

# Protective effects of methanolic extract form fruits of *Lycium ruthenicum* Murr on 2,2'-azobis (2-amidinopropane) dihydrochloride-induced oxidative stress in LLC-PK1 cells

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## ABSTRACT

**Background:** Fruits of *Lycium ruthenicum* Murr is a health food and also used as a folk to treat heart disease, abnormal menstruation and menopause in Tibetan, China. However; whether *L. ruthenicum* Murr fruits methanolic extracts (LFME) protect LLC-PK1 porcine renal tubules cells from AAPH-induced oxidative damage has not been investigated. **Objective:** To investigate the protective effects of *L. ruthenicum* Murr fruits methanolic extracts (LFME) against 2, 2'-azobis (2-amidinopropane) dihydrochloride (AAPH)-induced oxidative damage in renal proximal tubule LLC-PK1 cells. **Materials and Methods:** LLC-PK1 cells were co-incubated with AAPH (1mM) and different concentrations of LFMW together for 24 h. Cell viability was determined by MTT assay. Total intercellular reactive oxygen species (ROS) levels and lipid peroxidation were measured using a fluorescent probe 2', 7'-dichlorofluorescein-diacetate (DCFH-DA) and the TBA reactive substance (TBARS) assay, respectively. The endogenous antioxidant enzymes including catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GSH-px) and intercellular glutathione (GSH) levels were determined using commercial assay kits according to the manufacturer's instructions. **Results:** LFME did not show a significant cytotoxic effect and increased the viability of LLC-PK1 cells in a concentration-dependent manner. LFME also decreased the total intercellular levels of ROS, reduced lipid peroxidation and increased the GSH levels as well as the activities of endogenous antioxidant enzymes to protect LLC-PK1 cells against AAPH-induced oxidative damage. **Conclusion:** The results from the present study indicated that LFME is an effective ROS scavenger to protect LLC-PK1 cells against AAPH-induced oxidative damage through decreasing ROS generation, reducing lipid peroxidation and up-regulation of endogenous GSH levels and antioxidant enzymes.

**Key words:** Antioxidant enzymes, LLC-PK1 cells, *Lycium ruthenicum* Murr, oxidative stress

## INTRODUCTION

Chronic kidney disease (CKD) is a common and serious public health problem that adversely affects human health, limits longevity, increase costs to health-care system worldwide and poor prognosis of morbidity and mortality.<sup>[1]</sup> Some evidences indicated that the prevalence of CKD gradual increase is accounted for about 7 to 11.5%

in general population worldwide.<sup>[2,3]</sup> Reactive oxygen species (ROS) are produced in mammalian cells during energy production in mitochondria by reducing oxygen during aerobic respiration, including superoxide (O<sub>2</sub><sup>-</sup>), peroxide (O<sub>2</sub><sup>-2</sup>), hydroxyl radical(OH), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and peroxynitrite (ONOO<sup>-</sup>) play a key role in the pathogenesis of cell damage in renal-related diseases such as acute renal failure, rhabdomyolysis, obstructive nephropathy, hyperlipidemia and glomerular damage to chronic renal failure and hemodialysis.<sup>[1-7]</sup> In generally, the accumulated ROS are eliminated by endogenous antioxidant defense system including antioxidant enzymes catalase (CAT), superoxide dismutase (SOD), glutathione

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peroxidase (GSH-px) and glutathione S-transferase (GST) and other low molecular weight antioxidants such as glutathione, vitamin C and vitamin E.<sup>[8]</sup> In addition, dietary intake of foods rich in antioxidants, such as phenolic compounds is able to increase the levels of antioxidants in the body and improve the antioxidant defense system to reduce the risk of renal dysfunction induced by ROS.<sup>[4,9]</sup>

*Lycium ruthenicum* Murr is a perennial frutex native to northwest part of China, which grows widely distributes in Qinghai-Tibet plateau, central Asia and Caucasia.<sup>[10]</sup> *L. ruthenicum* Murr is rich in proteins, polysaccharides, unsaturated fats, flavones, amino acids, essential elements, pigments, ascorbic acid and tocopherols.<sup>[11,12]</sup> The ripe fruit of *L. ruthenicum* Murr has been recorded as a folk medicine used for treatment of heart disease, abnormal menstruation and menopause in Tibetan medical classic “Jing Zhu Ben Cao” and “Si Bu Yao Dian”.<sup>[10,13]</sup> Recently, some studies have reported that the fruits of *L. Murr* was rich in anthocyanins, in particular pentunidin-3-O-rutinoside (trans-p-coumaroyl)-5-O-glucoside and petundin-3-O-galactoside-5-O-glucoside.<sup>[10,14]</sup> *L. ruthenicum* Murr also showed anti-tumor, hypolipidemic and antioxidant effects.<sup>[10,13,14]</sup> However, the beneficial activities of *L. ruthenicum* Murr have not been studied. The present study was designed to investigate the ability of *L. ruthenicum* Murr to provide protection against 2, 2'-azobis (2-amidinopropane) dihydrochloride (AAPH)-induced oxidative damage compared in LLC-PK1 cells, an epithelial cell line derived from pig kidney that has retained characteristics of the proximal tubular epithelium and is widely used as a cell model to study ROS-induced renal damage.<sup>[15]</sup> Additionally, the mechanisms underlying these effects were elucidated.

## MATERIALS AND METHODS

### Chemicals and reagents

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), 2', 7'-dichlorodihydrofluorescein diacetate (DCFH-DA), epigallocatechin gallate (EGCG) and 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin solution and 0.05% trypsin-0.53mM EDTA were purchased from Grand Island Biological Company (Grand Island, NY, USA). Other chemicals used were of standard analytical grade.

### Plant extracts preparation

The wild fresh fruits of *Lycium ruthenicum* Murr were purchased from a local market in Terim, Xinjiang, China in October 2012. The collected samples were stored in a

heat preservation box with efficient ice bag in accordance with the method distributed by Zheng et al.<sup>[10]</sup> Two hundred grams of fresh *L. ruthenicum* Murr fruits were freeze dried and then ground to a fine powder. A tenth-fold volume of methanol (80%, v/v) was added to the powdered samples and extracted third by stirring overnight. *L. ruthenicum* Murr fruits methanolic extracts (LFME) were concentrated by heat evaporation, freeze-drying and stored at -20°C until further study.

### Cell culture

LLC-PK1 porcine renal tubules cells (ATCC® CL-101™) were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were routinely maintained in DMEM medium supplemented with FBS (10%, v/v) and 1% penicillin-streptomycin in a humidified CO<sub>2</sub> incubator (model 3154; Forma Scientific, Inc., Marietta, OH, USA) with 5% CO<sub>2</sub> at 37°C.

### Cell treatment and viability assay

Cell viability was determined by an MTT assay. LLC-PK1 cells were seeded in 96-well plates (Nunc, Rochester, NY, USA) at a density of 1 × 10<sup>4</sup> cells/well. The cells were incubated with the LFME (10, 25, 50 and 100 µg/ml) and AAPH (1mM) together for 24 h. After incubation, 100 µl MTT reagent (0.5 mg/ml) was added to each well and the cells were incubated in a humidified incubator at 37°C to allow the MTT to be metabolized. After 4 h, 100 µl DMSO was added to each well to dissolve the formazan crystals. Absorbance of the samples was measured at a wavelength of 490 nm using a microplate reader (model 680; Bio-Rad, Hercules, CA, USA).

### Measurement of lipid peroxidation

Lipid peroxidation was quantitated using a TBA reactive substance (TBARS) assay.<sup>[16]</sup> Briefly, the cultured cells were washed with cooled phosphate-buffered saline (PBS), scraped into TCA (2.8%, w/v) and sonicated 3 times for 10 second intervals at 40 V setting over ice. The suspension (200 µl) was mixed with 1 ml TBA (0.67%, w/v) and 1 ml TCA (25%, w/v), heated (30 min at 95°C) and centrifuged (3,000 × g for 10 min at 4°C). The TBA reacted with the products of lipid oxidative degradation, yielding red complexes. Absorbance was measured at 532 nm using a UV-2401PC spectrophotometer (Shimadzu, Kyoto, Japan). Protein contents were determined using a Thermo scientific pierce bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions.

### Assessment of total intracellular ROS generation

Intracellular ROS levels were measured using the fluorescent probe DCFH-DA. The treated LLC-PK1 cells were washed with calcium- and magnesium-free PBS and

incubated in DCFH-DA (20  $\mu$ M) containing serum- and phenol red-free DMEM at 37°C for 30 min. The medium was then removed and the cells were washed twice with PBS. Fluorescence was measured using a FLUOstar OPTIMA fluorescence plate reader (BMG Labtech, Ortenberg, Germany); excitation was read at 485 nm and emission was detected at 535 nm. Relative ROS production was expressed as the percentage of fluorescence for the treated samples over fluorescence for the appropriate controls:  $(\text{fluorescence}_{\text{treatment}} / \text{fluorescence}_{\text{control}}) \times 100$ .

### Antioxidant enzyme activity and GSH levels

LLC-PK1 cells grown in a 10-cm cell culture dish (Nunc, Rochester, NY, USA) were incubated with LFME and AAPH (1mM) for 24 h at 37°C. The cells were washed with PBS, removed by scraping and centrifuged (3,000  $\times$  g for 10 min at 4°C). The resulting cell pellet was stored at -80°C. The pellets were then thawed, resuspended in 300  $\mu$ l cold lysis buffer (PBS and 1mM EDTA), homogenized and centrifuged (12,000  $\times$  g for 10 min at 4°C). Protein contents were determined using a Thermo scientific pierce BCA protein assay kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Cellular catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GSH-px) and glutathione (GSH) levels were determined using commercial assay kits (Beyotime Institute of Biotechnology, Jiangsu, China) according to the manufacturer's instructions. Enzyme activities and GSH contents were expressed as units (U) of enzyme activity and  $\mu$ mol of GSH per mg protein, respectively.

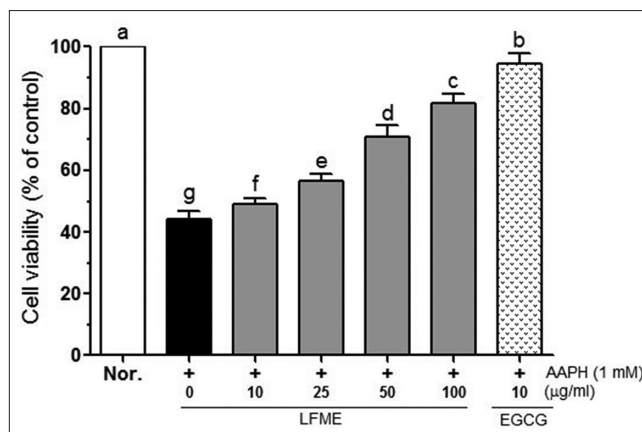
### Statistical analysis

Data are presented as the mean  $\pm$  standard deviation (SD). Differences between the mean values for individual groups were assessed with a one-way ANOVA and Duncan's multiple range tests.  $P < 0.05$  were considered statistically significant. The SAS v9.1 statistical software package (SAS Institute Inc., Cary, NC, USA) was used to conduct the analysis.

## RESULTS

### Cell viability

To determine the potential cytotoxic activity of LFME, LLC-PK1 cells were first treated with the LFME (10, 25, 50 and 100  $\mu$ g/ml) for 24 h and cell viability was measured with an MTT assay. LFME did not showed significantly cytotoxicity in LLC-PK1 cells (cell viability >90%, data not shown). Therefore, concentrations of 10, 25, 50 and 100  $\mu$ g/ml were used for further studies. As shown in Figure 1, AAPH (1mM) induced death of the LLC-PK cells (cell viability was 44.2%). LFME significantly reduced the AAPH-induced LLC-PK1 cell damage in a concentration-dependent manner ( $P < 0.05$ ). At 100  $\mu$ g/ml,



**Figure 1:** Effects of *Lycium ruthenicum* Murr fruits methanolic extracts (LFME) on cell viability in 1 mM 2, 2'-azobis(2-amidinopropane) dihydrochloride-treated LLC-PK1 cells. Data represent mean  $\pm$  SD. <sup>a-g</sup>Mean values with different letters on the bars are significantly different ( $P < 0.05$ ) according to Duncan's multiple range test. EGCG: Epigallocatechin gallate

LFME had showed the highest cell viability (81.61%) and it weaker than that in 10  $\mu$ g/ml EGCG-treated cells (cell viability was 94.5%).

### Total intercellular ROS concentration

As shown in Figure 2, AAPH significantly increased the total intercellular ROS levels (to 250.9%) compared to that in normal cells. In the presence of AAPH, LFME significantly reduced ROS generation in a concentration-dependent manner ( $P < 0.05$ ). Following treatment with 10  $\mu$ g/ml of EGCG completely prevented the increased ROS levels induced by AAPH compared with that in 100  $\mu$ g/ml LFME treated cells. These results suggest that LFME is an effective ROS scavenger.

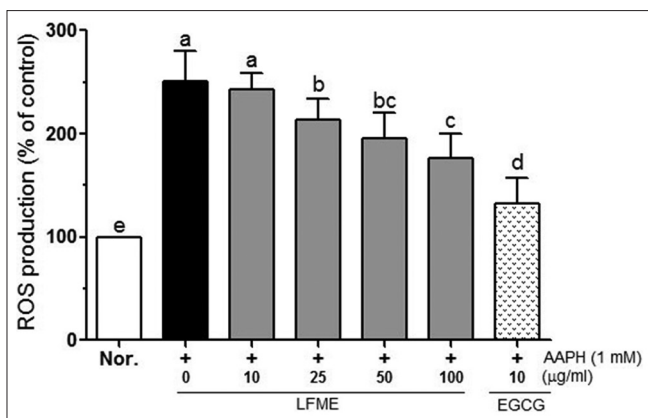
### Lipid peroxidation levels

As shown in Figure 3, AAPH significantly increased the MDA generation (to 1.49 nmol/mg protein) compared to the untreated cells (0.35 nmol/mg protein). However, treatment with LFME reduced MDA generation in a concentration-dependent manner. Cells treated with 10  $\mu$ g/ml EGCG exhibited significantly decreased MDA levels (0.62 nmol/mg protein) than that in 100  $\mu$ g/ml LFME-treated LLC-PK1 cells (0.70 nmol/mg protein).

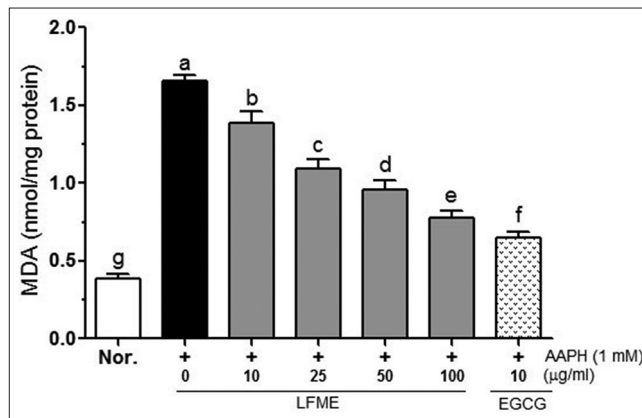
### Intercellular GSH levels

As shown in Figure 4, the intercellular levels of GSH were significantly ( $P < 0.05$ ) decreased by 1 mM AAPH. Following treatment with different concentrations of LFME (10, 25, 50 and 100  $\mu$ g/ml) was able to reduce the AAPH-induced decreasing of GSH in LLC-PK1 cells. 10  $\mu$ g/ml EGCG (2.13  $\mu$ mol/mg protein) showed a better activity to increase the GSH levels than that found in 100  $\mu$ g/ml LFME-treated cells (1.98  $\mu$ mol/mg protein).

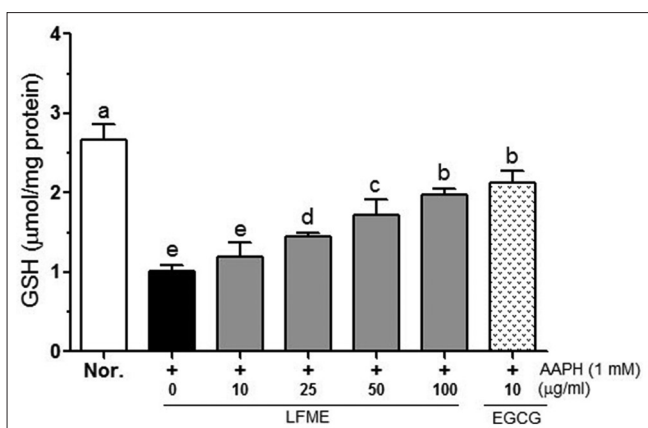




**Figure 2:** Effects of *Lycium ruthenicum* Murr fruits methanolic extracts (LFME) on reactive oxygen species in 1 mM 2, 2'-azobis (2-amidinopropane) dihydrochloride-treated LLC-PK1 cells. Data represent mean  $\pm$  SD. <sup>a-e</sup>Mean values with different letters on the bars are significantly different ( $P < 0.05$ ) according to Duncan's multiple range test. EGCG: Epigallocatechin gallate



**Figure 3:** Effects of *Lycium ruthenicum* Murr fruits methanolic extracts on malondialdehyde in 1 mM 2, 2'-azobis (2-amidinopropane) dihydrochloride-treated LLC-PK1 cells. Data represent mean  $\pm$  SD. <sup>a-g</sup>Mean values with different letters on the bars are significantly different ( $P < 0.05$ ) according to Duncan's multiple range test. EGCG: Epigallocatechin gallate



**Figure 4:** Effects of *Lycium ruthenicum* Murr fruits methanolic extracts on glutathione in 1 mM 2,2'-azobis(2-amidinopropane) dihydrochloride-treated LLC-PK1 cells. Data represent mean  $\pm$  SD. <sup>a-e</sup>Mean values with different letters on the bars are significantly different ( $P < 0.05$ ) according to Duncan's multiple range test. EGCG: Epigallocatechin gallate

**Table 1: Effect of *Lycium ruthenicum* Murr fruits methanolic extracts on the levels of catalase, superoxide dismutase and glutathione peroxidase in 2,2'-azobis (2-amidinopropane) dihydrochloride-treated LLC-PK1 cells**

Groups	U/mg protein		
	CAT	SOD	GSH-px
Normal	1.36 $\pm$ 0.07 <sup>a*</sup>	6.53 $\pm$ 0.26 <sup>a</sup>	1.71 $\pm$ 0.16 <sup>a</sup>
AAPH (1mM)	0.61 $\pm$ 0.05 <sup>e</sup>	3.03 $\pm$ 0.24 <sup>f</sup>	0.71 $\pm$ 0.13 <sup>d</sup>
AAPH+EGCG (10 µg/ml)	1.17 $\pm$ 0.04 <sup>b</sup>	5.63 $\pm$ 0.34 <sup>b</sup>	1.67 $\pm$ 0.13 <sup>a</sup>
AAPH+LFME (10 µg/ml)	0.66 $\pm$ 0.07 <sup>de</sup>	3.21 $\pm$ 0.26 <sup>f</sup>	0.84 $\pm$ 0.07 <sup>cd</sup>
AAPH+LFME (25 µg/ml)	0.75 $\pm$ 0.07 <sup>d</sup>	3.80 $\pm$ 0.10 <sup>e</sup>	1.09 $\pm$ 0.09 <sup>bc</sup>
AAPH+LFME (50 µg/ml)	0.91 $\pm$ 0.05 <sup>c</sup>	4.42 $\pm$ 0.26 <sup>d</sup>	1.24 $\pm$ 0.21 <sup>b</sup>
AAPH+LFME (100 µg/ml)	1.07 $\pm$ 0.04 <sup>b</sup>	5.03 $\pm$ 0.31 <sup>b</sup>	1.51 $\pm$ 0.12 <sup>b</sup>

\*Data represent mean $\pm$ SD. <sup>a-f</sup> Means with the different letters are significantly different ( $P < 0.05$ ) by Duncan's multiple range tests. CAT: Catalase; SOD: Superoxide dismutase; GSH-px: Glutathione peroxidase; EGCG: Epigallocatechin gallate; LFME: Lycium ruthenicum Murr fruits methanolic extracts; AAPH: 2,2'-azobis (2-amidinopropane) dihydrochloride

### Antioxidant enzyme activities

The effect of LFME on antioxidant enzyme activities in AAPH-treated LLC-PK1 cells are shown in Table 1. AAPH (1 mM) significantly ( $P < 0.05$ ) decreased CAT activity (to 0.61 U/mg protein) in LLC-PK1 cells compared to untreated cells (1.36 U/mg protein). Co-incubated with different concentrations of LFME effectively increased the CAT activity from 0.66 to 1.07 U/mg protein in LLC-PK1 cells treated with AAPH. In addition, 10 µg/ml EGCG markedly increased the CAT activity (1.17 U/mg protein) than that in 100 µg/ml LFME-treated cells. SOD activity in the LLC-PK1 cells treated with 1mM AAPH was also significantly ( $P < 0.05$ ) decreased (3.03 U/mg protein) compared to the untreated cells (6.53 U/mg protein). Co-incubated with the different concentrations of LFME for 24 h, the SOD activity was increased from 3.21 to 5.03 U/mg proteins in AAPH-treated LLC-PK1 cells. At

a concentration of 100 µg/ml, LFME helped restore SOD activity (5.03 U/mg protein), but its activity was weaker than that in cells treated with 10 µg/ml EGCG (5.63 U/mg protein). GSH-px activity was significantly reduced by 1 mM AAPH (0.71 U/mg protein) compared to the untreated LLC-PK1 cells (1.71 U/mg protein). Treatment with LFME was concentration-dependently increased the GSH-px activity in AAPH-treated LLC-PK1 cells. 10 µg/ml EGCG was effectively increased the GSH-px (1.67U/mg protein) than that in cells treated with 100 µg/ml LFME (1.51 U/mg protein).

### DISCUSSION

The fruits of *L. ruthenicum* Murr are not only as a health food but also as a folk medicine used for treatment of

heart disease, abnormal menstruation and menopause in Tibetan, China. The fruits of *L. ruthenicum* Murr are rich in vitamin C,  $\gamma$ -vitamin E,  $\delta$ -vitamin E and antocyanins.<sup>[11,12]</sup> Cheng *et al.*,<sup>[12]</sup> also reported the fruits of *L. ruthenicum* Murr contained high levels of poly-unsaturated fatty acids (PUFAs) including linoleic acid and oleic acid. However, whether *L. ruthenicum* Murr fruits methanolic extracts (LFME) protect LLC-PK1 porcine renal tubules cells from AAPH-induced oxidative damage has not been investigated. Therefore, the present study was to investigate the potential protective effect of LFME in AAPH-stimulated LLC-PK1 cells.

Many studies have suggested that ROS-induced oxidative stress plays an important role in the pathogenesis of chronic kidney disease.<sup>[1,6,7]</sup> AAPH is a well-known ROS donor, which generates peroxy radicals and molecular oxygen, eventually causing the oxidation of some biomacromolecules (such as lipid, protein and DNA) in living organs.<sup>[17]</sup> In the present study, incubation with 24 h of AAPH (1 mM) significantly induced the oxidative damage and also increasing the total intercellular ROS generations and lipid peroxidation levels (MDA productions) in LLC-PK1 cells. However, co-incubated with different concentrations (10, 25, 50 and 100  $\mu\text{g}/\text{ml}$ ) of LFME significantly reduced the AAPH-induced oxidative damage in LLC-PK1 cells [Figure 1].

Attenuation of the intercellular ROS levels by treatment with some antioxidants such as vitamin E, omega-3 PUFAs, N-acetyl cysteine (NAC), allopurinol and coenzyme Q10 are able to prevent and control the oxidative stress-induced renal diseases.<sup>[1]</sup> In order to evaluate the role of the ROS in the protective activity of LFME, the effect on AAPH-induced ROS generations was analyzed using a H2DCF-DA assay. We observed LFME concentration-dependently reduced the total intercellular ROS levels in LLC-PK1 cells treated with AAPH [Figure 2]. Furthermore, treatment with EGCG, epigallocatechin (EGC), curcumin and *Brassica rapa* root extracts are able to reduce the ROS generation to protect oxidative stress-induced cell damage in LLC-PK1 cells.<sup>[18-21]</sup> Our results suggested that the protective effect of LFME may associate with its great ROS scavenger activity.

3, 4-methylenedioxyamphetamine (MDA), a stable end product of the reaction between free radicals and cellular PUFAs, is an appropriate factor for evaluating the degree of ROS-induced cell damage.<sup>[22]</sup> Increasing of MDA levels has been reported frequently associated with the oxidative stress-induced renal cell damage in CKD such as end-stage renal failure.<sup>[23-25]</sup> Previous studies have demonstrated that AAPH is closely linked with increased peroxidation of lipid in cell membrane not only in renal cells<sup>[17]</sup> but also in other

cells including rat basophile leukemia cells,<sup>[26]</sup> and human cell lines including red blood cells,<sup>[27]</sup> intestinal Caco-2 cells<sup>[28]</sup> and liver cells.<sup>[29]</sup> We found co-incubated with LFME concentration-dependently reduced the AAPH-induced MDA production in LLC-PK1 cells [Figure 3]. It is noticed that 10  $\mu\text{g}/\text{ml}$  EGCG showed a better activity to inhibit MDA generation than that in 100  $\mu\text{g}/\text{ml}$  LFME-treated LLC-PK1 cells. Inhibition of MDA generation is able to attenuate AAPH-induced oxidative damage in LLC-PK1 cells.<sup>[17]</sup> These results indicate that LFME reduced ROS-induced lipid peroxidation in AAPH-treated LLC-PK1 cells.

Kidney is an important organ to serve homeostatic functions such as maintaining acid-base balance, regulation of electrolytes and regulation of blood pressure. Under normal physiological conditions, the kidney is easy to generate ROS including  $\text{O}_2^-$ ,  $\text{H}_2\text{O}_2$ , ONOO $^-$  and  $\cdot\text{OH}$ .<sup>[8]</sup> The accumulated ROS is able to eliminate by endogenous antioxidant enzymes such as CAT, SOD, GSH-px and GST and non-enzymatic low molecular weight antioxidants such as GSH, vitamin C and vitamin E.<sup>[8]</sup> GSH is a main endogenous non-enzyme antioxidant in renal proximal tubules.<sup>[30]</sup> The depletion of intercellular GSH may increase MDA generations and decreased the activities of endogenous antioxidant enzymes such as SOD, CAT, GSH reductase (GR) and GSH-px.<sup>[31,32]</sup> Maintaining the normal levels of GSH is able to restore cellular antioxidant defense system, block lipid peroxidation and protect renal cells to against nephrotoxic agents (such as cisplatin,  $\text{H}_2\text{O}_2$ )-induced oxidative stress.<sup>[21,33]</sup> In the present study, we found co-incubated with the different concentrations of LFME significantly increased the GSH levels in LLC-PK1 cells treated with AAPH [Figure 4]. The normal levels of intercellular GSH is useful to enhance the activity of protective enzymes NAD (P) H: Quinone oxidoreductase 1 (NQO1) and  $\gamma$ -glutamyl cysteine ligase ( $\gamma\text{GCL}$ ) and maintaining the normal function of mitochondria to protect LLC-PK1 cells against ROS-induced oxidative damage.<sup>[34,35]</sup>

In addition, we also found LFME attenuated the decrease in the activity of endogenous antioxidant enzymes CAT, SOD and GSH-px in LLC-PK1 cell exposed to AAPH for 24 h [Table 1]. SOD catalyzes the conversion of  $\text{O}_2^-$  into  $\text{H}_2\text{O}_2$ ; the  $\text{H}_2\text{O}_2$  is further reduced to  $\text{H}_2\text{O}$  by CAT and GSH-px.<sup>[36]</sup> Over expression of SOD was able to protect human embryonic kidney 293 (HEK 293) cells against ROS-induced oxidative damage.<sup>[37]</sup> Treatment with CAT and SOD helps prevent ROS-induced damage in LLC-PK1 cells.<sup>[38,39]</sup> Treatment with some nature plant extracts is able to increase the activity of antioxidant enzymes such as SOD, CAT, GSH-px and GR to against ROS-induced oxidative damage in LLC-PK1 cells and in rats, respectively.<sup>[21,40]</sup>

In conclusions, the results from the present study clearly demonstrated that LFME could protect LLC-PK1 cells against AAPH-induced oxidative damage by modulating ROS generation, MDA production and GSH levels, as well as up-regulated endogenous antioxidant enzyme activities.

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