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In-vitro antioxidant activity of flavonoids from *Acer okamotoanum*

Ji Hyun Kim[1], Hyun Young Kim[2], Sanghyun Lee[3], Eun Ju Cho[1,*]

[1]Department of Food Science and Nutrition & Kimchi Research Institute, Pusan National University, Busan 46241, Korea

[2]Department of Food Science, Gyeongnam National University of Science and Technology, Jinju 52725, Korea

[3]Department of Integrative Plant Science, Chung-Ang University, Anseong 17546, Korea

*Corresponding Author: ejcho@pusan.ac.kr

Abstract

Degenerative diseases are commonly associated with excess free radicals. *Acer okamotoanum*, a plant endemic to Korea, is reported to have anti-oxidant, anti-cancer, and anti-viral activities. We previously isolated flavonoids from the ethyl acetate fraction of *A. okamotoanum* such as quercitrin (QU), isoquercitrin (IQ), and afzelin (AF). In the present study, the *in vitro* antioxidant activity of flavonoids such as QU, IQ, and AF isolated from the ethyl acetate fraction of *A. okamotoanum* were investigated by measuring the free radical scavenging activity including 1,1-diphenyl-2-picrylhydrazyl (DPPH), hydroxyl radical ($^{\cdot}OH$), and superoxide anion (O_2^{-}). The flavonoids (QU, IQ, and AF) concentration-dependently showed a DPPH radical scavenging activity. In particular, QU and IQ showed a higher DPPH radical scavenging activity than that of AF. In addition, the flavonoids (QU, IQ, and AF) at 10 μg/mL showed over an 80% scavenging effect against $^{\cdot}OH$ radical production. Furthermore, the O_2^{-} radical scavenging activity of the flavonoids, QU, IQ, and AF increased in a dose-dependent manner. Particularly, IQ exerted the strongest scavenging activities against $^{\cdot}OH$ and O_2^{-} radicals among the other flavonoids. These results indicate that the flavonoids from *A. okamotoanum*, in particular IQ, would have a protective activity against oxidative stress induced by free radicals, and potentially be considered as a natural antioxidant agent.

Keywords: afzelin, anti-oxidant, free radical, isoquercitrin, quercitrin

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Introduction

Degenerative diseases such as cancer, diabetes, obesity, and Alzheimer's disease are associated with the over-production of free radicals in the body (Di Domenico et al., 2015). The accumulation of free radical in the cells damages biological molecules such as proteins, lipids, and deoxyribonucleic acid (Stanner et al., 2004; Halliwell, 2012). Hence, to remove free radicals, antioxidant enzymes in the body are activated including superoxide dismutase, catalase, and reduced glutathione. In addition, the consumption of dietary antioxidants can also provide added protection from free radicals (Halliwell,

2012). Dietary antioxidants are known to reduce the risk of several diseases caused by free radicals by supplying electrons to damaged cells (Halliwell, 2012). Secondary metabolites obtained from plants such as polyphenols, flavonoids, and vitamin C have been shown to confer antioxidant effects, and are regarded to confer lower side effects and toxicity, compared with other synthetic antioxidants such as butylated hydroxyanisole, butylated hydroxytoluene, and tert-butylhydroquinone (Liao and Yin, 2000; Stanner et al., 2004). Therefore, natural antioxidants from plants have been consistently studied as preventive therapy for degenerative diseases.

Acer okamotoanum is a plant endemic in Korea reported to have various biological activities including anti-cancer, anti-oxidant, and cognitive improvement effects (Jin et al., 2008; Takayama et al., 2013; Choi et al., 2017). Previous studies show that *A. okamotoanum* contains several active compounds such as flavonol glycoside gallate ester, cleomiscosins A and C (Kim et al., 1998; Jin et al., 2007). In addition, we have previously isolated flavonoids (Fig. 1) from the ethyl acetate fraction of *A. okamotoanum* such as quercitrin (QU), isoquercitrin (IQ), and afzelin (AF) (Lee et al., 2018), however, their free radical scavenging activities are yet to be determined. Therefore, in this study, we investigated the *in vitro* anti-oxidant activities of the flavonoids isolated from *A. okamotoanum*, namely, QU, IQ, and AF, by measuring their scavenging activities against the free radicals 1,1-dephenyl-2-picrylhydrazyl (DPPH), hydroxyl radical ($^{\cdot}OH$), and superoxide anion (O_2^-).

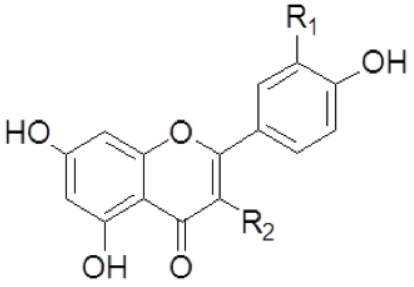
Materials and Methods

Preparation of flavonoids

QU, IQ, and AF were isolated from the ethyl acetate fraction of the aerial parts of *A. okamotoanum* by open column chromatography and were identified by spectroscopic analysis (Lee et al., 2018).

Reagents

DPPH and 2-deoxy-ribose were purchased from Sigma (St. Louis, MO, USA) and H_2O_2 was purchased from Junsei (Tokyo, Japan). $FeSO_4 \cdot 7H_2O$ was purchased from Daejung Chemicals & Metals Co. Ltd (Siheung, Korea), EDTA disodium salt dehydrate and phosphoric acid were obtained from Samchun Pure Chemical Co. Ltd (Pyeongtaek, Korea). The



Compound	R_1	R_2
QU	OH	*O*-Rham
IQ	OH	*O*-Glc
AF	H	*O*-Rham

Fig. 1. The structures of flavonoids from *Acer okamotoanum*.
QU, quercitrin; IQ, isoquercitrin; AF, afzelin; *O*-Rham, *O*-Rhamnoside; *O*-Glc, *O*-glucoside.

thiobarbituric acid (TBA) was from Acros Organics (New Jersey, USA), trichloroacetic acid (TCA) was from purchased Kanto Chemical Co. Inc (Tokyo, Japan). Phenezine methosulfate (PMS), NADH disodium salt, and nitrotetrazolium blue chloride (NBT) were from purchased Bio Basic Co. (Toronto, Canada).

DPPH radical scavenging activity

The DPPH radical scavenging activity were determined according to the method described by Hatano et al. (1989). Each sample was added to DPPH solution in the 96 well plate, and then incubated for 30 min at room temperature in the absence of light. The absorbance was measured at 540 nm using a microplate reader (Thermo Fisher Scientific, Vantaa, Finland). The DPPH radical scavenging activity was expressed as IC_{50} and a percentage (%) compared to the control as follow.

DPPH scavenging activity (%) = $(Abs_c - Abs_s)/Abs_c \times 100$

Abs_c: Absorbance of control, Abs_s: Absorbance of sample

Hydroxyl radical ($^{\cdot}OH$) scavenging activity

Hydroxyl radical ($^{\cdot}OH$) scavenging activity was determined according to the method described by Gutteridge (1987). Each sample was added to the reaction mixture containing 10 mM $FeSO_4 \cdot 7H_2O$-EDTA, 10 mM 2-deoxyribose, and 10 mM H_2O_2, and then incubated for 4 h at 37ºC without light. After, 1% TBA solution and 2.8% TCA solution were added to the mixture and heated for 20 min at 100ºC. The absorbance was measured at 490 nm using a microplate reader (Thermo Fisher Scientific, Vantaa, Finland). The $^{\cdot}OH$ radical scavenging activity was recorded as a percentage (%) compared to the control.

$^{\cdot}OH$ scavenging activity (%) = $(Abs_c - Abs_s)/Abs_c \times 100$

Abs_c: Absorbance of control, Abs_s: Absorbance of sample

Superoxide anion (O_2^-) scavenging activity

The O_2^- radical scavenging activities were measured according to the method described by Ewing and Janero (1995). Each sample was added to 0.1 M Tris-Hcl (pH 7.4), 100 μM PMS, 500 μM NBT, and 500 μM NADH, and then incubated for 10 min at room temperature without light. The absorbance was measured at 560 nm using a microplate reader (Thermo Fisher Scientific, Vantaa, Finland). The O_2^- radical scavenging activity was recorded as a percentage (%) compared to the control.

O_2^- scavenging activity (%) = $(Abs_c - Abs_s)/Abs_c \times 100$

Abs_c: Absorbance of control, Abs_s: Absorbance of sample

Statistical analysis

Data were presented as mean ± standard deviation (SD). Analysis of variance (ANOVA) followed with Duncan's multiple test was used for statistical analysis. $p < 0.05$ was considered statistically significant.

Results and Discussion

Scavenging of free radicals is vital in the prevention of the deleterious effects caused by the accumulation of free radicals that often leads to various degenerative diseases such as diabetes, cardiovascular disease and Alzheimer's disease (Di Domenico et al., 2015; Singh et al., 2015). Numerous studies have reported the antioxidant activities of different extracts and

compounds isolated from various plant sources. Particularly, flavonoids are among the major classes of plant compounds that are shown to exhibit strong antioxidative activity in biological systems by acting as scavengers of free radicals (Lapshina et al., 2015). In our previous study, the flavonoids that we isolated from ethyl acetate fraction of *A. okamotoanum*, namely, QU, IQ, and AF, had potent aldose reductase scavenging activities (Lee et al., 2018). However, *in vitro* radical scavenging activity of the three flavonoids has not yet been elucidated. Therefore, in the present study, we investigated the antioxidant effects of the flavonoids isolated from *A. okamotoanum* by measuring their free radical scavenging activities.

DPPH is a stable nitrogen-centered free radical which acts as a free radical scavenger or a hydrogen donor (Habu and Ibeh, 2015). Antioxidants react with the DPPH radical directly and restore it by transferring electrons or hydrogen. A change from the violet color of the DPPH radical in its reduced form to yellow can be used to spectrophotometrically determine and predict the antioxidant activities of various compound and plant extracts (Huang et al., 2005). As shown in Table 1, we examined the DPPH radical scavenging activity of the flavonoids from *A. okamotoanum* including QU, IQ, and AF. The DPPH radical scavenging activity of the three flavonoids evaluated increased in a dose-dependent manner. At a concentration of 25 μg/mL, the DPPH radical scavenging effects of QU and IQ were 76.51 ± 0.45%, and 75.71 ± 0.06%, respectively, suggesting their promising role as free radical scavengers. Furthermore, the IC_{50} values of QU and IQ were 3.67 ± 0.05 μg/mL and 3.79 ± 0.07 μg/mL, respectively.

·OH is the most reactive and toxic radical, and it is strongly related to several human diseases such as neurodegenerative diseases and obesity (Rahman et al., 2015). ·OH can react with biological molecules such as DNA, proteins, lipids, and membrane phospholipids, leading to the generation of free radicals, which in turn quickly reacts with oxygen to form peroxides (Halliwell and Gutteridge, 1984). Therefore, the removal of ·OH is the most effective defense against various diseases. In the ·OH assay, ·OH is formed by incubating Fe^{3+}-EDTA premixture with H_2O_2, causing 2-deoxy-ribose degradation and generating a malondialdehyde (MDA)-like product (Gutteridge, 1987). Table 2 showed the ·OH radical scavenging activity of flavonoids from *A. okamotoanum*. The ·OH radical scavenging activity of flavonoids showed over 80% scavenging activity at 10 μg/mL. Particularly, IQ showed the highest ·OH radical scavenging effects among the flavonoids. The previous research investigated that IQ showed higher *in vitro* anti-oxidant activity than QU in the Fe^{2+}-binding, electron-transfer-based ferric ion reducing antioxidant power (Li et al., 2016). The OH group of IQ showed increases the electron-transfer and ferric ion chelating abilities (Li et al., 2016).

The O_2^- radical is produced in biological systems during cellular respiration (Lushchak, 2014) and O_2^- is converted into a highly reactive radical in the presence of iron or during the incomplete metabolism of oxygen (Kirkinezos amd Moraes,

Table 1. DPPH radical scavenging activity of flavonoids from *Acer okamotoanum.*

Treatment (μg/mL)	Scavenging activity (%)		
	QU	IQ	AF
1	24.39 ± 1.00d	23.16 ± 1.14d	-
5	60.54 ± 0.43c	63.51 ± 0.97c	4.39 ± 0.35c
10	74.04 ± 0.45b	71.00 ± 0.20b	12.63 ± 0.53b
25	76.51 ± 0.45a	75.71 ± 0.06a	18.77 ± 0.35a
IC_{50}[z] (μg/mL)	3.67 ± 0.05	3.79 ± 0.07	726.74 ± 59.08

Values are means ± SD (n = 6).
DPPH, 1,1-diphenyl-2-picrylhydrazyl; QU, quercitrin; IQ, isoquercitrin; AF, afzelin.
[z]IC_{50} is the concentration in μg/mL required to inhibit the formation of DPPH radical by 50%.
a - d: Different letters are significantly different ($p < 0.05$) by Duncan's multiple range test.

Table 2. Hydroxyl radical (˙OH) scavenging activity of flavonoids from *Acer okamotoanum.*

Treatment (μg/mL)	Scavenging activity (%)		
	QU	IQ	AF
1	55.70 ± 2.51c	66.73 ± 2.73b	59.05 ± 0.93d
5	79.10 ± 0.67b	83.83 ± 1.86a	81.49 ± 0.39c
10	83.45 ± 1.26a	84.67 ± 3.50a	84.72 ± 0.28b
25	85.48 ± 1.61a	87.77 ± 0.96a	88.50 ± 1.81a
IC_{50}[z](μg/mL)	0.53 ± 0.06	0.12 ± 0.04	0.37 ± 0.02

Values are means ± SD (n = 6).
QU, quercitrin; IQ, isoquercitrin; AF, afzelin.
[z]IC_{50} is the concentration in μg/mL required to inhibit the formation of ˙OH radical by 50%.
a - d: Different letters are significantly different ($p < 0.05$) by Duncan's multiple range test.

Table 3. Superoxide anion (O_2^-) scavenging activity of flavonoids from *Acer okamotoanum.*

Treatment (μg/mL)	Scavenging activity (%)		
	QU	IQ	AF
1	0.52 ± 1.92d	3.30 ± 0.26d	4.34 ± 0.43d
5	18.94 ± 0.13c	23.44 ± 0.34c	11.50 ± 0.50c
10	38.51 ± 0.23b	46.76 ± 0.13b	25.31 ± 0.17b
25	59.56 ± 0.08a	64.46 ± 0.18a	43.69 ± 0.25a
IC_{50}[z] (μg/mL)	17.46 ± 0.01	12.89 ± 0.04	49.70 ± 0.29

Values are means ± SD (n = 6).
QU, quercitrin; IQ, isoquercitrin; AF, afzelin.
[z]IC_{50} is the concentration in μg/mL required to inhibit the formation of O_2^- radical by 50%.
a - d: Different letters are significantly different ($p < 0.05$) by Duncan's multiple range test.

2001). Highly reactive radical generated by the excess of O_2^-, such as H_2O_2, ˙OH, and peroxynitrite damage biomolecules, resulting in various diseases in the body (Stanner et al., 2004). Therefore, the removal or neutralization of O_2^- radicals is necessary to protect the cells from their deleterious effects. In our study, the O_2^- radical scavenging activity of flavonoids increased in a dose-dependent manner (Table 3). The concentration at 25 μg/mL, the O_2^- radical scavenging activities of IQ was higher than other flavonoids. These results indicated that flavonoids from *A. okamotoanum* may have protective activity against O_2^- radical. The previous study also reported that QU, IQ, and AF have strong O_2^- radical scavenging activity (Vellosa et al., 2015; Li et al., 2016).

Conclusion

The present study demonstrated that the flavonoids isolated from the ethyl acetate fraction of *A. okamotoanum,* namely, QU, IQ, and AF inhibited the free radicals DPPH, ˙OH, and O_2^- in a dose-dependent manner. IQ exhibited the highest free radical scavenging activity among the flavonoids examined. Our study showed that the flavonoids from *A. okamotoanum* possess antioxidant potential and it might be useful against diseases relating to oxidative stress generated by free radicals.

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