

Protective Effects of the Fermented *Laminaria japonica* Extract on Oxidative Damage in LLC-PK1 Cells

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ABSTRACT: This study investigated the protective effect of the butanol (BuOH) fraction from fermented *Laminaria japonica* extract (BFLJ) on AAPH-induced oxidative stress in porcine kidney epithelial cells (LLC-PK1 cells). *L. japonica* was fermented by *Aspergillus oryzae* at $35 \pm 1^\circ\text{C}$ for 72 h. Freeze-dried fermented *L. japonica* was extracted with distilled water, and the extracted solution was mixed with ethanol and then centrifuged. The supernatant was subjected to sequential fractionation with various solvents. The BuOH fraction was used in this study because it possessed the strongest antioxidant activity among the various solvent fractions. The BuOH fraction of fermented *L. japonica* had a protective effect against the AAPH-induced LLC-PK1 cells damage and increased cell viability while reducing lipid peroxidation formation and increased activities of antioxidant enzymes such as superoxide dismutase and glutathione peroxidase. The inhibitory effect of BFLJ on lipid peroxidation formation had a higher value of 0.11 ± 0.01 nmol MDA at 100 $\mu\text{g}/\text{mL}$ concentration in comparison with intact BuOH fraction showing 0.22 ± 0.08 nmol MDA at the same concentration. Furthermore, BFLJ treatment increased glutathione concentration. GSH concentration in the cell treated with BFLJ of 100 $\mu\text{g}/\text{mL}$ was $1.80 \text{ pmol}/\text{L} \times 10^5$ cells. These results indicate that BFLJ protects the LLC-PK1 cells against AAPH-induced cell damage by inhibiting lipid peroxidation formation and increasing antioxidant enzyme activities and glutathione concentration.

Keywords: *Laminaria japonica*, fermentation, antioxidant activity

INTRODUCTION

Seaweeds have demonstrated free radical scavenging activities, and thus may help slow aging and prevent some chronic diseases. Almost all seaweed species have substantial ability to scavenge hydroxyl radicals (1) and are considered to be a rich source of antioxidants (2). Sulfated polysaccharides from edible seaweeds potentially could be used as natural antioxidants by the food industry (3). In particular, brown algae display a variety of biological activities, including antioxidant (4), anti-inflammatory properties (5), anti-coagulant (6) and anti-hyperlipidemic (7).

Laminaria japonica, a kind of brown algae, has long been used as a food to promote health. Recently, as a dietary supplement, *L. japonica* has been known for several biological activities: scavenging activity against DPPH radicals (8), antimutagenic activity (9) and down-regulation of blood glucose in diabetic rats (10). *L. japonica* usually contains, in abundance, alginic acid, fucoidan and laminaran polysaccharides; these polysaccharides have been reported to exhibit a variety of biological activities (3,11).

Fermentation is a chemical reaction that splits complex organic compounds into relatively simple substances. *L. japonica*'s active compounds may be packed in its rigid structural matrix. During fermentation, the active compounds in *L. japonica* will be exposed and these may have effectiveness such as antioxidant activity.

Antioxidant activity is intensively focused due to the currently growing demand from the functional food industry. Almost all photosynthesizing plants including seaweeds are exposed to a combination of light and high oxygen concentrations, which lead to the formation of free radicals and other strong oxidizing agents, but they seldom suffer any serious photodynamic damage during metabolism. This fact implies that their cells have some protective antioxidative mechanisms and compounds (12). Recently, the potential antioxidant compounds were identified as some pigments and polyphenols. Those compounds are widely distributed in plants and seaweeds and are known to exhibit high antioxidant activity.

Previous studies from our laboratory have shown that fermented *L. japonica* extract had higher radical scavenging and antioxidant activities than intact *L. japonica* (13).

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However, only a few studies have investigated the protective effects of fermented *L. japonica* on oxidative damage in the cell. Hence, the present study was conducted with an aim to examine whether BFLJ, could ameliorate the AAPH-induced oxidative injury in LLC-PK1 cells by assessing the lipid peroxidation, glutathione concentration and antioxidant enzymes.

MATERIALS AND METHODS

Material

Laminaria japonica was purchased at a local market in Busan, Korea and used in this study.

Chemicals

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), DPPH, nitroblue tetrazolium (NBT), ethylenediaminetetra acetic acid disodium salt (Na_2EDTA), linoleic acid, ascorbic acid and other reagents of analytic grade were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Sample preparation and extraction

Freeze-dried *L. japonica* was fermented by *Aspergillus oryzae* at $35 \pm 1^\circ\text{C}$ for 72 h. Fresh and fermented *L. japonica* was extracted with distilled water. The extract solutions were mixed with ethanol and centrifuged. The supernatant is the ethanol soluble fraction, non-polysaccharide fraction and the residue is ethanol insoluble precipitation, and a polysaccharide fraction (14). Ethanol soluble fraction was subjected to sequential fractionation with dichloromethane, ethyl acetate, butanol and water. Our preliminary study showed that the butanol fraction possessed the strongest antioxidant activity among those various solvent fractions. Therefore, the butanol fraction of intact (BLJ) or fermented (BFLJ) *L. japonica* extract was dried and placed in a plastic bottle, and then stored at -80°C (15,16).

Cell culture and treatment

Commercially available porcine kidney epithelial cells (LLC-PK1 cells, passages 7~35) were maintained at 37°C in a humidified atmosphere of 5% CO_2 in culture plates with a 5% FBS-supplemented DMEM/F-12 medium. After confluence had been reached, the cells were seeded into 24-well plates (4×10^5 cells/well) or 10-mm dishes (5×10^6 cells/dish). Two hours later, 1 mM of AAPH was added to all of the wells and pre-incubated for 24 h, followed by adding *L. japonica* extracts incubated under routine conditions for 24 h. The proper concentration of AAPH and the incubation time was determined by the preliminary experiment (17).

Cell viability

The MTT assay of cell viability was performed following a well-described procedure with minor modifications (18). Cells were plated in 24-well cell culture plates at a density of 4×10^5 cells per 24-well. At the end of culture, 100 μL MTT solution (5 mg/mL in PBS) was added to each well containing 1 mL medium. After 4 h of incubation, the media were removed and formazan crystals were solubilized with 300 μL DMSO. The absorbance of each well was then read at 540 nm using a microplate reader.

Determination of lipid peroxidation

Lipid peroxidation was measured by thiobarbituric acid reactive substances (TBARS) production (19). Cells (4×10^4 cells/well) in 24-well plates were first incubated with glucose (5.5 mM and 30 mM) for 48 h, and then incubated with or without the indicated concentrations of *L. japonica* extracts (25, 50, and 100 $\mu\text{g}/\text{mL}$) for 20 h. 200 μL of each medium supernatant was mixed with 400 μL of TBARS solution then boiled at 95°C for 30 min. The absorbance at 532 nm was measured and TBARS concentrations extrapolated from the 1,1,3,3-tetraethoxypropane serial dilution standard curve and TBARS values were then expressed as equivalent nmoles of malondialdehyde (MDA).

Glutathione concentration

Glutathione (GSH) was measured by an enzymatic recycling procedure of Tietze (20) in which GSH is sequentially oxidized by 5,5-dithiobis-(2-nitrobenzoic acid) and is reduced by NADPH in the presence of glutathione reductase. The medium was removed from the cultured HUVECs and the cells were washed twice with PBS. One milliliter of PBS was added and cells were scraped. Cell suspensions were sonicated three times for 5 sec on ice then the cell sonicates were centrifuged at 2,300 g for 10 min. 400 μL of cell supernatant was mixed with 200 μL of 5% sulfosalicylic acid then centrifuged at 2,300 g for 10 min. A 50 μL aliquot of supernatant was mixed with 100 μL of the reaction mixture (100 mmol/L sodium phosphate buffer with 1 mmol/L EDTA (pH 7.5), 1 mmol/L dithionitrobenzene, 1 mmol/L NADPH, 1.6 U GSH reductase). The rate of 2-nitro-5-thiobenzoic acid formation was spectrophotometrically monitored at wavelength of 412 nm. GSH content was determined by extrapolation from the standard curve obtained from known amounts of GSH.

Antioxidant enzyme assays

Cells (5×10^6 cells/dish) in 10-mm dishes were pre-incubated for 2 h. 1 mM of AAPH was added to all of the well and pre-incubated 24 h, then incubated with or without the indicated concentrations of *L. japonica* ex-

tracts (25, 50, 100 $\mu\text{g}/\text{mL}$) for 24 h. The medium was removed and the cells were washed twice with PBS. One milliliter of 50 mmol/L potassium phosphate buffer with 1 mmol/L EDTA (pH 7.0) was added and cells were scraped. Cell suspensions were sonicated three times for 5 sec on ice each time then cell sonicates were centrifuged at 10,000 g for 20 min at 4°C. Cell supernatants were used for antioxidant enzyme activities. The protein concentration was measured by using the method of Bradford (21) with bovine serum albumin as the standard. Superoxide dismutase (SOD) activity was determined by monitoring the auto-oxidation of pyrogallol (22). A unit of SOD activity was defined as the amount of enzyme that inhibited the rate of oxidation of pyrogallol. Glutathione peroxidase (GSH-px) activity was measured by using the method of Lawrence and Burk (23). One unit of GSH-px was defined as the amount of enzyme that oxidizes 1 nmol of NADPH per consumption per minute.

Statistical analysis

The data are represented as mean \pm SD. The statistical analysis was performed with SAS program (SAS Institute Inc., Cary, NC, USA). The values were evaluated by one-way analysis of variance (ANOVA) followed by post-hoc Duncan's multiple range tests.

RESULTS AND DISCUSSION

Cell viability

Fig. 1 shows the effects of BLJ and BFLJ on the cell viability. LLC-PK1 cells were cultured with 0.01, 0.05, 0.1, 1, and 10 mg/mL of BLJ or BFLJ for 1 day and then cell viability was examined by MTT assay. Results showed that BLJ and BFLJ did not have significant cytotoxicity on LLC-PK1 cells; the cytotoxicity of BFLJ itself was not

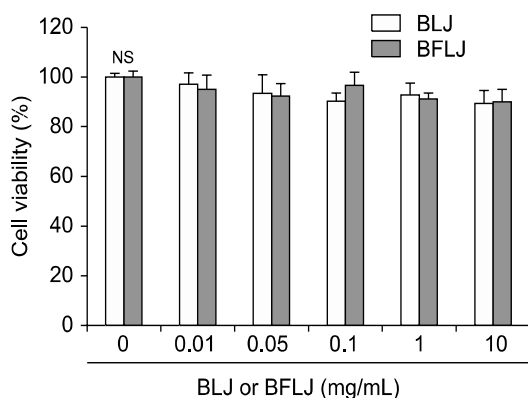


Fig. 1. Cytotoxicity of BFLJ on LLC-PK1 cells. Cells were pre-treated with various concentrations of BLJ or BFLJ for 1 day and cell viability was determined by the MTT assay. Data are mean \pm SD (n=3). NS: no significant. BLJ, *Laminaria japonica* BuOH fraction; BFLJ, fermented *Laminaria japonica* BuOH fraction.

observed. Therefore, we evaluated the protective effect of BFLJ in AAPH-treated LLC-PK1 cells. The cells were pretreated with 1 mM of AAPH for 24 h, then BLJ or BFLJ at 25, 50, and 100 $\mu\text{g}/\text{mL}$ was added. The cell survival was determined after 24 h. As shown in Fig. 2, cell viability was notably decreased in cells treated with AAPH only. However, treatment with BLJ or BFLJ resulted in increase of cell survival in a dose-dependent manner.

AAPH causes a diverse array of pathological changes. Therefore, an AAPH-intoxication experiment may be a promising assay system for the biological activities of antioxidants. In addition, LLC-PK1, a renal-tubular epithelial cell line, is susceptible to oxidative stress, resulting in cell death or injury. Previously reported, AAPH led to the decreased viability of LLC-PK1 renal epithelial cells (24). Treatment with 1 mM of AAPH decreased viability of LLC-PK1 cells to 24.85%. Treatment with more than 50 $\mu\text{g}/\text{mL}$ BFLJ increased cell viability in AAPH-induced LLC-PK1 cells, and by 86.48% in the cells treated 100 $\mu\text{g}/\text{mL}$ of BFLJ.

Fermentation consists of modifying food by microorganisms that grow, reproduce and consume part of the substrate while enriching it with the products of their metabolism. The fermentation of *L. japonica* was suspected to result in various compositional and functional changes by different kinds of microorganism. The presence of BFLJ with AAPH recovered the viability of LLC-PK1 cells more than 80%. The BuOH fraction of fermented *L. japonica* extracts showed higher cell viability than BuOH fraction of *L. japonica* extracts overall. Thus, we could guess that the compounds increasing cell viability might be produced during fermentation of *L. japonica*. But further studies are needed to identify the antioxidant compounds still in question after this study.

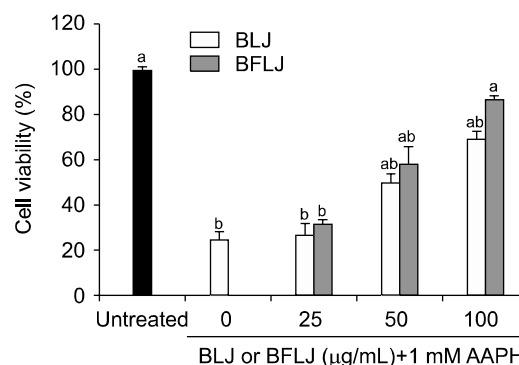


Fig. 2. Effects of BFLJ on cell viability in AAPH-treated LLC-PK1 cells. The cells were seeded into 24-well plates (4×10^5 cells/well), two hours later, 1 mM of AAPH was added to all of the well and pre-incubated for 24 h, then incubated with BLJ or BFLJ (25, 50, and 100 $\mu\text{g}/\text{mL}$) for 24 h. Untreated is negative control without AAPH-treatment. Each value is expressed as mean \pm SD (n=3). A value sharing the same superscript is not significantly different at $P < 0.05$. BLJ, *Laminaria japonica* BuOH fraction; BFLJ, fermented *Laminaria japonica* BuOH fraction.

Lipid peroxidation

Fig. 3 shows the effect of BFLJ on the AAPH-induced lipid peroxidation formation in LLC-PK1 cells. A sharp increase in MDA level of the culture medium of LLC-PK1 cells exposed to AAPH (1 mmol/L) was observed compared with untreated cells (0.18 ± 0.02 vs. 0.72 ± 0.05 nmol/L). The concentrations of MDA were decreased by addition of BLJ or BFLJ in a dose-dependent manner. BFLJ showed higher protective effects against lipid peroxidation than BLJ overall. When the cells were treated with 100 $\mu\text{g}/\text{mL}$ BFLJ, TBARS were significantly decreased by 0.15 ± 0.01 nmol MDA ($P < 0.05$). Sevanian and Hochstein (25) suggested that lipid peroxidation was regarded as a type of cell damage mediated by free radicals. In this study, AAPH induced lipid peroxidation in LLC-PK1 cells and BFLJ inhibited TBARS formations effectively.

Lipid peroxidation in biological systems has long been thought to be a toxicological phenomenon, resulting in pathological consequences. Hence, measurement of lipid peroxidation end products such as TBARS provides a good index of cell destruction. The studies have shown that AAPH-induced cytotoxicity is mediated, at least in part, through increases in the amount of lipid peroxidation of cell membranes (26,27). We demonstrated that lipid peroxidation was notably increased in LLC-PK1 cells pretreated with AAPH, but the addition of BLJ or BFLJ inhibited lipid peroxidation in a dose dependent manner, especially BFLJ, which showed a higher inhibitory effect than BLJ. Our results indicate that the inhibition of lipid peroxidation may be one of the possible actions that are responsible for the protective effect of BFLJ against AAPH toxicity in LLC-PK1 cells.

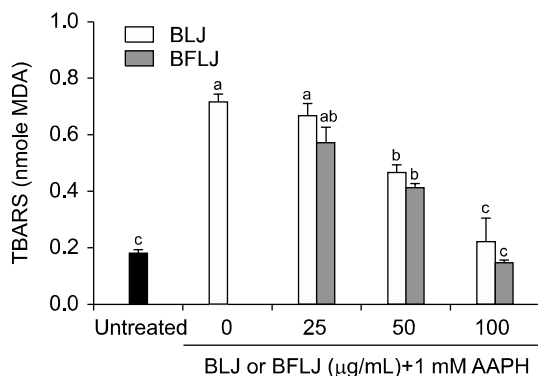


Fig. 3. Effects of BFLJ on TBARS generation in AAPH-treated LLC-PK1 cells. The cells were seeded into 24-well plates (4×10^5 cells/well), two hours later, 1 mM of AAPH was added to all of the well and pre-incubated for 24 h, then incubated with BLJ or BFLJ (25, 50, and 100 $\mu\text{g}/\text{mL}$) for 24 h. Untreated is negative control without AAPH-treatment. Each value is expressed as mean \pm SD ($n=3$). BLJ, *Laminaria japonica* BuOH fraction; BFLJ, fermented *Laminaria japonica* BuOH fraction. ^{a-c}Means with different superscripts are significantly different at $P < 0.05$.

GSH concentration

GSH is the most abundant low molecular weight thiol compound in cells and plays an important role in antioxidant defense and detoxification. Fig. 4 showed the effect of BFLJ on GSH concentration when LLC-PK1 cells exposed to AAPH. AAPH caused a decrease of 0.77 pmol/L $\times 10^5$ cell of GSH compared with untreated 1.39 pmol/L $\times 10^5$ cell respectively. GSH concentration in the cell treated with BFLJ of 100 $\mu\text{g}/\text{mL}$ was 1.80 pmol/L $\times 10^5$ cell. Treatment of the BFLJ showed higher GSH concentration than that of BLJ.

GSH provides primary defense against oxidative stress by its ability to scavenge free radicals and participates in the reduction of H_2O_2 catalyzed by GSH peroxidase (28). Decrease in GSH can compromise cell defenses against oxidative damage and may lead to cell death (29). Incubation of LLC-PK1 cells with AAPH caused a decrease in GSH. Treatment of AAPH with BFLJ 100 $\mu\text{g}/\text{mL}$ resulted in a significant increase in GSH concentration ($P < 0.05$). An increase in GSH concentration by BFLJ treatment could lead to decreased oxidative stress and thus could be part of the mechanism for the defensive effect of BFLJ against oxidative stress because GSH plays an important role in the protection of cells against oxidative stress.

Antioxidant enzyme activities

Cells are protected from activated oxygen species by endogenous antioxidant enzymes such as SOD and GSH-px. The effects of BLJ or BFLJ on antioxidant enzyme activities in AAPH treated LLC-PK1 cells are shown in Table 1. Pretreatment with 1 mM AAPH for 24 h significantly ($P < 0.05$) decreased SOD activity of LLC-PK1 cells compared with untreated cells. BLJ or BFLJ treatment appeared to have increased the antioxidant enzyme levels.

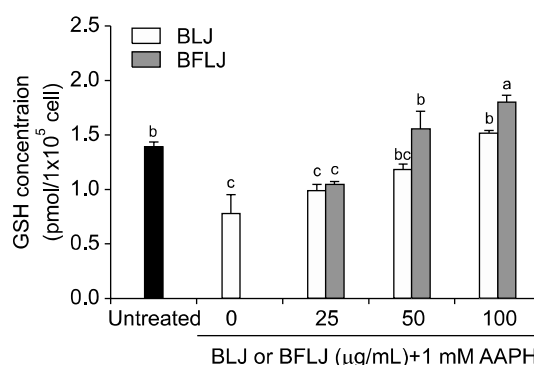


Fig. 4. Effects of BFLJ on GSH concentration in AAPH-treated LLC-PK1 cells. The cells were seeded into 24-well plates (4×10^5 cells/well), two hours later, 1 mM of AAPH was added to all of the well and pre-incubated for 24 h, then incubated with BLJ or BFLJ (25, 50, and 100 $\mu\text{g}/\text{mL}$) for 24 h. Untreated is negative control without AAPH-treatment. Each value is expressed as mean \pm SD ($n=3$). BLJ, *Laminaria japonica* BuOH fraction; BFLJ, fermented *Laminaria japonica* BuOH fraction. ^{a-c}Means with different superscripts are significantly different at $P < 0.05$.

Table 1. Effects of BFLJ on antioxidant enzyme activities in AAPH-treated LLC-PK1 cells

	Untreated	1 mM AAPH	BLJ or BFLJ ($\mu\text{g/mL}$)+1 mM AAPH					
			BLJ			BFLJ		
			25	50	100	25	50	100
SOD (unit/mg protein)	61.29 \pm 4.51 ^a	10.34 \pm 2.27 ^c	15.92 \pm 8.14 ^c	37.18 \pm 9.93 ^{bc}	45.63 \pm 9.01 ^b	14.96 \pm 8.13 ^c	42.05 \pm 3.01 ^b	51.03 \pm 0.77 ^{ab}
GSH-px (unit/mg protein)	5.70 \pm 0.59 ^a	2.57 \pm 0.20 ^c	2.12 \pm 0.13 ^c	4.33 \pm 0.13 ^b	5.16 \pm 0.10 ^{ab}	2.40 \pm 0.11 ^c	3.48 \pm 1.44 ^b	5.61 \pm 0.15 ^a

The cells were seeded into 10-mm dishes (5×10^6 cells/dish), two hours later, 1 mM of AAPH was added to all of the wells and pre-incubated for 24 h, then incubated with BLJ or BFLJ (25, 50, and 100 $\mu\text{g/mL}$) for 24 h. Untreated is the negative control without AAPH-treatment.

Each value is expressed as mean \pm SD (n=3).

SOD, superoxide dismutase; GSH-px, glutathione peroxidase; BLJ and BFLJ, See the abbreviations in Fig. 1.

^{a-c}Means with different superscripts in the same row are significantly different at $P < 0.05$.

SOD levels were significantly increased to 51.03 ± 0.77 unit/mg protein at concentration 100 $\mu\text{g/mL}$ in comparison to BLJ (45.63 ± 9.01 unit/mg, $P < 0.05$).

GSH-px activity in AAPH pretreated LLC-PK1 cells was significantly decreased to 2.57 ± 0.20 unit/mg protein compared to 5.70 ± 0.59 unit/mg protein in untreated cells. However, addition of BLJ or BFLJ to the cells increased GSH-px activity in a dose-dependent manner. At concentrations of 25, 50, and 100 $\mu\text{g/mL}$ BFLJ were 2.40 ± 0.11 , 3.48 ± 1.44 , and 5.61 ± 0.15 unit/mg protein, respectively. The superoxide anion radical scavenging activity of SOD is effective only when it is followed by the actions of GSH-px, because the activity of SOD generates H_2O_2 , which needs to be further scavenged by GSH-px. In the present study, GSH-px activity was also significantly increased ($P < 0.05$) in the presence of BFLJ. These elevations of antioxidant enzyme activities by BFLJ might support the idea that fermented *L. japonica* contains a high content of antioxidant compound to help the attenuation of oxidative damage.

Seaweed has been considered as a third generation biomass for bioethanol production (30). The carbohydrate contents of seaweed are in a range of 30~70%. Carbohydrates in brown seaweed such as *L. japonica* consist of alginate, laminaran, fucoidan and mannitol (31). Especially, fucoidans, the family of sulfated heteropolysaccharides, composed mainly of 1,3-linked fucose residues, are constituents of brown algae (32-34). Several biological activities have been attributed to the fucoidans, such as antioxidant, anticoagulant, antithrombotic, antiinflammatory, antitumoral and antiviral (35-42). Degradation of these polysaccharides is slow and requires specific enzymes. Fermentation is a chemical reaction that splits complex organic compounds into relatively simple substances. *L. japonica's* active compounds may be packed in its rigid structural matrix. During fermentation, the active compounds in *L. japonica* will be exposed and these may have effectiveness such as antioxidant activity. So, this study was carried out to increase and improve the

active carbohydrate compounds by the fermentation using *Aspergillus oryzae*. The fermented *L. japonica* extract showed higher antioxidant activities such as lipid peroxidation, glutathione concentration and antioxidant enzyme activities than the intact *L. japonica* extract on 100 $\mu\text{g/mL}$ concentration. In particular, the inhibition of lipid peroxidation was predominant in the cell treated BFLJ. BFLJ reduced lipid peroxidation formation and increased activities of antioxidant enzymes such as superoxide dismutase and glutathione peroxidase. Also, BFLJ treatment increased glutathione concentration. In our previous study, BFLJ increased free radical scavenging activity comparison with BLJ. Therefore, we demonstrated that BFLJ can protect LLC-PK1 cells from AAPH-induced oxidative damage by increasing radical scavenging and antioxidant activity.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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