

Protective Effect of *Sasa borealis* Leaf Extract on AAPH-Induced Oxidative Stress in LLC-PK1 Cells

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Abstract

This study was designed to investigate the protective effect of *Sasa borealis* leaf extract on 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH)-induced oxidative stress in LLC-PK1 cells (porcine kidney epithelial cells). The butanol fraction from *Sasa borealis* leaf extract (SBBF) was used in this study because it possessed strong antioxidant activity and high yield among fractions. Exposure of LLC-PK1 cells to 1 mM AAPH for 24 hr resulted in a significant decrease in cell viability, but SBBF treatment protected LLC-PK1 cells from AAPH-induced cell damage in a dose dependant manner. To determine the protective action of SBBF against AAPH-induced damage of LLC-PK1 cells, we measured the effects of SBBF on lipid peroxidation and antioxidant enzymes activities of AAPH treated cells as well as scavenging activities on superoxide anion radical and hydroxyl radical. SBBF had a protective effect against the AAPH-induced LLC-PK1 cellular damage and decreased lipid peroxidation and increased activities of antioxidant enzymes such as superoxide dismutase and glutathione peroxidase. Furthermore, SBBF showed strong scavenging activity against superoxide anion radical. The IC₅₀ value of SBBF was 28.45 ± 1.28 µg/mL for superoxide anion radical scavenging activity. The SBBF also had high hydroxyl radical scavenging activity (IC₅₀=31.09 ± 3.08 µg/mL). These results indicate that SBBF protects AAPH-induced LLC-PK1 cells damage by inhibiting lipid peroxidation, increasing antioxidant enzyme activities and scavenging free radicals.

Key words: oxidative stress, antioxidant activity, *Sasa borealis*, LLC-PK1 cells

INTRODUCTION

Cell injury and lipid peroxidation caused by free radical-mediated oxidative stress may be of critical importance in various pathological phenomena (1,2). AAPH, which can generate free radicals that react rapidly with oxygen molecules to yield peroxy radicals, causes a diverse array of pathological changes. It has been reported that AAPH leads to the decreased viability of LLC-PK1 cells (3); LLC-PK1 cells are renal-tubular epithelial cells that are susceptible to oxidative stress, which results in their death or injury.

Oxidative stress is caused by an imbalance between antioxidant systems and the production of oxidants, including reactive oxygen species (ROS). The human body has several mechanisms of defense against free radical-mediated oxidative stress, including radical scavenging activity. The presence of antioxidants also helps the body's defense system; therefore, antioxidants that prevent damage caused by free radicals are considered to be worthy of study. Although several synthetic antioxidants have been suggested for the prevention and treatment of diseases, the various side effects and tox-

icities have become an issue (1). Natural antioxidants have advantages over synthetic ones, since natural antioxidants are considered to be safe by the consumer. Also, fewer safety tests are required by the legislation if the food component is generally recognized as safe (4).

Sasa borealis (Hackel) Makino is a kind of *Bambusa-ceae*, bamboo tree, whose leaves are used by Koreans for hypertension, diabetes, jaundice, fever, cancer and eczema (5). There is currently intense attention in developing new antioxidant agents from plants used for alternative medicines (6). Accordingly, *Sasa borealis* leaves may be a novel plant for alternative medicinal uses.

However, there are few studies investigating the protective effects of *Sasa borealis* leaves on oxidative damage in the cell. In our previous studies (7,8), the butanol fraction of *Sasa borealis* leaves extract appeared to be a potential therapeutic agent for free radical induced cell damage. The aim of this work was to investigate whether the butanol fraction of *Sasa borealis* leaf extract can protect LLC-PK1 cells from AAPH toxicity. In addition, to evaluate the protective action of these extracts, we examined their effects on the lipid peroxidation of

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AAPH-treated cells and their scavenging activity on free radicals.

MATERIALS AND METHODS

Materials

The *Sasa borealis* leaves were purchased at Korean herb Research Institute (Jeju, Korea). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), AAPH and bovine serum albumin were purchased from Sigma (St. Louis, MO, USA). All other chemicals and solvents used were analytical grade from Sigma. The butanol fraction of *Sasa borealis* leaves extract (SBBF) was dissolved in phosphate buffered saline (PBS; Invitrogen, Carlsbad, CA, USA) and used in concentrations of up to 50 µg/mL (10, 25, and 50 µg/mL) in this study.

Sample preparation and extraction

Freeze-dried *Sasa borealis* leaves were extracted with 70% methanol and the extract was subsequently subjected to sequential fractionation with dichloromethane, ethylacetate, butanol and water. In our previous study, SBBF had strong antioxidant activity and high yield among the fractions, with an IC₅₀ value of 22.12 ± 5.23 µg/mL in DPPH radical scavenging activity (8). Therefore, SBBF was dried and placed in a plastic bottle, and then stored at -80°C until used (9).

Cell culture

Commercially available porcine renal tubular epithelial cells (LLC-PK1 cells, passages 7~35) were maintained at 37°C in a humidified atmosphere of 5% CO₂ 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 × 10⁵ cells/well) or 10-mm dishes (5 × 10⁶ cells/dish). Two hours later, 1 mM AAPH was added to each of the wells, pre-incubated for 24 hr, and then incubated with SBBF (10, 25, 50 µg/mL) for 24 hr (10). The proper concentration of AAPH and the incubation time were determined by preliminary experiments.

Cell viability

The MTT assay of cell viability was performed following a well-described procedure with minor modifications (11). Cells were plated in 24-well cell culture plates at a density of 4 × 10⁵ cells per 24 wells. At the end of the culture, 100 µg of MTT solution (5 mg/mL in PBS) was added to each well containing 1 mL medium. After 4 hr of incubation, the media were removed and formazan crystals were solubilized with 300 µg DMSO. The absorbance of each well was then read at 540 nm using a microplate reader.

Determination of lipid peroxidation

Lipid peroxidation was measured as thiobarbituric acid reactive substances (TBARS) production (12). Cells (4 × 10⁴ cells/well) in 24-well plates were first incubated with 1 mM AAPH for 24 hr, and then incubated with SBBF for 24 hr. 200 µg of each medium supernatant was mixed with 400 µg of TBARS solution and then boiled at 95°C for 30 min. The absorbance at 532 nm was measured and TBARS concentrations were extrapolated from the 1,1,3,3-tetraethoxypropane serial dilution standard curve. TBARS values were then expressed as equivalent nmoles of malondialdehyde (MDA).

Superoxide anion radical scavenging activity

The scavenging potential for superoxide anion radical was analyzed via a hypoxanthine/xanthine oxidase generating system coupled with nitroblue tetrazolium (NBT) reduction following the method of Kirby and Schmidt (13). The reaction mixture contained 125 µg of buffer (50 mM K₂HPO₄/KOH, pH 7.4), 20 µg of 15 mM Na₂EDTA in buffer, 30 µg of 3 mM hypoxanthine in buffer, 50 µg of xanthine oxidase in buffer (1 unit per 10 mL buffer) and 25 µg of plant extract in buffer (a diluted sonicated solution of 10 µg per 250 µg buffer). The absorbance of the solution was measured at 540 nm. Superoxide scavenging activity was expressed as % inhibition compared to the blank.

Hydroxyl radical scavenging activity

The reaction mixture contained 0.45 mL of 0.2 M sodium phosphate buffer (pH 7.0), 0.15 mL of 10 mM 2-deoxyribose, 0.15 mL of 10 mM FeSO₄-EDTA, 0.15 mL of 10 mM H₂O₂, 0.525 mL of H₂O, and 0.075 mL of sample solution. The reaction was started by the addition of H₂O₂. After incubation at 37°C for 4 hr, the reaction was stopped by adding 0.75 mL of 2.8% trichloroacetic acid and 0.75 mL of 1.0% of 2-tribarbitric acid in 50 mM NaOH. The solution was boiled for 10 min, and then cooled in water. The absorbance of the solution was measured at 520 nm. Hydroxyl radical scavenging activity was evaluated as the inhibition rate of 2-deoxyribose oxidation by ·OH (14).

Antioxidant enzyme assays

Cells (5 × 10⁶ cells/dish) in 10-mm dishes were pre-incubated for 2 hr. 1 mM of AAPH was added to all of the wells, pre-incubated for 24 hr, and then incubated with or without the indicated concentrations of SBBF for 24 hr. The medium was removed and the cells were washed twice with PBS. One mL 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 on ice three times for 5 sec each time and then cell sonicates were centrifuged at $10,000 \times g$ 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 (15), with bovine serum albumin as the standard. Superoxide dismutase (SOD) activity was determined by monitoring the auto-oxidation of pyrogallol (16). A unit of SOD activity was defined as the amount of enzyme that inhibited the rate of oxidation of pyrogallol by 50%. Glutathione peroxidase (GSH-px) activity was measured by using the method of Lawrence and Burk (17). One unit of GSH-px was defined as the amount of enzyme that oxidizes 1 nmol of NADPH per minute.

Statistical analysis

The data are expressed 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

LLC-PK1 cells were cultured with 0.01, 0.1, 0.5 and 1 mg/mL of SBBF for 24 hr and then cell viability was examined by the MTT assay (Fig. 1). Results showed that SBBF was not significantly cytotoxic to LLC-PK1 cells and the cytotoxicity of SBBF itself was not observed. Therefore, we evaluated the protective effect of SBBF in AAPH-treated LLC-PK1 cells. The cells were pretreated with 1 mM of AAPH and pre-incubated for 24 hr, then SBBF at 10, 25 and 50 $\mu\text{g}/\text{mL}$ was added.

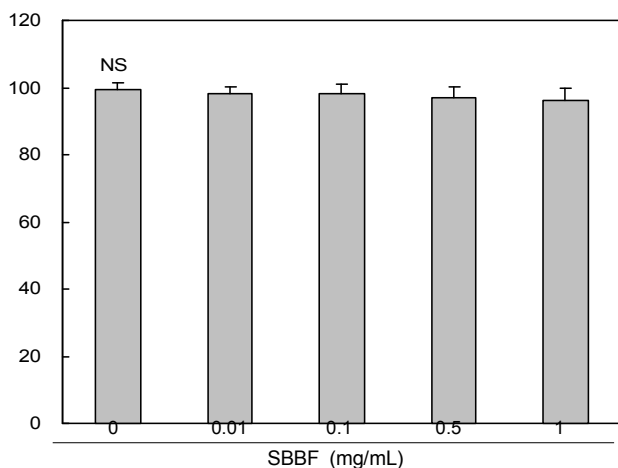


Fig. 1. Cytotoxicity of SBBF on LLC-PK1 cells. Cells were treated with various concentrations of SBBF for 1 day and cell viability was determined by the MTT assay. Each value is expressed as mean \pm SD ($n=3$). NS: not significant. SBBF: butanol fraction from *Sasa borealis* leaf extract.

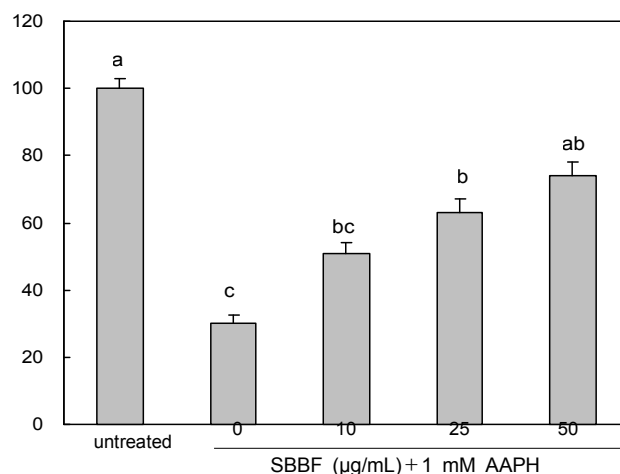


Fig. 2. Protective effects of SBBF against AAPH-induced cell damage. The cells were seeded into 24 well plates (4×10^7 cells/well), two hours later 1 mM of AAPH was added to all of the well and preincubated for 24 hr, then incubated with SBBF (10, 25, and 50 $\mu\text{g}/\text{mL}$) for 24 hr. Untreated is negative control without AAPH treatment. Each value is expressed as mean \pm SD ($n=3$). Values sharing the same superscript are not significantly different at $p < 0.05$. SBBF: See the abbreviations in Fig. 1.

The cell survival was determined after 24 hr. As shown in Fig. 2, cell viability was notably decreased in cells treated with AAPH only. However, treatment with SBBF resulted in increase of cell survival in a dose dependent manner. At a concentration of 50 $\mu\text{g}/\text{mL}$, SBBF revised cell viability from 30.21% in cell treated with AAPH only to 74.21%.

LLC-PK1 renal tubular epithelial cells are susceptible to free radicals (18) and an *in vitro* model of oxidative damage, in which LLC-PK1 cells are exposed to free radicals, would appear useful for searching for agents that can provide effective protection. Although various kinds of free radical initiators are known, AAPH has been used easily and successfully as a radical initiator. The free radicals generated from AAPH ultimately cause physicochemical alterations and cellular damage through the processes of oxidation reaction (19,20). The exposure of LLC-PK1 cells to 1 mM AAPH for 24 hr resulted in a marked decrease in cell viability. However, SBBF treatment exerted significant increase ($p < 0.05$) in cell viability in a dose dependent manner, suggesting that SBBF protected LLC-PK1 cells from AAPH-induced cytotoxicity.

Lipid peroxidation

To investigate the protective action of SBBF against AAPH induced cytotoxicity, we examined the effects of SBBF on lipid peroxidation of LLC-PK1 cells. Fig. 3 shows the inhibitory effect of SBBF on AAPH-induced lipid peroxidation of LLC-PK1 cells. The lipid perox-

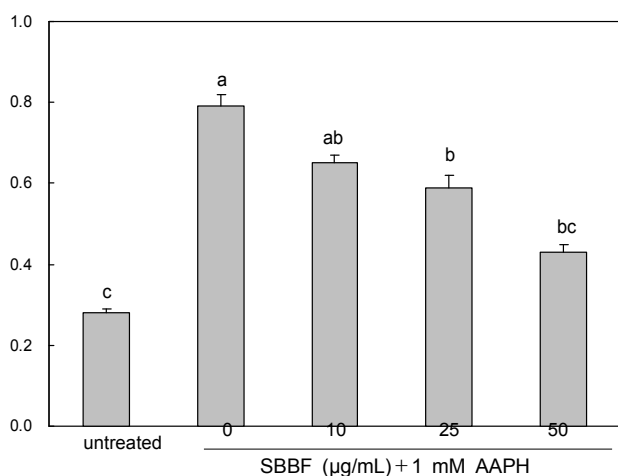


Fig. 3. Inhibitory effect of SBBF on AAPH-induced lipid peroxidation of LLC-PK1 cells. The cells were seeded into 24 well plates (4×10^5 cells/well), two hours later 1 mM of AAPH was added to all of the well and preincubated for 24 hr, then incubated with SBBF (10, 25, and 50 $\mu\text{g/mL}$) for 24 hr. Untreated is negative control without AAPH treatment. Each value is expressed as mean \pm SD ($n=3$). Values sharing the same superscript are not significantly different at $p < 0.05$. SBBF: See the abbreviations in Fig. 1.

idation was markedly increased in the cells treated with AAPH, as shown by TBARS of 0.79 ± 0.03 nmol MDA in the LLC-PK1 cells exposed to 1 mM AAPH compared with TBARS of 0.28 ± 0.01 nmol MDA in untreated cells. However, treatment with SBBF significantly inhibited the lipid peroxidation in a dose dependent manner ($p < 0.05$), indicating TBARS levels of 0.65 ± 0.02 , 0.59 ± 0.03 , and 0.43 ± 0.02 nmol MDA at 10, 25 and 50 $\mu\text{g/mL}$, respectively.

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. Previous studies have shown that AAPH-induced cytotoxicity is mediated, at least in part, through increases in the amount of lipid peroxidation of cell membranes (21,22). In this study, we demonstrated that lipid peroxidation was notably increased in LLC-PK1 cells pretreated with AAPH, but the addition of SBBF inhibited lipid peroxidation in a dose dependent manner. Therefore, our results indicate that the inhibition of lipid peroxidation may be one of the possible actions that are responsible for the protective effect of SBBF against AAPH toxicity in LLC-PK1 cells.

ROS scavenging activity

Since lipid peroxidation is associated with overproduction of ROS, we examined the scavenging activity of SBBF on ROS, such as superoxide anion radical and hydroxyl radical. Superoxide anion radical scavenging

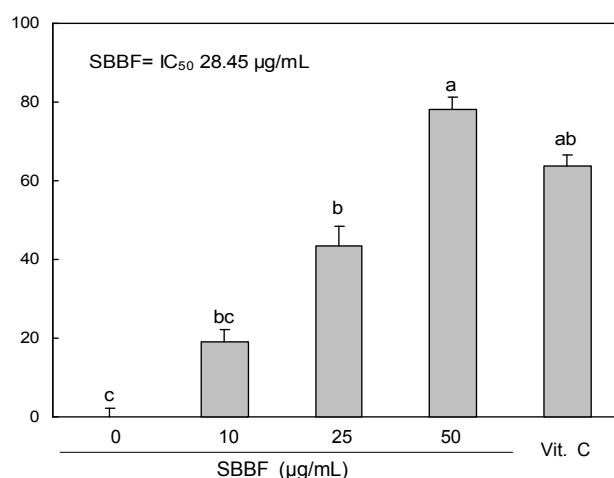


Fig. 4. Scavenging activity of SBBF on the superoxide anion radical. IC_{50} : The concentration of sample required for 50% inhibition, Vit. C: vitamin C. The final concentration of vitamin C in the reaction mixture was 10 $\mu\text{g/mL}$. Each value is expressed as mean \pm SD ($n=3$). Values sharing the same superscript are not significantly different at $p < 0.05$. SBBF: See the abbreviations in Fig. 1.

activity of SBBF was estimated using xanthine-xanthine oxidase system (NBT method) and presented in Fig. 4. The superoxide anion radical scavenging activity was notably increased in the presence of 10, 25 and 50 $\mu\text{g/mL}$ of SBBF. The percentage of superoxide anion radical scavenging activity with 50 $\mu\text{g/mL}$ of SBBF was 78.14%. The fenton reagent as a source of hydroxyl radical was used to test the scavenging activity of SBBF on hydroxyl radical. The SBBF also showed high hydroxyl radical scavenging activity (Fig. 5). Addition of SBBF with concentrations of 10, 25 and 50 $\mu\text{g/mL}$ showed

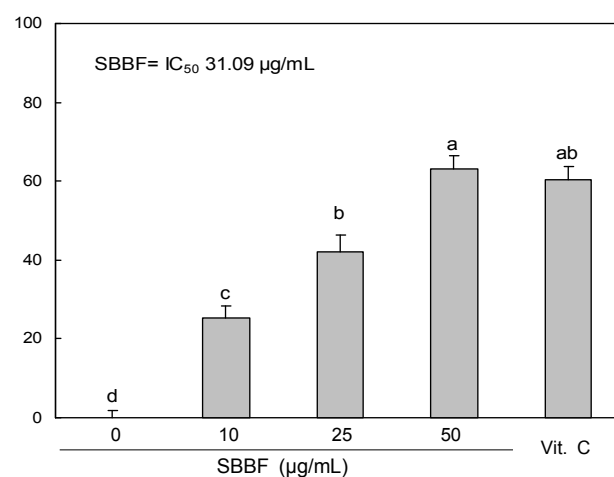


Fig. 5. Scavenging activity of SBBF on hydroxyl radical. IC_{50} : The concentration of sample required for 50% inhibition, Vit. C: vitamin C. The final concentration of vitamin C in the reaction mixture was 10 $\mu\text{g/mL}$. Each value is expressed as mean \pm SD ($n=3$). Values sharing the same superscript are not significantly different at $p < 0.05$. SBBF: See the abbreviations in Fig. 1.

strong hydroxyl radical scavenging activity, which were 25.32, 42.11 and 63.21%, respectively.

It is known that superoxide anion radical is associated with several pathophysiological processes, and that it can not only directly initiate lipid peroxidation, but also be converted into more reactive radical species, such as hydroxyl radical, that also initiate lipid peroxidation (23). In this study, the superoxide anion radical scavenging activity was significantly increased in the presence of 10, 25 and 50 $\mu\text{g/mL}$ of SBBF. Previous studies have shown that superoxide radicals can be converted into hydrogen peroxide by SOD and the hydrogen peroxide can produce highly reactive hydroxyl radicals in the presence of metal ions (24). In our investigation, SBBF over 10 $\mu\text{g/mL}$ also showed significant hydroxyl radical scavenging activity ($p < 0.05$).

Hydroxyl radical is a reactive free radical formed in biological systems and has been implicated as a damaging species in free radical pathology, capable of damaging almost every molecule found in cells (25). Additionally, hydroxyl radicals are known to be capable of abstracting hydrogen atoms from the membrane and bringing about peroxidic reactions of lipids (26). The results of this study indicate that SBBF has noticeable effects of O_2^- and OH scavenging activity, with IC_{50} values of superoxide anion and hydroxyl radical scavenging activity of SBBF at 28.45 and 31.09 $\mu\text{g/mL}$, respectively. The removal of hydroxyl radical as well as superoxide anion radical may play important roles in antioxidant defense of LLC-PK1 cells.

According to a recent study (27), the butanol fraction of *Sasa borealis* leaves had a higher antioxidant activity than butylated hydroxytoluene (BHT) in electron donating ability, superoxide dismutase-like ability, reducing power and lipid peroxidation inhibitory activity. Many researchers have reported positive correlation between free radical scavenging activity and antioxidant compounds (28). This radical scavenging activity of the SBBF could be related to the nature of antioxidant compounds, thus contributing to their electron transfer/hydrogen donating ability (29). It was reported that the SBBF contained an active compound, isoorientin, which

is the nature of flavonoids (30,31). It is supposed that this compound could react as a scavenging or inducing substance of reactive oxygen species.

Antioxidant enzyme activities

Cells are protected from activated oxygen species by endogenous antioxidant enzymes such as SOD and GSH-px. The effects of SBBF on antioxidant enzyme activities in AAPH-treated LLC-PK1 cells are shown in Table 1. Pretreatment with 1 mM AAPH for 24 hr significantly ($p < 0.05$) decreased SOD activity of LLC-PK1 cells compared with untreated cells. Treatment of LLC-PK1 cells with SBBF increased SOD activity in 1 mM AAPH-pretreated cells. After the cells were treated with 10, 25 and 50 $\mu\text{g/mL}$ of SBBF, they resulted in a significant ($p < 0.05$) increase of SOD activity, with SOD activity of 23.51 ± 4.52 , 33.52 ± 4.99 and 49.51 ± 5.97 unit/mg protein, respectively. GSH-px activity in AAPH pretreated LLC-PK1 cells was significantly decreased to 3.01 ± 0.93 unit/mg protein compared to 6.91 ± 1.91 unit/mg protein in untreated cells. However, addition of SBBF to the cells increased GSH-px activity in a dose-dependent manner. GSH-px activity at concentrations of 10, 25 and 50 $\mu\text{g/mL}$ SBBF were 3.91 ± 1.98 , 4.55 ± 0.89 and 5.51 ± 1.01 unit/mg protein, respectively.

Treatment with SBBF restored the antioxidant enzyme activities, such as SOD and GSH-px, in AAPH-pretreated LLC-PK1 cells. SBBF significantly increased the SOD activity ($p < 0.05$). SOD, the endogenous scavenger, catalyzes the dismutation of the highly reactive superoxide anion to H_2O_2 (32). 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 SBBF. These elevations of antioxidant enzyme activities by SBBF might support the idea that it contains a high content of antioxidant compound to help the attenuation of oxidative stress.

In conclusion, we demonstrated that SBBF could protect LLC-PK1 cells from AAPH-induced cellular dam-

Table 1. Effects of SBBF on antioxidant enzyme activities in AAPH-induced LLC-PK1 cells

	Untreated	SBBF ($\mu\text{g/mL}$) + 1 mM AAPH			
		0	10	25	50
SOD (unit/mg protein)	59.43 ± 2.25^a	15.35 ± 4.01^c	23.51 ± 4.52^{bc}	33.52 ± 4.99^b	49.51 ± 5.97^{ab}
GSH-px (unit/mg protein)	6.91 ± 1.91^a	3.01 ± 0.93^c	3.91 ± 1.98^{bc}	4.55 ± 0.89^b	5.51 ± 1.01^{ab}

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 wells and preincubated for 24 hr, then incubated with SBBF (10, 25, 50 $\mu\text{g/mL}$) for 24 hr. Untreated is negative control without AAPH treatment. Each value is expressed as mean \pm SD ($n=3$). Values sharing the same superscript within a row are not significantly different at $p < 0.05$. SBBF: See the abbreviations in Fig. 1.

age, which might, in part, be linked to an inhibitory effect of SBBF on lipid peroxidation, its ROS radical scavenging activity and its increases in antioxidant enzyme activities.

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