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Antioxidative and Radical Scavenging Properties of Extracts from *Geum japonicum*

Kang-Jin Cho*, Jong-Bum Kim, Jung-Bong Kim, Dong-Gil Kim[1], Haeng-Ran Kim[2] and Young-Soo Hwang

Division of Biochemistry, National Institute of Agricultural Science and Technology, Suwon 441-707, Korea
[1]Hamyang Medicinal Crop Exp. Station, Gyeongnam Rural Development Office, Hamyang, Gyeongsangnam-do 676-820, Korea
[2]Division of Home Management, National Rural Living Science Institute, Rural Development of Administration, Suwon 441-100, Korea

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Extracts from *Geum japonicum* (Bammu in Korean) were analyzed for their antioxidative activities and scavenging effects on free radicals. The ethyl acetate fraction of *G. japonicum* methanol extract (EFGJ) showed a remarkable scavenging activity on the 1,1-diphenyl-2-picrylhydrazyl radical. EFGJ also showed excellent antioxidative activity on linoleic acid during long-period storage and on rat liver microsome peroxidation system, and good anti-peroxidation effect on lipid in Rancimat system using lard, palm oil, and perilla oil, as compared with BHT and α-tocopherol. Varying effects of antioxidative activity of the EFGJ on various fatty acids were observed. The prevention of linoleic acid and linolenic acid peroxidation was superior to α-tocopherol, but inferior to BHT. The methanol extract of *G. japonicum* did not show mutagenicity as revealed through SOS chromotest. Based on the results, the extracts of *G. japonicum* may be useful source as natural antioxidants.

Key words: Geum japonicum, *antioxidant, free radical, rancimat analysis.*

Antioxidant is a substance that siginificantly delays or prevents chain breaking of lipid-peroxidation or oxidation of biomolecules by a reactive oxygen.[1,2] The reactive oxygen species (ROS) are generated as a by-product of normal metabolism and attack biological molecules, leading to cell or tissue injury.[3] The ROS often encountered in biological systems include the radicals (hydroxyl, $OH^{\cdot}$; superoxide, $O_2^{\cdot}$; peroxyl, $RO_2^{\cdot}$), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$),[4] among others. All these activated oxygen species are extremely reactive and cytotoxic in all organisms. Therefore, biomolecules (DNA, protein, lipids, and carbohydrates) are damaged by the ROS in the cell. Oxidative stress was suggested to play a role in heart disease, malaria, neurodegenerative disease, AIDS, cancer, and in the aging process.[5-7] In addition, unsaturated fatty acids in food are peroxidized easily by the ROS, which leads to the loss of nutritional quality and a reduction in the shelf life of various lipid-containing food.[1]

Pharmacologists and food manufacturers have long been searching for antioxidants. A small and simple molecular substance with antioxidative activity has been synthesized and employed for food processing. Of the synthetic antioxidants, butylated hydroxyanisole (BHA), 3,5-di-tert-butyl-4-hydroxytoluene (BHT), α-tocopherol, and propyl gallate (PG) are permitted to be used for food singly or in combination at a level not exceeding 200 ppm in several countries including USA and Canada.[1] However, the possible toxicity of the synthetic antioxidant makes it unacceptable to consumers who prefer natural products. Several researches have been concentrated on identifying natural antioxidants for the past two decades.[8-14]

Recently, many chinese medicinal plants have been screened in our laboratory for their antioxidative effects.[15] Powerful antioxidative activities were detected in the extracts of *Agastache rugosa* and *Geum japonicum*. In *A. rugosa*, the antioxidant was identified as rosmarinic acid, while in *G. japonicum*, the antioxidant yet remains unknown. *G. japonicum* is one of the perennial and flowering plants used as a diuretic and an astringent in Japan and China.[16] Its potent functions have also been reported, such as immunodficiency virus (HIV-1) protease inhibitors[17] and antiviral effects.[18] However, little information is available on its antioxidative activity.

This paper describes the antioxidative activity of EFGJ through several systems including scavenging of free radical, inhibition of lipid-peroxidation, and microsome membrane oxidation *in vitro*, and examination of its possible mutagenicity.

*Corresponding author
Phone: 82-31-290-0374; Fax:82-31-290-0391
E-mail: cho374@rda.go.kr

Abbreviations: EFGJ, *G. japonicum* methanol extract; BHA, butylated hydroxyanisole; BHT, 3,5-di-tert-butyl-4-hydroxytoluene; DPPH, α, α'-diphenyl-β- picrylhydrazyl.

Materials and Methods

Materials. *Geum japonicum* THUNBERG plants (Bammu in Korea) were grown at Hamyang Medicinal Plant Experimental Station (Rural Development Office, Gyeongsangnam-do), and harvested in October. The stems and leaves were dried under air condition and used for extracts. Sprague-Dawley male rat (approximately 200 g) was sacrified for preparation of microsome to test antioxidative activity *in vitro*. *E. coli* PQ37 was used for mutagenicity test.

Extraction of *Geum japonicum*. Aerial parts of *G. japonicum* THUNBERG were extracted with methanol, and the extracts were dried in rotary evaporator, dissolved in H_2O, and fractionated using hexane, methylene chloride, ethyl acetate, and butanol. The extracts and fractions were dried in a rotary evaporator and dissolved in a proper solvent for the antioxidative activity test

Free radical scavenging test. DPPH (α,α'-diphenyl-β-picrylhydrazyl) solution (100 μM) was prepared by dissolving DPPH in ethanol. Two mililiters of DPPH solution was added to 100 μl sample resolved in DMSO. Ten minutes after incubation at room temperature, the absorbance at 490 nm was determined and the SC_{50} calculated.[10]

Fatty acid autoxidation. Autoxidation of fatty acid in alcohol solution was assayed using Ferric-Thiocyanate method.[19] Linoleic acid was used for long-time storage. Palmitic, stearic, oleic, linoleic, and linolenic acids were employed for specificity of antioxidant to fatty acids. Each antioxidant was dissolved in ethanol and added to fatty acid solution mixtures to a final concentration of 0.005%. The fatty acid solution mixtures contained 2 m*l* of fatty acid solution (2.5 g of fatty acid in 100 m*l* of ethanol), 4.0 m*l* of 0.05 M phosphate buffer (pH 7.0), 1.9 m*l* of distilled water, 0.1 m*l* of Tween 20, and adjusted to a final volume of 10 ml with ethanol. The mixtures in the test tube were incubated at 40°C, and sampled from the 9th day with 6-day interval. For generated free radical assay, 0.1 m*l* of the incubated mixtures, 9.7 m*l* of 75% ethanol, and 0.1 m*l* of 30% ammonium thiocyanate were premixed, to which 0.1 m*l* of 0.02 M $FeCl_2$ in 3.5% HCl was added. After 5 min, the absorbances were determined at 490 nm using a microplate bio-kinetic reader (EL 312e, Bio-Tek instrument, Winooski, VT).

Rancimat test. Antioxidative activity was also measured using Rancimat 679 (METROHM AG, CH-9100 Herisau, Swiss). Thirty grams of fat or oil (lard, palm oil, and perilla oil) were placed in reaction tubes. The extracts and controls were added to the reaction tubes to a 0.005% final concentration. The tubes were then heated at 130°C with aeration. The indices were calculated[20] as follows:

Index = initiation time of sample/ initiation time of control

Microsomal lipid peroxidation. Microsomes were prepared according to the method of Hogeboom.[21] Sprague-Dawley male rats were sacrificed. Their livers were removed, washed three times in ice-cold 0.25 M sucrose, sliced, and homogenized with a homogenizer in 8 volume of liver weight homogenizing buffer [0.25 M sucrose, 5 mM Tris-HCl (pH 7.4), and 0.1 mM EDTA]. The homogenate was centrifuged at 10,000 rpm (Hanil supra 28k, Korea) for 10 min. The supernatant was placed in a new tube, centrifuged at 100,000 × g for 60 min, then discarded. The precipitate was washed with the homogenizing buffer three times, resuspended in a homogenizing buffer, and stored at -70°C.

The microsome was diluted with 0.1 M Na_2HPO_4/KH_2PO_4, pH 7.4 to 1.5 mg protein/m*l*. Lipid peroxidation test was carried out following the method of Ohakawa *et al.*[22] Reaction mixtures were prepared by mixing 0.5 m*l* of 100 mM Tris-HCl (pH 7.5), 0.1 m*l* of 4 mM $FeSO_4 \cdot 7H_2O$, 0.1 m*l* of 2 mM ascorbic acid, and 0.005% of sample, then incubated at 37°C for 60 min. The reaction was stopped by adding 0.25 m*l* of 3 M TCA/2 N HCl (1 : 1) mixture, then centrifuged at 3,500 × g for 10 min. The supernatant (1 m*l*) was placed in a test tube, 0.25 m*l* of 0.67% butylated hydroxyanisole (TBA) added, boiled at 100°C for 20 min, and cooled to room temperature. Absorbance was measured at 530 nm. The antioxidative activity was calculated as follows:

Antioxidative activity(%) = (AC-AS) × 100/(AC-AB)

AC: Absorbance of control (without sample)

AS: Absorbance of test mixture

AB: Absorbance of blank (without sample and $FeSO_4 \cdot 7H_2O$)

SOS chromotest.[23,24] *E. coli* PQ37 was cultured at 37°C overnight. The following day, 1/10 volume of culture solution was added to 5 m*l* LB medium, and incubated with shaking for 2 h. Subsequently, 1 m*l* of the new culture solution was placed in 3 m*l* LB medium. The dilute (0.4 m*l*) was placed into a new tube, 20 μ*l* (10 mg/m*l*) sample and 3.58 m*l* LB were added, and incubated at 37°C for 2 h. The cultured solution (0.2 m*l*) was placed into a new tube, to which 0.4 m*l* of ONPG solution (4 mg/m*l*) and 1.8 m*l* of buffer B (16.1 g Na_2HPO_4, 5.5 g $NaH_2PO_4 \cdot H_2O$, 0.75 g KCl, 0.25 g $MgSO_4 \cdot 7H_2O$, 1 g SDS, 2.7 m*l* β-mercaptoethanol/*l*) were added. After incubation at 28°C for 30 min, 1 M Na_2CO_3 (1.6 m*l*) was added to stop the enzymatic reaction. The absorbances at 420 and 550 nm were measured, and the unit calculated. Mitomycin C (0.4 μg/10 μl) was used as the positive control and DMSO as the negative control.

$$\text{Unit} = \frac{1000 \times (A_{420} - 1.75 \times A550)}{T \times V \times A_{600}}$$

T: reaction time (min)

V: reaction volume (ml)

A_{600}: concentration of *E. coli* PQ37

Results

Free radical scavenging test. DPPH is generally used to determine the antioxidative activity. Under the existence of antioxidant, the free radical was reduced to 1,1-diphenyl-2-picrylhydrazine, and the purple color disappeared gradually. At a given time, the change in color was measured at A_{490}. The

Table 1. SC_{50} of the ethyl acetate fraction of *G. japonicum* methanol extracts.

Exracts and fractions	Free radical scavenging activities (SC_{50}, μg/ml*)
Control	
BHA	6
BHT	89
α-Tocopherol	49
MeOH extracts	25
Hexane fraction	>1000
$MeCl_2$ fraction	>1000
EtOAc fraction	7
BuOH fraction	>1000

*SC_{50} (μg/ml) denotes the concentration of the sample required to scavenge 50% of 100 μM DPPH radical.

SC_{50} was calculated on the basis of A_{490} (Table 1), and the SC_{50} values of α-tocopherol, BHT, BHA, and EFGJ were 49, 89, 6, and 7, respectively. The EFGJ was at least 7 and 12.5 times more powerful than the commercial synthetic antioxidant α-tocopherol and BHT, respectively. Considering that EFGJ still contain impurities, the antioxidative activity of pure EFGJ might be much stronger than this.

Fatty acid autoxidation. To evaluate the applicability of EFGJ to long-term storage, the inhibition of lipid-peroxidation of linoleic acid was tested. Linoleic acid is one of the most important and abundant plant fatty acids. The fatty acid contains two unsaturated double bonds, which are easily oxidized. EFGJ, α-tocopherol, BHT, and BHA were added to linoleic acid and incubated at 40°C for 69 days. The peroxidation of linoleic acid containing α-tocopherol was initiated for 21 days, after which the oxidation occurred rapidly. On the other hand, the antioxidative effect of EFGJ continued until 69 days, similar to that of BHT. α-Tocopherol was not able to inhibit peroxidation of linoleic acid after 21 days, while BHT and EFGJ were able to effectively prevent the oxidation of the linoleic acid for over 2 months (Fig. 1). This shows the excellent storage effect of EFGJ compared to α-tocopherol.

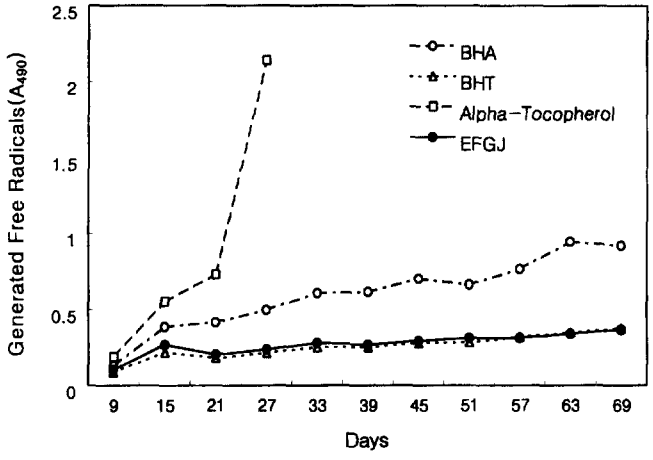

Fig. 1. Antioxidative effects of EFGJ on linolenic acid. The generated free radical was determined by the ferric-thiocyanate method. Final concentration of samples: 0.005%.

Table 2. Antioxidative indices of the ethyl acetate fraction of *G. japonicum* methanol extracts on lard, palm oils and perilla oil.

Samples	Antioxidative indices		
	Lard	Palm oil	Perilla oil
BHT	1.35	1.39	0.93
α-Tocopherol	1.35	1.63	0.8
Rosmarinic acid	2.21	2.17	0.86
EFGJ	1.66	2.29	1.03

Antioxidative index: the induction period of oil containing sample divided by the induction period of control oil, which is determined by Rancimat analysis at 130°C.

Rancimat test. Lard with saturated fatty acid, perilla oil with unsaturated fatty acid and palm oil with medium level unsaturated fatty acid were used as substrate to test the antioxidative activity. The antioxidative indices of α-tocopherol were 1.35, 1.63, and 0.8 against lard, palm oil, and perilla oil, while those of EFGJ were 1.66, 2.29, and 1.03, respectively (Table 2). The antioxidative activity of EFGJ generally was better than that of α-tocopherol. Rosmarinic acid showed the best antioxidative activity for lard. Anti-peroxidation activity of EFGJ on palm and perilla oil was better than that of rosmarinic acid.

Inhibition of microsomal lipid peroxidation. For the rat liver microsome, *in vitro* test was done, and the antioxidative activity of EFGJ against animal liver fatty acid was also tested. BHT and EFGJ showed about 90% antioxidative activity, while that of α-tocopherol was about 10%. This shows that the antioxidative activity of EFGJ was 8 times powerful compared to that of α-tocopherol (Fig. 2).

Inhibition of fatty acid peroxidation. The inhibitory effect of EFGJ was tested on the major fatty acids of plant, such as palmitic, stearic, oleic, linoleic, and linolenic acids. In general, free radicals were easily produced in multi-double fatty acids than in saturated one. As linoleic acid with two double bonds and linolenic acid with three double bonds were

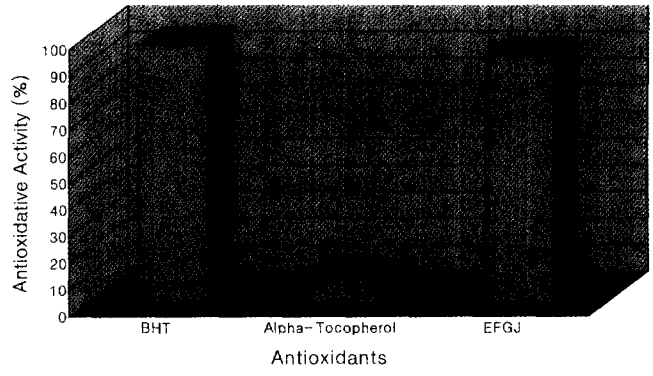

Fig. 2. Antioxidative activities of EFGJ using *in vitro* system (rat liver microsome). Final concentration of samples: 0.005%.

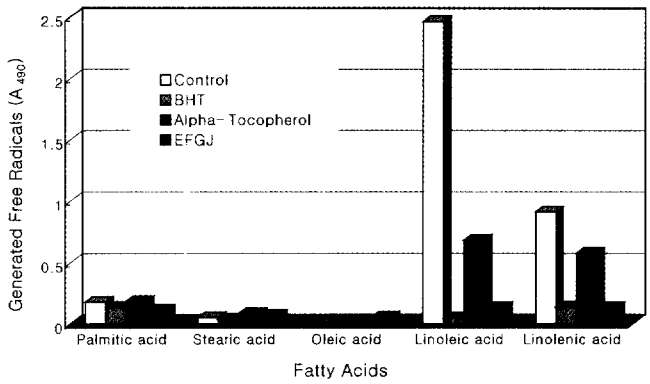

Fig. 3. Antioxidative effect of EFGJ on fatty acids. Major plant fatty acids (palmitic acid, stearic acid, oleic acid, linoleic acid and linolenic acid) were used for the test. Final concentration of sample: 0.005%.

Table 3. Mutagenicity test on methanol extracts from *G. japonicum* by SOS Chromotest.

Samples	Mutagenicity
	β-Galactosidase activities (unit)
DMSO (negative control)	33
Mitomycin C (positive control)	143
MeOH extracts of *G. japonicum*	36

with antioxidants, they were more resistant to peroxidation (Fig. 3). EFGJ and BHT strongly inhibited peroxidation of linoleic and linolenic acids.

SOS chromotest. SOS chromotest was carried out to test the mutagenicity of the EFGJ. Mitomycin C was used as the positive control. Mitomycin C forms a cross-link between DNA strands and interacts with amino group of purine, which results in the change of DNA. The quinone part of mitomycin C interacts with O_2 and produces superoxide anion and hydrogen peroxide, which induce the excision of DNA strand. Once the damage occured by the excision of DNA strand, SOS repair genes are turned on to repair the damaged DNA. Based on this mechanism, mitomycin C is generally used as a control in SOS chromotest. If a substance has a positive effect in mutagenicity, even though it reveals a good function in any other physiological tests, it cannot be used in human. The induction of β-glucosidase activity was 33, 36, and 143 units in DMSO, EFGJ, and Mitomycin C, respectively (Table 3). These data suggest that the EFGJ does not contain any substance affecting the mutagenicity.

Discussion

The unsaturated double bond in lipid is attacked easily by ROS, resulting in the production of semioxidized radicals. The radicals produces another radicals so that the peroxidation reaction occurs continuously in a chain reaction. Therefore, it is important to prevent and delay the initiation of semioxidized radical in living cell. Antioxidant, which plays a role of scavenger for the radical, thus has a significant physiological function.

The SC_{50} is the concentration $\mu g \cdot ml^{-1}$ of the sample required to scavenge 50% of free radicals. It is a criterion of the power of antioxidant, lower the value more powerful the antioxidative activity. Based on SC_{50}, the antioxidative activity of EFGJ is 7 and 12.5 times more powerful than those of BHT and α-tocopherol, respectively (Table 1). Considering that the sample contained some impurities, we can speculate that EFGJ must have very powerful antioxidant. Rancimat analysis of EFGJ also showed good antioxidative activity particularly on palm oil (Table 2). The antioxidative index of EFGJ on lard is 1.3 and 1.8 times higher than those of control BHT and α-tocopherol, respectively. Even though rosmarinic acid has good antioxidative activity on lard, EFGJ is better than rosmarinic acid on palm oil. Linoleic acid was oxidized more rapidly than the other four fatty acids. The inclination of the antioxidants on linolenic acid was similar to that on the linoleic acid. The increasing order of antioxidative activity was α-tocopherol, EFGJ, and BHT (Fig. 3), indicating that EFGJ should be a very useful antioxidant source for unsaturated fatty acids.

The negative side effects must also be considered. If the substance proposed as a good antioxidant has carcinogenic properties, it cannot be used in food and medicines. Hence, mutagenicity test was done via SOS chromotest. For this purpose, *E. coli* PQ37 was employed, which was especially designed and developed by Quillerdat. This contains the gene cassette that Rec A gene promoter is linked to the 5' regulatory region of GUS gene in the chromosome of *E. coli* PQ37.[26] Therefore, if DNA is damaged, the SOS genes are turned on to repair the damaged DNA. If DNA damage occurs by EFGJ, the β-galactosidase activity will increase. The result shows that the activity was induced by Mitomycin C at a significant level, but the levels of the activities by negative control DMSO and EFGJ were almost the same (Table 3). It indicates that EFGJ does not have mutagenic property.

Based on the results, it can be concluded that EFGJ has strong antioxidative properties and does not cause DNA damage. EFGJ may be used in food and medicines. However, feasibility of the substance must be evaluated through various types of *in vitro* tests to assess unexpected negative effects. In the future, we will attempt to isolate the substance and determine its structure. In addition, its applicability for food processing and human health will be considered and tested.

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