furthermore, we aimed to develop a food ingredient that can help prevent various oxidative stress-related diseases, by measuring the various antioxidant, antidiabetic, and anti-inflammation-related bioactivities of EPS powder (EPSP) obtained by the optimized processing method.

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## MATERIALS AND METHODS

### Chemicals

Linoleic acid, gallic acid, quercetin, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS),  $\alpha$ -amylase,  $\alpha$ -glucosidase, 4-nitrophenyl- $\alpha$ -D-glucopyranoside (*p*-NPG), *N*-hippuryl-histidyl-leucine (HHL), 1,2,3,4,6-penta-*O*-galloyl- $\beta$ -D-glucose (PGG), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and lipopolysaccharides (LPS) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Grape seeds oligomeric proanthocyanidins were purchased from United States Pharmacopeia (Rockville, MD, USA), and Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Corning (New York, NY, USA).

### Materials and sample preparation

Evening primrose (*Oenothera erythrosepala* Borbas) seeds were washed, and the sample extracted using a twin-gear household juicer (Angelia 8000ss, Angel Co., Ltd., Busan, Korea). Samples were prepared by fruit housing, multi-housing, standard housing, and grinding housing. To select the optimal processing method, EPS was pretreated as follows: raw, mixing, roasting, steaming, and powdering. Raw EPS refers to seed without any processing; mixed EPS refers to seed processed physically using a mixer; roasted EPS refers to seed roasted on a pan at 150~160 °C; steamed EPS refers to seed steamed at 121°C for 15 min; and EPSP refers to seed after extraction with a household juicer equipped with grinding housing.

### Vitamin E and fatty acid analyses

Vitamin E analysis was performed using a high performance liquid chromatography (HPLC; HPLC 1100 Series, Agilent Technologies, Inc., Santa Clara, CA, USA), following the method described by the Korean Food Standards Codex (MFDS, 2019). Fatty acid analysis was performed using gas chromatography (Agilent 7890 GC, Agilent Technologies, Inc.), following the method described by the Korean Food Standards Codex (MFDS, 2019).

### Lipid peroxidation assays

To analyze the lipid peroxidation inhibitory activity, samples were mixed with 2.5% linoleic acid, 0.2 M potassium phosphate buffer (pH 7.0), and distilled water to produce the reaction solution. The degree of oxidation was then measured using the ferric thiocyanate method (Ohkawa et al., 1979).

### Solvent fractionation

To measure physiological activity of the EPSP, we per-

formed solvent fractionation using 100 g EPSP methanol extract dissolved in 500 mL distilled water. The resulting solution was placed into a 2 L separating funnel, and *n*-hexane was added to collect the separated organic solvent layer for extraction. The process was repeated three times, and the collected hexane fraction was concentrated using the rotary evaporator (rotavapor R-100, BÜCHI Labortechnik AG, Flawil, Switzerland). The same extraction process was performed for the dichloromethane (CH<sub>2</sub>Cl<sub>2</sub>), ethyl acetate (EtOAc), and butanol (BuOH) fractions.

### Antioxidant content

The total phenolic content (TPC), total flavonoid content (TFC), and total proanthocyanidin content (TPCC), all of which show a close association with antioxidant activity, were measured using the methods described by Folin and Denis (1912), Davis (1947), and Sun et al. (1998), respectively. The quantities of each were estimated from the calibration curves using gallic acid, quercetin, and grape seed extracts, respectively, as the references.

### Antioxidant activities

DPPH and ABTS radical scavenging activities were measured following the method described by Blois (1958) and Re et al. (1999), respectively. The reducing power was measured by ferric reducing antioxidant power assays (Benzie and Strain, 1996). A calibration curve was obtained using FeSO<sub>4</sub>·7H<sub>2</sub>O as the reference substance, and the results were expressed in  $\mu$ M FeSO<sub>4</sub> equivalent/g.

### SOD-like activities

To measure the SOD-like activity, we used commercial SOD assay kits (Dojindo Molecular Technologies, Inc., Kumamoto, Japan).

### $\alpha$ -Amylase and $\alpha$ -glucosidase inhibitory activities

To measure  $\alpha$ -amylase and  $\alpha$ -glucosidase inhibitory activities, we followed the method described by Hwang et al. (2014). To measure  $\alpha$ -amylase inhibitory activity, samples were mixed with 1.0 U/mL  $\alpha$ -amylase solution, 200 mM potassium phosphate buffer (pH 6.9) and 0.5% soluble starch, and left to react at 37°C for 10 min. Next, the 3,5-dinitrosalicylic acid solution was added to the mixture, incubated at 100°C for 10 min, and then the absorbance was measured at 540 nm. To measure  $\alpha$ -glucosidase inhibitory activity, samples were mixed with 0.3 U/mL  $\alpha$ -glucosidase, 67 mM potassium phosphate buffer (pH 6.8) and 10 mM *p*-NPG, and left to react at 37°C for 10 min. The reaction was terminated by adding 1 M Na<sub>2</sub>CO<sub>3</sub> and the absorbance measured at 405 nm using a spectrophotometer (Multiskan GO, Thermo Scientific, Vantaa, Finland).

### Angiotensin I-converting enzyme (ACE) inhibitory activities

ACE inhibitory activity was measured by using the method described by Cushman and Cheung (1971) with modifications. ACE solution, 0.1 M sodium borate buffer (pH 8.3) and HHL were added to the sample, and left to react at 37°C for 1 h. Next, 1 N HCl was added to terminate the reaction, followed by EtOAc was added. This mixture was then mixed by vortex for 15 s, and centrifuged (12,000 g at 4°C for 5 min). Then, 1 mL of the supernatant was collected, dried, and then supplemented with 1 mL distilled water. After mixing, the absorbance was measured at 228 nm.

### PGG contents

PGG content were analyzed according to the method described by Li et al. (2011), using HPLC (Waters e2695, Waters Corporation, Milford, MA, USA) with a Zorbax ODS column (250×4.6 mm, 5 μm, Agilent Technologies, Inc.). The mobile phase consisted of 0.3% acetic acid (solvent A) and 95% acetonitrile containing 0.3% acetic acid (solvent B). The linear gradient conditions were: 0~20 min, 0~50% B; 20~25 min, 50% B. The flow rate was maintained at 1.0 mL/min, and the PGG content of 10 μL sample was detected at 220 nm using a UV detector (Waters 2489, Waters Corporation).

### Cell culture

RAW264.7 cells (Korean Cell Line Bank, Seoul, Korea) were cultured in DMEM containing 5% FBS, 2 mM glutamine, and penicillin-streptomycin (100 μg/mL) at 37°C with 5% CO<sub>2</sub> (MCO-15AC, Sanyo Electric Co., Ltd., Gunma, Japan). EA.hy926 cells (American Type Culture Collection, Manassa, VA, USA) were incubated cultured in DMEM containing 10% FBS, 2 mM glutamine, and penicillin-streptomycin (100 μg/mL) at 37°C with 5% CO<sub>2</sub>.

### MTT assays

The cytotoxicity of EPSP solvent fractions were measured by MTT assay according to the method described by Hansen et al. (1989). Cell viability was expressed as % compared with the blank control.

### Cytoprotective effects

RAW264.7 or EA.hy926 cells were cultured for 24 h, and then the culture media was replaced with serum-free media. Cells were treated with varying concentrations of the EPSP solvent fraction. After culture for 1 h, cells were treated with 10 mM H<sub>2</sub>O<sub>2</sub>, and then incubated for a further 24 h. Culture media was then removed, and cell viability was measured MTT assays.

### Nitric oxide (NO) assays

The amounts of NO produced by RAW264.7 and EA.hy

926 cells were measured by formation of NO<sub>2</sub><sup>-</sup>, by following the method described by Yoon et al. (2009). NO production in cells was induced by LPS, and NO inhibitory activity was measured. The cell culture supernatant was mixed with the same volume of Griess reagent, and the absorbances were measured at 540 nm.

### Statistical analysis

All analyses were performed at least three times, and the results were expressed as mean±standard deviation (SD). The significance of differences between mean values was analyzed through Duncan's multiple range test of one-way ANOVA and Student's *t*-tests, using the SPSS (version 20.0 for Windows, SPSS Inc., Chicago, IL, USA). Significance tests were performed with *P*<0.05 confidence intervals.

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## RESULTS AND DISCUSSION

### Quality property analysis of EPS based on the juice extraction conditions and processing methods

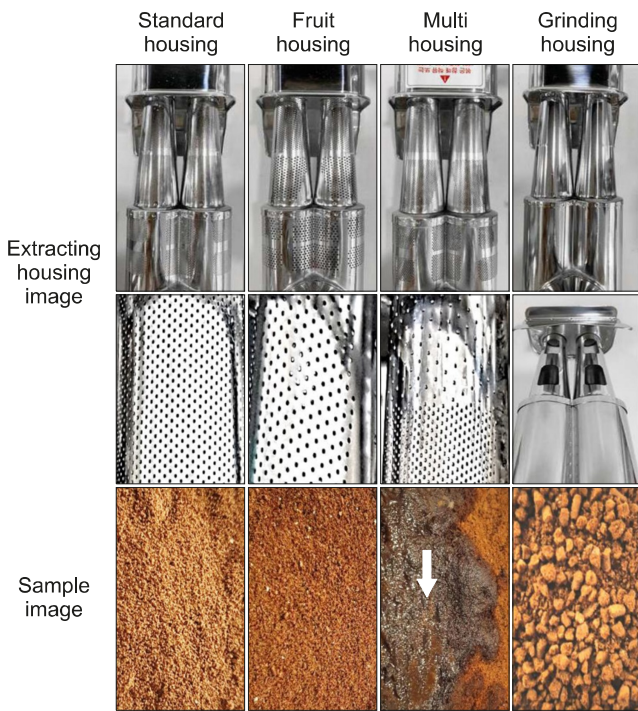
Proximate composition analysis demonstrated EPS contained high amounts of carbohydrates (59.91~65.18%), followed by the protein (13.03~17.91%), moisture (10.34~11.91%), fat (5.26~5.83%), and ash (4.62~6.01%) (data not shown). EPS is a food ingredient with verified functionality, attributed to beneficial fatty acids such as GLA. However, the low fat content may makes it highly challenging to collect a large amount of these beneficial fatty acids. Thus, to find a way to maximize the physiological effects of balanced nutrient intakes, we examined changes to EPS bioactivity according to juice extraction conditions and processing methods. During juice extraction using a household juicer, the low-speed rotation of the cone-shaped screw produces pressure, and the multi-step compression disperses the pressure in three steps. The process is performed at 82 times/min low-speed rotation, and does not generate heat, minimizing heat-induced nutrient destruction while assuring an outstanding yield. To examine changes in EPS quality by the juice extraction conditions, the EPS was extracted using the household juicer with fruit, multi, standard, and grinding housing (Fig. 1). Standard housing is the most commonly used housing, and is mainly used for vegetable and seed extractions. Fruit housing has larger holes than the standard housing, and can therefore be used for extraction of foods with high moisture content such as fruits. Multi-housing is suitable for extraction of multiple types of food, including commonly used vegetable, fruit, and seeds. Grinding housing has an open bottom, making it suitable for grinding such foods as garlic and pepper.

EPS extraction using standard housing and multi-housing showed a rapid temperature rise to 75~80°C within

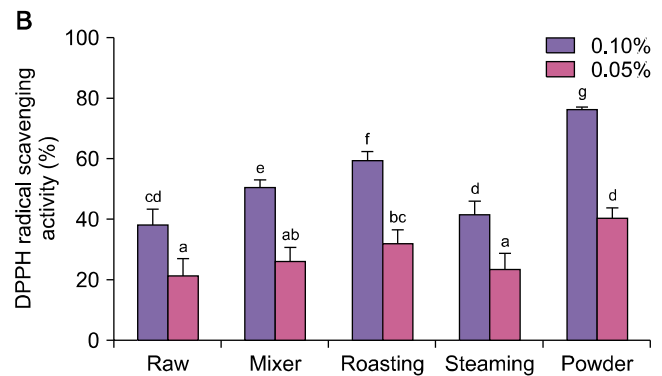
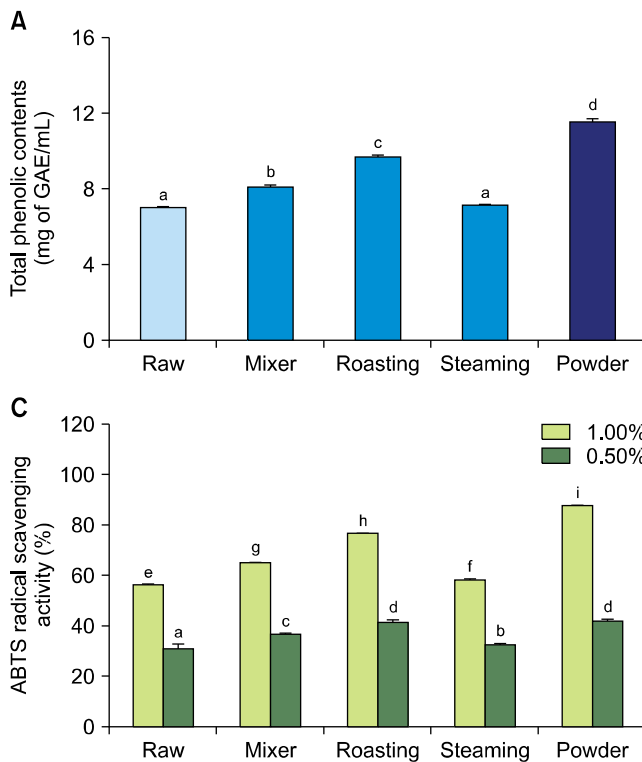
2~4 min, generated excess smoke, and the housing burst. EPS extraction using the fruit housing produced a powder-form sample, with a maximum temperature of

≤49°C and no sudden increase in temperature. EPS extraction using the multi-housing produced an oily sample via the housing micro-holes, and generated a sample in a mixed form of oil and powder. EPS extraction using the grinding housing produced a powdery sample without any pomace or temperature increase. Two sample types were obtained using the different housings: powder form, and a mixed form of oil and powder (Fig. 1). The mixed form exhibited a 20~35% yield and a high temperature increase during extraction, whereas the powdered form exhibited a high yield of 60~80% and a minimal temperature increase during the extraction. These results indicated that problem of low EPS yield and increased temperature-induced nutrient destruction can be avoided. Therefore, the powder-form sample collected using the grinding housing was selected as the extraction condition for the final processing method.

The TPC of EPS extracts was increased compared with raw EPS following all tested processing methods except steaming (Fig. 2A). The EPSP showed the highest increase in TPC (1.65-fold higher than in raw EPS). Similarly, radical scavenging activity (DPPH and ABTS) of EPS extracts were increased compared with raw EPS, with highest levels observed for EPSP. Indeed, for EPSP, DPPH radical scavenging activity was 75.91% [2.00-fold increase compared with raw EPS (Fig. 2B)], and ABTS radical scavenging activity was 87.59% [1.56-fold increase compared with raw EPS (Fig. 2C)]. Based on these results, a twin-gear household juicer could ideally be used with grinding housing for the extraction as a novel processing method that maximizes intake of beneficial nutrients



**Fig. 1.** Images of the equipment and samples used in this study. All extract housing except grinding housing had a round hole of a different size on the surface. The grinding housing has a large square hole at the bottom. The fruit housing, standard housing and grinding housing produced powdered samples. The multi-housing produced a powder-oil sample, with the arrow indicating oil in the powder-oil mixed sample.



**Fig. 2.** Phenolic contents and antioxidant activity of evening primrose seeds by processing method. (A) Total phenolic contents, (B) 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, and (C) 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radical scavenging activity. Values are mean±SD (n=3). Different letters (a-i) indicate significant differences among samples ( $P<0.05$ ), as calculated by Duncan's multiple range test.

without cooking.

The mixed EPS and EPSP samples were extracted in powdered forms without heat treatment and using a mixer and a household juicer, respectively. Compared with mixing EPS in a mixer, a better antioxidant content and activity could be obtained in EPSP. In later experiments, the four sample types (raw, roasting, steaming, and powdered EPS) were used to compare vitamin E, fatty acid and antioxidant contents, and antioxidant activity.

#### Vitamin E and fatty acid contents of EPS samples

Vitamin E analysis showed highest contents of  $\gamma$ -tocopherol in the EPS samples, with significantly higher level in EPSP compared with the other samples. Furthermore, the fatty acid content was significantly higher level in EPSP than other EPS samples (Table 1). In EPSP, there were high contents of the unsaturated fatty acids: linoleic acid (73.079%), GLA (9.308%), and oleic acid (7.589%). Long-chain polyunsaturated fatty acids (LC-PUFAs), such as linoleic acid and linolenic acid, are known to improve

blood lipid metabolism and exhibit anti-inflammatory effects, which exhibit preventive effects on conditions such as hypertension and diabetes (Abedi and Sahari, 2014). While vitamin E compounds found in cellular lipids block free radical chain reactions, they are nontoxic and serve as powerful inhibitors, reportedly preventing oxidative damage on lipids by reducing peroxy radicals (Le Prell et al., 2007). EPSP was shown to contain an abundance of antioxidant vitamin E compounds compared with EPS extracted by other processing methods. Based on these results, EPSP could be predicted to exhibit high antioxidant activity.

#### Antioxidant content and activity of EPS samples

To estimate the antioxidant contents of EPS methanol concentrates, we measured the TPC, TFC, and TPCC contents (Table 1). The results showed that TPC was highest in the EPSP (1.11-fold higher than in raw EPS). The TFC and TPCC were also highest in EPSP compared with EPS extracted by other methods, with 1.57-fold higher TFC and 1.25-fold higher TPCC contents than in

**Table 1.** Vitamin E contents, fatty acid compositions, and antioxidant activities of evening primrose seeds by processing method

	Evening primrose seeds			
	Raw	Roasting	Steaming	Powder
Vitamin E (mg/100 g)				
$\alpha$ -tocopherol	35.26±0.02 <sup>a</sup>	34.60±0.32 <sup>a</sup>	35.25±1.07 <sup>a</sup>	36.55±0.61 <sup>b</sup>
$\beta$ -tocopherol	0.76±0.08 <sup>a</sup>	0.76±0.04 <sup>a</sup>	0.81±0.02 <sup>a</sup>	1.00±0.07 <sup>b</sup>
$\gamma$ -tocopherol	52.97±3.33	53.57±1.38	54.35±0.35	56.32±2.11
$\delta$ -tocopherol	1.21±0.04 <sup>a</sup>	1.24±0.03 <sup>a</sup>	1.27±0.03 <sup>a</sup>	1.34±0.03 <sup>b</sup>
Fatty acid (g/100 g)				
Palmitic acid (C <sub>16:0</sub> )	0.697±0.005	1.051±0.020	1.038±0.003	1.171±0.017
Palmitoleic acid (C <sub>16:1</sub> )	0.005±0.000	0.008±0.000	0.008±0.000	0.009±0.000
Stearic acid (C <sub>18:0</sub> )	0.194±0.004	0.297±0.004	0.294±0.001	0.330±0.001
Oleic acid (C <sub>18:1</sub> , Cis)	0.791±0.005	1.182±0.019	1.150±0.010	1.308±0.008
Linoleic acid (C <sub>18:2</sub> , Cis)	7.500±0.081	11.388±0.122	11.233±0.068	12.599±0.078
Linolenic acid (C <sub>18:3n-3</sub> )	0.016±0.000	0.025±0.001	0.024±0.000	0.029±0.001
$\gamma$ -Linolenic acid (C <sub>18:3n-6</sub> )	0.938±0.014	1.448±0.011	1.435±0.006	1.605±0.010
Arachidic acid (C <sub>20:0</sub> )	0.041±0.001	0.061±0.001	0.062±0.001	0.069±0.001
Gadoleic acid (C <sub>20:1</sub> )	0.021±0.001	0.033±0.001	0.033±0.001	0.037±0.001
Heneicosanoic acid (C <sub>20:2</sub> )	0.009±0.001	0.015±0.001	0.014±0.000	0.016±0.001
Eicosadienoic acid (C <sub>22:0</sub> )	0.026±0.000	0.036±0.001	0.039±0.001	0.042±0.002
Lignoceric acid (C <sub>24:0</sub> )	0.015±0.000	0.021±0.000	0.023±0.000	0.025±0.001
Saturated fatty acid	0.972±0.009 <sup>a</sup>	1.465±0.026 <sup>b</sup>	1.456±0.002 <sup>b</sup>	1.638±0.005 <sup>c</sup>
Unsaturated fatty acid	9.281±0.094 <sup>a</sup>	14.098±0.154 <sup>c</sup>	13.887±0.083 <sup>b</sup>	15.603±0.097 <sup>d</sup>
Total fatty acid	10.253±0.102 <sup>a</sup>	15.563±0.179 <sup>b</sup>	15.342±0.084 <sup>b</sup>	17.240±0.102 <sup>c</sup>
Anti-oxidant activity				
TPC (mg GAE/g)	206.41±4.40 <sup>a</sup>	221.52±2.27 <sup>b</sup>	205.36±2.85 <sup>a</sup>	229.64±5.99 <sup>c</sup>
TFC (mg QE/g)	88.56±13.96 <sup>a</sup>	121.83±2.20 <sup>b</sup>	85.61±2.27 <sup>a</sup>	139.46±7.52 <sup>c</sup>
TPCC (mg/g)	77.80±2.52 <sup>a</sup>	92.72±5.44 <sup>b</sup>	80.28±4.25 <sup>a</sup>	97.21±3.08 <sup>b</sup>
DPPH radical scavenging (IC <sub>50</sub> value)	36.38±2.83 <sup>b</sup>	34.13±1.45 <sup>b</sup>	35.23±1.73 <sup>b</sup>	28.05±2.19 <sup>a</sup>
ABTS radical scavenging (IC <sub>50</sub> value)	117.04±8.32 <sup>c</sup>	69.49±3.37 <sup>ab</sup>	75.37±3.02 <sup>b</sup>	64.83±4.10 <sup>a</sup>

Mean±SD (n=3).

Means with different letters (a-d) within a row are significantly different ( $P<0.05$ ), as calculated by Duncan's multiple range test. TPC, total phenolic content; GAE, gallic acid equivalents; TFC, total flavonoid content; QE, quercetin equivalents; TPCC, total proanthocyanidin content; DPPH, 2,2-diphenyl-1-picrylhydrazyl; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt.

raw EPS. In addition, DPPH and ABTS radical scavenging activities were highest in EPSP, followed by EPS extracted by roasting and steaming, and raw EPS. These results indicate that the radical scavenging activities were significantly higher in EPSP than samples derived from the other processing methods.

Phenolic compounds consist of an aromatic benzene ring with one or more hydroxyl substituents, and are under investigation as outstanding antioxidant materials. The natural phenolic compounds may react with ROS, which are fundamental causes of various diseases and exhibit antioxidant, anti-inflammatory, anti-allergic and anticancer activities (Lewandowska et al., 2014). Higher levels of antioxidant content and activity were demonstrated in EPSP compared with EPS extracted by other processing methods. This data imply that EPSP may be beneficial for helping to prevent oxidative damage caused by ROS accumulation, a leading cause of aging and diseases.

#### Lipid peroxidation inhibitory activity of EPSP during storage period

While EPSP was stored at room temperature, changes in lipid peroxidation inhibitory activity in the control led to a continuously increasing absorbance due to rancidity, whereas the positive control ( $\alpha$ -tocopherol) only showed increasing absorbance due to rancidity after 14 days (Fig. 3). In contrast, the EPSP retained lipid peroxidation inhibitory activity without any increases in absorbance for 49 days. This data indicate that EPSP has higher lipid peroxidation inhibitory activity compared with  $\alpha$ -tocopherol, and is therefore able to maintain antioxidant activity for longer periods of time.

#### Antioxidant and antidiabetic activities of EPSP solvent fractionated samples

Analysis of EPSP solvent fractionation showed that the EtOAc fraction exhibited significantly higher levels of all three antioxidant contents than the other fractions (Table 2). The ABTS and DPPH radical scavenging activities were also highest in the EtOAc fraction of EPSP (Table 2). Furthermore, highest reducing activity was exhibited for the EtOAc fraction, followed by the BuOH and water fractions, and lowest reducing activity for the  $\text{CH}_2\text{Cl}_2$  and hexane fractions (Table 2). In addition, very low SOD-like activity was exhibited for the hexane fraction, whereas the highest activity was exhibited for the EtOAc fraction, and activities  $\geq 97\%$  were exhibited for the BuOH and water fractions (Table 2).

In agreement with results of the current study, in a previous study, highest levels of polyphenols were observed in EtOAc and BuOH fractions (Park et al., 2020). Indeed, in our study, the EPSP EtOAc fraction contained an abundance of antioxidant compounds and exhibited

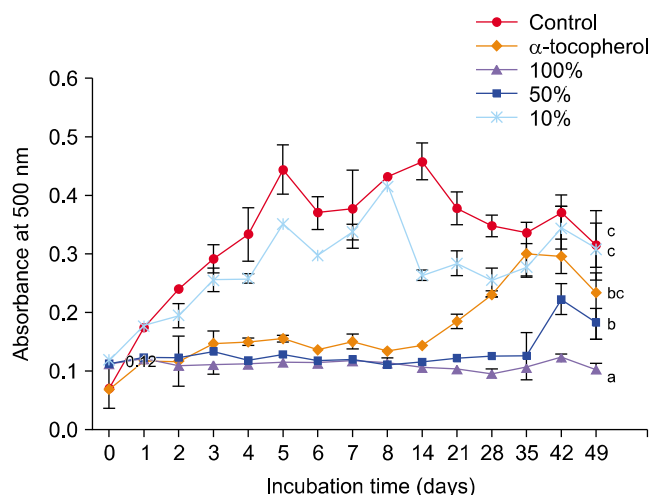


Fig. 3. Lipid peroxidation inhibitory activity of evening primrose seed powder. Values are mean  $\pm$  SD (n=3). Different letters (a-c) indicate significant differences among samples ( $P < 0.05$ ), as calculated by Duncan's multiple range test.

high antioxidant activity across all measured parameters, presumably attributed to the high content of antioxidant compounds.

#### Antidiabetic activities of EPSP solvent fractionated samples

$\alpha$ -Glucosidase inhibitory activity showed a concentration-dependent inhibitory activity in all EPSP fractions (Table 2). For the EtOAc, BuOH, and water fractions,  $\alpha$ -glucosidase inhibitory activity was  $\geq 90\%$  for all concentrations, indicating high inhibitory activity. In contrast, the  $\text{CH}_2\text{Cl}_2$  fraction showed a markedly decreased  $\alpha$ -glucosidase inhibitory activity of 48.08% at 10% concentration. Furthermore,  $\alpha$ -amylase inhibitory activity was highest in the EtOAc fraction (64.57% activity at 100% concentration), following by the BuOH (55.44%) and water (55.43%) fractions; there were no significant difference in the inhibitory activities of BuOH and water.

Diabetes is a chronic disease, triggered by a combination of hereditary and the environmental sources that cause abnormally high blood sugar levels. As diabetes leads to complications such as cardiovascular diseases, renal failure, and neurological disorders, blood glucose level control is critical for patients with diabetes. One method is to suppress carbohydrate hydrolases such as  $\alpha$ -glucosidase and  $\alpha$ -amylase, while carbohydrate hydrolase inhibitors act to delay breakdown into glucose, thus effectively preventing carbohydrate-related diseases such as type II diabetes (Cardullo et al., 2020; Jini and Sharmila, 2020). PGG is an indicator of EPS extract functionality that might be helpful for suppressing postprandial increases in blood glucose levels. Using HPLC, we showed that only the EtOAc fraction contained PGG (237.82  $\mu\text{g}/\text{mg}$ ), and this content was 10.75-fold higher than the content of PGG in MeOH extracts prior to fractionation (22.12  $\mu\text{g}/\text{mg}$ ; data not shown) (Table 2). With

**Table 2.** Antioxidant, anti-hypertensive, and anti-diabetic activities of solvent fractions of evening primrose seed powder

	Hexane	CH <sub>2</sub> Cl <sub>2</sub>	EtOAc	BuOH	Water
TPC (mg GAE/g)	43.33±0.15 <sup>a</sup>	56.22±0.11 <sup>a</sup>	868.88±7.58 <sup>d</sup>	596.32±3.11 <sup>b</sup>	612.02±4.80 <sup>c</sup>
TFC (mg QE/g)	ND	ND	429.89±7.13 <sup>c</sup>	171.96±16.22 <sup>a</sup>	221.63±15.56 <sup>b</sup>
TPCC (mg/g)	ND	ND	59.57±0.40 <sup>c</sup>	31.82±0.35 <sup>a</sup>	34.13±0.95 <sup>b</sup>
ABTS radical scavenging (IC <sub>50</sub> value)	32,048.43±1,982.22 <sup>b</sup>	1,363.14±154.12 <sup>a</sup>	46.31±1.09 <sup>a</sup>	124.98±1.85 <sup>a</sup>	86.73±0.60 <sup>a</sup>
DPPH radical scavenging (IC <sub>50</sub> value)	9,280.05±133.53 <sup>c</sup>	594.63±20.16 <sup>b</sup>	13.02±0.25 <sup>a</sup>	29.97±1.38 <sup>a</sup>	38.07±16.78 <sup>a</sup>
Reducing power (μM FeSO <sub>4</sub> /g)	5.97±0.41 <sup>a</sup>	24.16±1.40 <sup>b</sup>	96.96±1.74 <sup>e</sup>	87.31±0.31 <sup>d</sup>	84.14±2.77 <sup>c</sup>
SOD-like activity (%)	29.15±3.88 <sup>a</sup>	74.53±0.48 <sup>b</sup>	100.88±2.09 <sup>d</sup>	97.79±0.68 <sup>c</sup>	97.67±0.90 <sup>c</sup>
α-Glucosidase inhibitory activity (%)					
10%	84.56±3.45 <sup>b</sup>	48.08±4.08 <sup>a</sup>	99.12±1.29 <sup>c</sup>	99.23±0.34 <sup>c</sup>	98.99±0.09 <sup>c</sup>
50%	96.06±3.11 <sup>ab</sup>	92.60±2.01 <sup>a</sup>	93.75±2.86 <sup>a</sup>	98.84±0.80 <sup>b</sup>	100.01±1.59 <sup>b</sup>
100%	98.82±1.73 <sup>a</sup>	97.39±1.31 <sup>a</sup>	97.36±4.94 <sup>a</sup>	97.92±0.57 <sup>a</sup>	99.17±1.36 <sup>a</sup>
α-Amylase inhibitory activity (%)					
10%	ND	ND	19.72±1.01 <sup>c</sup>	13.26±0.90 <sup>b</sup>	7.25±1.00 <sup>a</sup>
50%	ND	ND	53.34±1.90 <sup>b</sup>	43.94±4.47 <sup>a</sup>	39.39±3.16 <sup>a</sup>
100%	ND	ND	64.57±1.58 <sup>b</sup>	55.44±4.29 <sup>a</sup>	55.43±0.94 <sup>a</sup>
PGG (μg/mg)	ND	ND	237.82±5.84	ND	ND
ACE inhibitory activity (%)	10.70±1.68 <sup>b</sup>	10.20±0.64 <sup>b</sup>	24.74±1.86 <sup>c</sup>	3.88±0.55 <sup>a</sup>	3.83±0.34 <sup>a</sup>

Mean±SD (n=3).

Means with the different letters (a-e) within a row are significantly different ( $P<0.05$ ), as calculated by Duncan's multiple range test.

ND, not detected.

TPC, total phenolic content; TFC, total flavonoid content; TPCC, total proanthocyanidin content; GAE, gallic acid equivalents; QE, quercetin equivalents; PGG, 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose; DPPH, 2,2-diphenyl-1-picrylhydrazyl; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; SOD, superoxide dismutase; ACE, angiotensin I-converting enzyme; CH<sub>2</sub>Cl<sub>2</sub>, dichloromethane; EtOAc, ethyl acetate; BuOH, butanol.

its high PGG content, EtOAc fractions also showed high levels of α-glucosidase inhibitory activity and ≥50% α-amylase inhibitory activity; these results are predictive of a beneficial role of the fraction for suppressing postprandial increases in blood glucose levels.

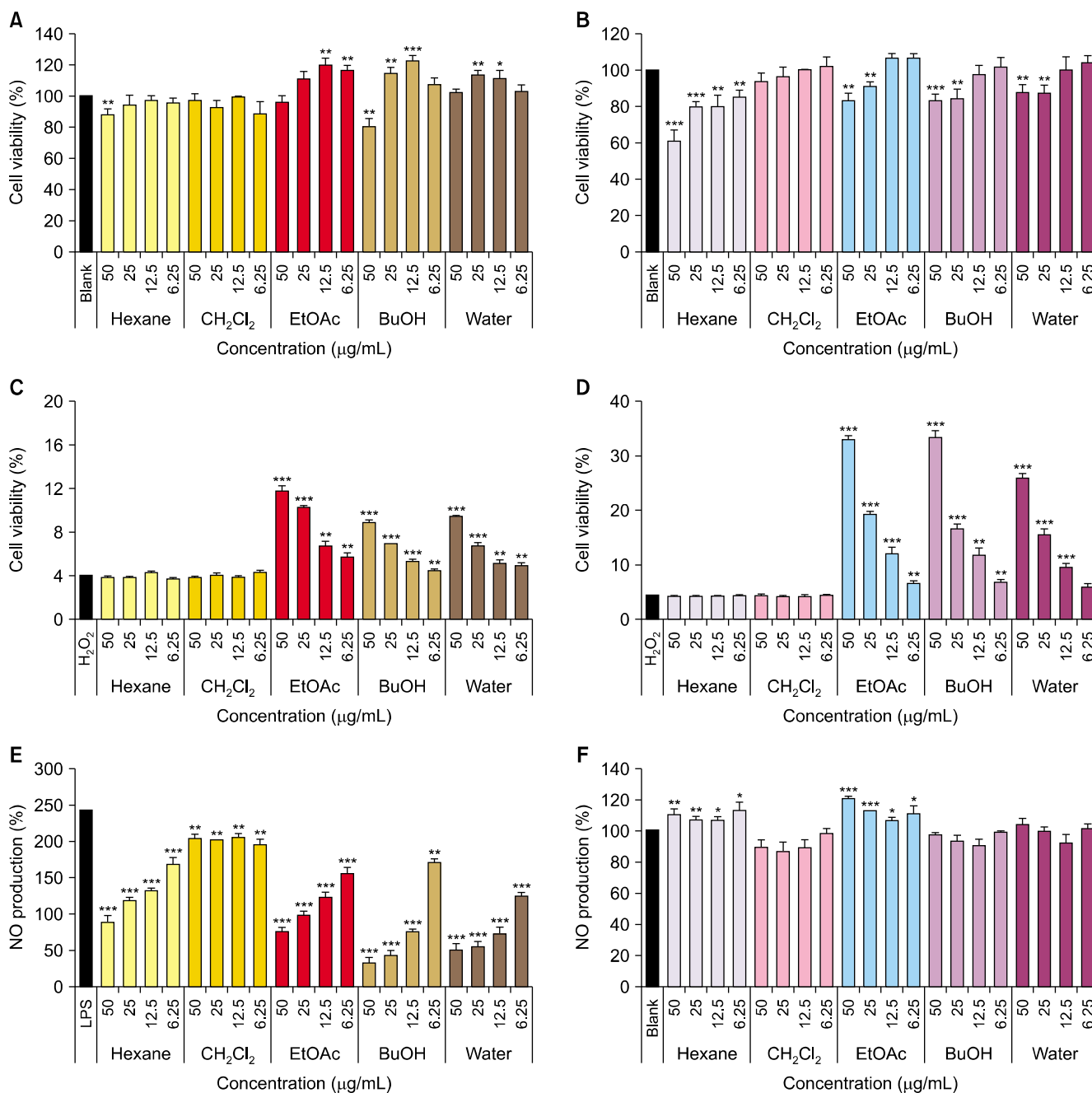
#### ACE inhibitory activity of EPSP solvent fractionated samples

ACE is an enzyme that raises blood pressure by activating angiotensin. Since persistent ACE activity might lead to continuous hypertension and cause diseases such as stroke, ACE inhibition is important for treatment of hypertension (Actis-Goretta et al., 2003). ACE inhibitory activity was significantly higher in the EtOAc fraction of EPSP, followed by the hexane, CH<sub>2</sub>Cl<sub>2</sub>, BuOH, and water fractions (Table 2). Known ACE activity inhibitors include low-molecular weight peptides and polyphenols that reportedly help improve hypertensive conditions (Actis-Goretta et al., 2003; Hügel et al., 2016). Actis-Goretta et al. (2003) reported that flavonoids-containing plant extracts have potential to function as ACE inhibitors, while flavonoids-rich foods show blood pressure-reducing effects in humans and rats by inhibition of ACE via flavan-3-ols and procyanidins. The EtOAc fraction showed highest ACE inhibitory activity compared with the other fractions, and exhibited high levels of TPC, TFC, and TPCC. These results indicate that the abundance of phenolic compounds in the EtOAc fraction is likely related to the high ACE inhibitory activity, which may help reduce the

risk of cardiovascular diseases by preventing hypertension.

#### Cytoprotective effect of the EPSP solvent fractionated samples

The cytoprotective effect of EPSP fractions in RAW264.7 cells and EA.hy926 cells was evaluated by measuring cellular cytotoxicity. In RAW264.7 cells, no EPSP fractions induced cellular cytotoxicity (cell viability ≥80%) (Fig. 4A), whereas in EA.hy926 cells, only the 50 μg/mL hexane fraction induced cytotoxicity (Fig. 4B). H<sub>2</sub>O<sub>2</sub> is a ROS that induces cellular damage through directly oxidizing biomolecules such as lipids, proteins, and DNA, ultimately leading to cell death (Wang et al., 2018). When RAW264.7 cells were treated with H<sub>2</sub>O<sub>2</sub> to induce cell damage, cell viability decreased to 4.06% (Fig. 4C), with significantly higher viabilities observed for the EtOAc, BuOH, and water fractions for all concentrations. Notably, the EtOAc fraction decreased cell viability to 11.76% at 50 μg/mL, indicating a 2.90-fold higher cytoprotective effect than for the H<sub>2</sub>O<sub>2</sub>-treated groups. For EA.hy926 cells, H<sub>2</sub>O<sub>2</sub> treatment reduced cell viability to 4.51% (Fig. 4D). However, a significantly higher cell viability was observed for the water fraction (12.5~50 μg/mL) compared with the H<sub>2</sub>O<sub>2</sub>-treated group, and cytoprotective effects were 7.30-fold and 7.37-fold higher for the EtOAc and BuOH fractions, respectively. Overall, the EtOAc, BuOH, and water fractions demonstrated cytoprotective effects, presumably resulting from the high an-



**Fig. 4.** Cell cytotoxicity, cell protective activity and nitric oxide (NO) production induced by solvent fractions of evening primrose seed powder. Cell cytotoxicity in (A) RAW264.7 cells and (B) EA.hy926 cells. Cell protective activity against 10 mM H<sub>2</sub>O<sub>2</sub> in (C) RAW264.7 cells and (D) EA.hy926 cells. NO production in (E) RAW264.7 cells and (F) EA.hy926 cells. CH<sub>2</sub>Cl<sub>2</sub>, dichloromethane; EtOAc, ethyl acetate; BuOH, butanol. Values are mean±SD (n=3). Significant differences among samples, as calculated by Student's *t*-test. \**P*<0.05, \*\**P*<0.01, and \*\*\**P*<0.001.

tioxidant content and high antioxidant activity suppressing cell damage induced by H<sub>2</sub>O<sub>2</sub>.

**Inhibitory effect of EPSP samples on NO production in LPS-induced RAW264.7 cells**

RAW264.7 cells are mainly engaged in inflammatory reactions, and generate NO by inducible NO synthase as a pro-inflammatory mediator (Jung et al., 2007). Although inflammatory reactions occur as protective mechanism to regenerate damaged tissues upon entry of foreign mate-

rial, when the reactions persist without termination, cancer cells may grow and exacerbate arteriosclerosis (Yuan et al., 2020). To estimate the anti-inflammatory activity of EPSP fractions in RAW264.7 cells, we determined NO production inhibitory activity. LPS-induced NO production was significantly reduced by administration of all EPSP fractions (Fig. 4E), the greatest effect observed from the EtOAc, BuOH, and water fractions. Oxidative stress can cause acute and chronic inflammatory diseases as it induces inflammatory reactions. In a previous study,



intake of nutrients with high antioxidant activity reduced inflammatory reactions and decreased oxidative stress (Nam et al., 2015). A high NO inhibitory effect was demonstrated for the EtOAc, BuOH, and water fractions that exhibited high antioxidant content and antioxidant activity, probably acting to inhibit NO production by reducing oxidative stress.

#### Effect of EPSP samples on NO production in EA.hy926 cells

NO production in EA.hy926 cells dilates blood vessels and plays a direct role in lowering blood pressure (Park et al., 2019). In EA.hy926 cells, treatment with hexane and EtOAc EPSP fractions significantly increased NO production compared with control cells (50 µg/mL EtOAc fraction: 1.20-fold vs control; hexane fraction: 1.10-fold vs control) (Fig. 4F). According to Schölkens et al. (1982), LC-PUFAs are the biosynthetic precursors of prostaglandin, which contributes to regulation of arterial blood pressure in blood vessel walls and the kidney. The study also reported that intake of evening primrose oil leads to reduced blood vessel reactivity following stimulation with renin and angiotensin II, with increased activity with prostacyclin, a powerful vasodilator. In addition, vitamin E compounds lowers blood pressure by increasing endothelial NO synthase activity (Newaz et al., 1999; Pitocco et al., 2013). EPSP contains ≥70% LC-PUFAs and significantly higher amounts of vitamin E than EPS extracts generated by other processing methods. Therefore, EPSP is predicted to help prevent hypertension by ACE inhibitory activity and by promoting NO production in blood vessels.

In conclusion, generating EPSP using a twin-gear household juicer with grinding housing overcomes the drawback of the low fat content in EPS, and produces a high yield. Furthermore, EPSP may prevent destruction of nutrients resulting from heat generation during food processing. In addition, extraction using grinding housing was shown to be a better processing method than mixing, roasting and steaming, with respect to enhancing antioxidant activity, and the contents of vitamin E and beneficial fatty acids. In an experiment to monitor long-term storage, we demonstrated high lipid peroxidation inhibitory activity of EPSP. Our results demonstrated that EPS extraction with grinding housing may be a reliable method to ensure a balanced intake of nutrients through increased EPS activity.

Of the EPSP fractions obtained using grinding housing, highest levels of antioxidant content, antioxidant activity and antidiabetic activity were exhibited for the EtOAc, BuOH, and water fractions. Specifically, in RAW264.7 cells, the EtOAc fraction showed higher anti-inflammatory activity than the other fractions, and a higher level of ACE inhibitory activity and increased NO production.

The LC-PUFAs and vitamins E compounds found in abundance in EPSP are likely to promote NO production in blood vessel to help prevent hypertension, and the high contents of antioxidant compounds and antioxidant activity are predicted to have beneficial roles in reducing the incidence of various chronic diseases by removing the ROS that induce oxidative stress.

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#### AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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#### REFERENCES

- Abedi E, Sahari MA. Long-chain polyunsaturated fatty acid sources and evaluation of their nutritional and functional properties. *Food Sci Nutr*. 2014. 2:443-463.
- Actis-Goretti L, Ottaviani JI, Keen CL, Fraga CG. Inhibition of angiotensin converting enzyme (ACE) activity by flavan-3-ols and procyanidins. *FEBS Lett*. 2003. 555:597-600.
- Benzie IFF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Anal Biochem*. 1996. 239:70-76.
- Blois MS. Antioxidant determinations by the use of a stable free radical. *Nature*. 1958. 181:1199-1200.
- Cardullo N, Muccilli V, Pulvirenti L, Cornu A, Pouységu L, Deffieux D, et al. C-glucosidic ellagitannins and galloylated glucoses as potential functional food ingredients with anti-diabetic properties: a study of  $\alpha$ -glucosidase and  $\alpha$ -amylase inhibition. *Food Chem*. 2020. 313:126099. <https://doi.org/10.1016/j.foodchem.2019.126099>
- Cushman DW, Cheung HS. Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. *Biochem Pharmacol*. 1971. 20:1637-1648.
- Davis WB. Determination of flavanones in citrus fruits. *Anal Chem*. 1947. 19:476-478.
- Farooq MA, Niazi AK, Akhtar J, Saifullah, Farooq M, Souri Z, et al. Acquiring control: the evolution of ROS-induced oxidative stress and redox signaling pathways in plant stress responses. *Plant Physiol Biochem*. 2019. 141:353-369.
- Folin O, Denis W. On phosphotungstic-phosphomolybdic compounds as color reagents. *J Biol Chem*. 1912. 12:239-243.
- Hansen MB, Nielsen SE, Berg K. Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. *J Immunol Methods*. 1989. 119:203-210.
- Hügel HM, Jackson N, May B, Zhang AL, Xue CC. Polyphenol protection and treatment of hypertension. *Phytomedicine*. 2016. 23:220-231.
- Hwang CR, Tak HM, Kang MJ, Suh HJ, Kwon OO, Shi JH. Antioxidant and antiobesity activity of natural color resources. *J Life Sci*. 2014. 24:633-641.
- Jini D, Sharmila S. Green synthesis of silver nanoparticles from *Allium cepa* and its *in vitro* antidiabetic activity. *Mater Today*

- Proc. 2020. 22:432-438.
- Jung KH, Ha E, Kim MJ, Won HJ, Zheng LT, Kim HK, et al. Suppressive effects of nitric oxide (NO) production and inducible nitric oxide synthase (iNOS) expression by *Citrus reticulata* extract in RAW 264.7 macrophage cells. *Food Chem Toxicol.* 2007. 45:1545-1550.
- Kawamura A, Ooyama K, Kojima K, Kachi H, Abe T, Amano K, et al. Dietary supplementation of gamma-linolenic acid improves skin parameters in subjects with dry skin and mild atopic dermatitis. *J Oleo Sci.* 2011. 60:597-607.
- Le Prell CG, Hughes LF, Miller JM. Free radical scavengers vitamins A, C, and E plus magnesium reduce noise trauma. *Free Radic Biol Med.* 2007. 42:1454-1463.
- Lewandowska U, Owczarek K, Szewczyk K, Podśędek A, Koziółkiewicz M, Hrabec E. Influence of polyphenol extract from evening primrose (*Oenothera paradoxa*) seeds on human prostate and breast cancer cell lines. *Postepy Hig Med Dosw.* 2014. 68:110-118.
- Li L, Shaik AA, Zhang J, Nhkata K, Wang L, Zhang Y, et al. Preparation of penta-*O*-galloyl- $\beta$ -D-glucose from tannic acid and plasma pharmacokinetic analyses by liquid-liquid extraction and reverse-phase HPLC. *J Pharm Biomed Anal.* 2011. 54:545-550.
- MFDS. 2019. Korean Food Standards Codex. [cited 2019 Mar 8]. Ministry of Food and Drug Safety, Cheongju, Korea. Available from: [https://www.foodsafetykorea.go.kr/foodcode/01\\_01.jsp](https://www.foodsafetykorea.go.kr/foodcode/01_01.jsp)
- Mittler R, Vanderauwera S, Gollery M, Van Breusegem F. Reactive oxygen gene network of plants. *Trends Plant Sci.* 2004. 9: 490-498.
- Nam JH, Seo JT, Kim YH, Kim KD, Yoo DL, Lee JN, et al. Inhibitory effects of extracts from *Arabis glabra* on lipopolysaccharide induced nitric oxide and prostaglandin E<sub>2</sub> production in RAW 264.7 macrophages. *Korean J Plant Res.* 2015. 28:568-573.
- Newaz MA, Nawal NN, Rohaizan CH, Muslim N, Gapor A.  $\alpha$ -Tocopherol increased nitric oxide synthase activity in blood vessels of spontaneously hypertensive rats. *Am J Hypertens.* 1999. 12:839-844.
- Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem.* 1979. 95:351-358.
- Park HJ, Kim JY, Kim HS, Lee SH, Jang JS, Lee MH. Synergistic effect of fruit-seed mixed juice on inhibition of angiotensin I-converting enzyme and activation of NO production in EA.hy926 cells. *Food Sci Biotechnol.* 2019. 28:881-893.
- Park HJ, Lee SM, Kim HS, Kim JY, Lee SH, Jang JS, et al. Hepatoprotective effect of meal replacement seeds juice based on sweet potato (MRSJ) against CCl<sub>4</sub>-induced cytotoxicity in HepG2 cells. *Food Sci Biotechnol.* 2020. 29:693-704.
- Pitocco D, Tesaro M, Alessandro R, Ghirlanda G, Cardillo C. Oxidative stress in diabetes: implications for vascular and other complications. *Int J Mol Sci.* 2013. 14:21525-21550.
- Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radic Biol Med.* 1999. 26:1231-1237.
- Schölkens BA, Gehring D, Schlotte V, Weithmann U. Evening primrose oil, a dietary prostaglandin precursor, diminishes vascular reactivity to renin and angiotensin II in rats. *Prostag Leukotr Med.* 1982. 8:273-285.
- Sun B, Ricardo-da-Silva JM, Spranger I. Critical factors of vanillin assay for catechins and proanthocyanidins. *J Agric Food Chem.* 1998. 46:4267-4274.
- Wang W, Zhang F, Li Q, Chen H, Zhang W, Yu P, et al. Structure characterization of one polysaccharide from *Lepidium meyenii* Walp., and its antioxidant activity and protective effect against H<sub>2</sub>O<sub>2</sub>-induced injury RAW264.7 cells. *Int J Biol Macromol.* 2018. 118:816-833.
- Yoon WJ, Ham YM, Yoo BS, Moon JY, Koh J, Hyun CG. *Oenothera laciniata* inhibits lipopolysaccharide induced production of nitric oxide, prostaglandin E<sub>2</sub>, and proinflammatory cytokines in RAW264.7 macrophages. *J Biosci Bioeng.* 2009. 107:429-438.
- Yuan HL, Zhao YL, Ding CF, Zhu PF, Jin Q, Liu YP, et al. Anti-inflammatory and antinociceptive effects of *Curcuma kwangsiensis* and its bioactive terpenoids *in vivo* and *in vitro*. *J Ethnopharmacol.* 2020. 259:112935. <https://doi.org/10.1016/j.jep.2020.112935>
- Zhu MT, Huang YS, Wang YL, Shi T, Zhang LL, Chen Y, et al. Comparison of (poly)phenolic compounds and antioxidant properties of pomace extracts from kiwi and grape juice. *Food Chem.* 2019. 271:425-432.