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Gallotannin suppresses calcium oxalate crystal binding and oxalate-induced oxidative stress in renal epithelial cells

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Abstract

Calcium oxalate monohydrate (COM) crystals bind avidly to the surface of proliferating and migrating renal endothelial cells, perhaps a key event in kidney stone formation. Oxalate-induced pre-oxidative stress can further promote crystal attachment cells. Natural products including gallotannins found in green teas have been studied as potentially novel treatments to prevent crystal retention and kidney stone formation. Gallotannin significantly inhibited COM crystal growth and binding to MDCK I renal epithelial cells at non-toxic concentrations and also delayed renal cell migration in a wound healing assay. Reverse transcription polymerase chain reaction (RT-PCR) analysis revealed that gallotannin significantly attenuated oxalate-induced mRNA expression of monocyte chemoattractant protein 1 (MCP-1), osteopontin (OPN), nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunit $p22^{phox}$ and $p47^{phox}$ in human primary renal epithelial cells (HRCs). Gallotannin also reduced HRC production of reactive oxygen species (ROS) and malondialdehyde (MDA) as well as enhanced antioxidant enzyme superoxide dismutase (SOD) activity in response to oxalate. Taken together, our findings suggest that gallotannin can contribute to nephrolithiasis prevention via direct effects on renal epithelial cells including suppression of COM binding and MCP-1 and OPN expression, along with augmenting antioxidant activity.

Keywords

gallotannin; renal epithelial cells; calcium oxalate monohydrate; MCP-1; osteopontin; ROS; SOD

INTRODUCTION

Nephrolithiasis or kidney stones are a common, painful condition caused by precipitation and retention of poorly soluble salts in the kidney. Calcium oxalate (CaOx) kidney stones are the most common type, accounting for up to 70%. Adhesion of newly formed CaOx monohydrate (COM) crystals to the apical surface of renal tubular epithelial cells could be an important initiating event, at least in the subset with more marked hyperoxaluria as a contributing factor (i.e., primary hyperoxaluria or enteric hyperoxaluria). Interaction of renal

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epithelial cells with COM crystals has been shown to stimulate expression of monocyte chemoattractant protein-1 (MCP-1) and osteopontin (OPN)[1] as well as increase generation of reactive oxygen species (ROS) from nicotinamide adenine dinucleotide phosphate (NADPH) as a second messenger system[2].

Recently, several studies have reported that natural products can prevent renal calcification in animal models of nephrolithiasis, in part by preventing hyperoxaluira-induced oxidative stress. Powdered green tea leaves significantly decreased CaOx deposit formation and increased superoxide dismutase (SOD) activity in an ethylene glycol (EG)-induced hyperoxaluric rat model[3] while *Bergenia ligulata* also ameliorated free radical formation and lipid peroxidation[4]. We recently reported that 1,2,3,4,6-penta-o-galloyl-beta-D-glucose (PGG) from gallnut of *Rhus chinensis* MILL reduced renal crystallization and oxidative renal injury in a hyperoxaluric rat model[5]. Gallotannin, polyphenolic hydrolysable tannin found in green tea, was also previously demonstrated to effectively block renal calcification in an ethylene glycol rat model. In the current study we investigated the effect of this natural product on the interaction of renal cells, oxalate, and COM crystals.

MATERIALS AND METHODS

Chemicals

Gallotannin (molecular weight = 1701.2, Fig. 1A) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Sodium oxalate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and heparin were purchase from Sigma Chemicals (St. Louis, MO).

Cell culture

Human primary renal epithelial cells (HRCs)[6] were maintained in Dulbecco's modified Eagle's medium (DMEM) (Welgene, Deagu, Korea) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 μg/ml). Madin-Darby Canine Kidney Cells type I (MDCK I), derived from the distal nephron, were kindly provided by Dr. John C Lieske at Mayo Clinic. The cells were maintained in DMEM containing 25 mM glucose supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml).

Cytotoxicity assay

The cytotoxicity of gallotannin was measured by MTT colorimetric assay. HRCs or MDCK I cells were seeded onto 96-well microplates at a density of 1×10^4 cells per well and treated with various concentrations of gallotannin for 24 h. MTT working solution (5 mg/ml in PBS) was added to each well and incubated at 37°C for 3 h. The optical density (OD) was then measured at 570 nm using a microplate reader (Sunrise, TECAN, Männedorf, Switzerland). Cell viability was calculated as a percentage of viable cells in gallotannin-treated group *versus* untreated control by the following equation.

$$\text{Cell viability}\ (\%) = [\text{OD}\ (\text{GAllotannin}) - \text{OD}\ (\text{Blank})] / [\text{OD}(\text{Control}) - \text{OD}\ (\text{Blank})] \times 100.$$

Crystal growth inhibition assay

Crystal growth inhibition (CGI) was assayed using the method of Asplin and colleagues[7]. COM seed crystal slurries (0.8 mg/ml.) were prepared by adding 32 mg of crystals to 40 ml of 90 mM NaCl, 10 mM Tris buffer, pH 7.2. The slurry was stirred for 48 h at approximately 1,500 rpm. Between 80 and 150 μl of seed slurry (amount varied daily to give consistent growth signal) was added to 2 ml of calcium oxalate solution consisting of 0.5 mM calcium, 0.5 mM oxalate, 90 mM NaCl and 10 mM Tris (pH 7.2) in a quartz cuvette at 37°C and magnetically stirred at 1,100 rpm. Oxalate consumption was initiated by the

addition of seed crystals and monitored for 420 seconds with a Varian UV-visible, continuously recording spectrophotometer (Varian Cary 3E UC-Visible spectrophotometer, Melbourne, Australia) at 214 nm. Data for analysis were ported from the spectrophotometer to a personal computer using data capture software (Cary Win UV (version 3.00) 2002 Varian Australia Pty Ltd, Melbourne, Australia). Buffer-exchanged totally urinary protein (20 μg) was added to experimental samples and an equal volume of buffer to the controls. The rate of oxalate consumption in a seeded crystal growth system followed second order kinetics[8]. Oxalate consumption was fitted to a nonlinear function (Eq 1):

$$Ct=(C_i-C_\infty)\,at/(1+at),$$

where C_t is the measured cuvette optical density at 214 nm, C_i is the initial and C_∞ the final stable reading after 4 h of incubation, a is a rate constant and t is time of incubation. The function was fitted to the data for C_t and allowed to calculate best fit for C_i and a. C_∞ was measured experimentally as 0.16 in the system and given as a constant. The rate constant a was calculated for the buffer-exchanged urine protein and concomitant controls, and growth rate (G) in the presence of urine proteins was expressed as a percentage of control growth (Eq 2):

$$G=(a_e/a_c)\times 100,$$

where e and c refer to the experimental and control cuvette results, respectively. To obtain the experimental rate constant a_e we used the average of the 2 experimental cuvette values. The units were a measure of residual growth after urine inhibition. Each sample and control was run in duplicates.

Calcium oxalate monohydrate (COM) crystal binding assay

The COM crystal binding assay was performed as previously described[9,10]. MDCK I cells were grown to confluency in 35-mm tissue culture dishes and serum-starved for 24 h. [^{14}C] COM crystals were incubated with gallotannin to pre-coat crystals in microcentrifuge tubes subjected to end-over-end rotation for 15 min at room temperature, and then washed 3 times with PBS. To assess the capacity to bind crystals, a [^{14}C] COM crystal suspension (8 μg/ml, 50 μl) was distributed homogeneously across the monolayer, where it settled on top of the cells under the force of gravity (41.6 μg/cm^2 of cells) for 2 min and rinsed three times with phosphate buffered saline (PBS). The monolayer was scraped and directly added into a 5.0-ml scintillation vial filled with 3.0 ml of scintillation cocktail (Emulsifier Safe Scintillation mixture; PerkinElmer). Radioactivity of adherent crystals was counted using a beta scintillation counter (Beckman Coulter, Fullerton, CA)[10]

Total RNA isolation and Reverse Transcription –Polymerase Chain Reaction (RT-PCR) analysis

Total RNA was prepared by using Trizol reagent according to the manufacturer's instructions. Total RNA (1.0 μg) was reverse transcribed using MMLV reverse transcriptase (Promega, Madison, WI) by incubation at 25°C for 10 min, at 42°C for 60 min and at 99C for 5 min. The synthesized cDNA was amplified using TaKaRa Taq DNA polymerase (TaKaRa Biotechnology, Shiga, Japan) and the following specific primers: *p22phox* (sense 5′-GTTTGTTTTGTGCCTGCTGGAGT-3′; antisense 5′-TGGGCGGCTGCTTGATGGT-3′), *p47phox* (sense 5′-ACCCAGCCAGCACTATGTGT-3′; antisense 5′-AGTAGCCTGTGACGTCGTCT-3′), *MCP-1* (sense 5′-GCTCGCTCAGCCAGATGCAAT-3′, antisense 5′-

TGGGTTGTGGAGTGAGTGTTC-3′, *OPN* (sense 5′-TGAGTCTGGAAATAACTAATGTGTTTGA-3′, antisense 5′-GAACATAGACATAACCCTGAAGCTTTT-3′, and *GAPDH* (sense 5′-GTGGATATTGTTGCCATCA-3′, antisense 5′-ACTCATACAGCACCTCAG-3′. PCR conditions were 30 cycles of 94°C for 30 sec, 59°C for 30 sec and 72°C for 30 sec, followed by 5 min incubation at 72°C. PCR products were run on 2% agarose gel and then stained with ethidium bromide (EtBr).

Western Blot Analysis

Cells were lysed in radio immunoprecipitation assay (RIPA) buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholic acid, 1 M ethylene diaminetetraacetic acid (EDTA), 1 mM Na3VO4, 1 mM NaF and protease inhibitors cocktail). Protein samples were quantified by using a Bio-Rad DC protein assay kit II (Bio-Rad, Hercules, CA, U.S.A.), separated by electrophoresis on 8 to 15% sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE) gel and electrotransferred onto a Hybond ECL transfer membrane (Amersham Pharmacia, Piscataway, NJ, U.S.A.). After blocking with 5% nonfat skim milk, the membrane was probed with antibodies for MCP-1, OPN, p22phox, p47phox (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), and β-actin (Sigma Aldrich Co., St. Louis, MO, U.S.A.) followed by exposing to horseradish peroxidase (HRP)-conjugated secondary anti-mouse or rabbit antibodies (AbD serotec, kidlington, U.K.). Protein expression was determined by using enhanced chemiluminescence (ECL) system (Amersham Pharmacia, Piscataway, NJ, U.S.A.).

Measurement of reactive oxygen species (ROS) production

ROS level was measured using 2,7-dichlorofluorescein diacetate (DCFDA) fluorescence dye. Cells were incubated with 1 μM DCFDA at 37°C for 30 min. Fluorescence intensities were measured by BD FACSCalibur flow cytometry (Becton Dickinson, Franklin Lakes, NJ).

Superoxide dismutase (SOD) and malondialdehyde (MDA) assays

SOD enzyme activity and MDA content were measured by using Bioxytech SOD-525 and MDA-586 Assay kits (OXIS International, Portland, OR), respectively.

Statistical analyses

All data were expressed as means ± SD. The statistically significant differences between control and gallotannin-treated cells were calculated by analysis of variance (ANOVA) test followed by a post hoc analysis (Tukey's or Dunnett's multiple comparison test) using Prism software 5 (GraphPad Software, Inc., San Diego, CA, U.S.A.).

RESULTS

Cytotoxicity of gallotannin in MDCK I cells and HRCs

To evaluate the cytotoxic effect of gallotannin on MDCK I cells and HRCs, an MTT assay was conducted. The cells were treated with increasing concentrations of gallotannin (0, 6.25, 12.5, 25, 50 or 100 μM) for 24 h. As shown in Fig. 1, gallotannin exerted weak cytotoxicity against MDCKs to 60% of untreated control at the concentration of ca. 12.5 μM, while it did not show any cytotoxicity against HRCs at the concentrations of <60 μM, indicating that MDCKs originated from mouse distal nephron were more susceptible to gallotannin compared to HRCs originated from human nephron. We also think the susceptibility of two cell lines can be different by origin, type and gene specificity.

Effects of gallotannin on COM crystal binding in MDCK I cells

To examine whether gallotannin can prevent the growth of CaOx crystals, a crystal growth inhibition (CGI) assay was performed. Gallotannin significantly increased the inhibitory activity to 0.05, 0.24 and 0.48 at the concentrations of 0.1, 1.0 and 10 μM, respectively (Fig. 2A) while the positive control molecule heparin showed an inhibitory activity of 0.47 at 10.29 U/ml. Furthermore, gallotannin significantly reduced the binding of pre-coated calcium oxalate monohydrate (COM) crystals to MDCK I cells in a dose-dependent manner (Fig. 2B). However, gallotannin-pretreatment of cells for 15 min or 24 h had no significant effect on the crystal binding (data not shown).

Effect of gallotannin on MCP-1 and osteopontin expression in HRCs

COM crystals were previously shown to promote the renal epithelial cell production of monocyte chemoattractant protein 1 (MCP-1) and osteopontin (OPN) [11,12]. Therefore we analyzed MCP and OPN mRNA expression in oxalate-treated human primary renal epithelial cells (HRCs) with or without gallotannin. As expected, the mRNA levels of MCP-1 and OPN were clearly increased in oxalate-treated cells (lane 2) compared with untreated control (lane 1) (Fig. 4). In contrast, gallotannin treatment remarkably suppressed oxalate-mediated expression of MCP-1 and OPN in a dose-dependent manner (Fig. 4, lanes 3 and 4). Nicotinamide adenine dinucleotiide phosphate (NADPH) oxidase has been proposed as a second messenger that signals expression of OPN and MCP-1[2]. In our study, gallotannin significantly reduced the expression of $p22^{phox}$ and $p47^{phox}$, major subunits of NADPH oxidase, in oxalate-treated HRCs (Figs. 3A, B). Consistently, gallotannin decreased oxalate-mediated overexpression of MCP-1, OPN, p22phox and p47phox at protein level by Western blotting (Fig. 3C). However, oxalate did not exert significant cytotoxicity against HRCs (data not shown), despite significant regulation of NADPH oxidase system. Also, regarding some gene expression less than basal level at mRNA level, we have to confirm the results in different cell lines and experimental design in the future.

Anti-oxidant activity of gallotannin in oxalate-treated HRCs

NADPH oxidase is an important source of reactive oxygen species (ROS) in renal diseases[13]. To assess the effect of gallotannin on oxalate-induced oxidative stress, HRCs were exposed to oxalate in the absence or presence of gallotannin for 24 h. As shown in Fig. 4A, ROS levels were significantly raised in the presence of oxalate. In contrast, gallotannin significantly reduced the production of ROS from 9.97% to 6.16 and 4.73% at 40 and 80 μM, respectively (Fig. 4A). Activity of the antioxidative enzyme superoxide dismutase (SOD) was significantly increased in oxalate/gallotannin-treated cells compared with oxalate control (Fig. 4B). In contrast, malondialdehyde (MDA) levels significantly declined after gallotannin treatment in a dose-dependent manner (Fig. 4C).

DISCUSSION

The incidence of nephrolithiasis has been increasing in the US over the past three decades[14]. Nephrolithiasis has multifactorial causes, including contribution by diet and genetics. One important result is supersaturation of the urine with salts that produce kidney stones, including CaOx. CaOx in turn can induce oxidative renal cell injury[15]. Recently, phytotherapeutic agents were proposed as useful alternative or a complementary therapies for the management of urolithiasis, in part due to anti-oxidative effects[16]. Gallotannins, hydrolysable tannins with polyesters of a sugar moiety (or other non-aromatic polyhydroxy compounds) and organic acids are chiefly contained in cheakpeas, cowpeas, mangos, persimmons, star fruit (*Averrhoa carambola* L), pecans and rhubarbs[17]. Despite their multi-biological activities including anticancer, anti-inflammatory and hepatoprotective effects,

the potential anti-nephrolithic activities of gallotannin (MW=1701.2) have not been investigated until now.

COM crystals bind avidly to the surface of renal endothelial cells, and oxalate-induced pre-oxidative stress can promote crystal attachment to renal epithelial cells[18,19]. Massive accumulation of COM crystals in kidney tissues can produce renal tubular necrosis that leads to kidney failure[20,21]. In the current study, the potential for gallotannins to protect renal cells against CaOx and/or oxalate was assessed. Gallotannin significantly inhibited COM crystal growth and binding to MDCK I renal epithelial cells at non-toxic concentrations *in vitro*, implying a potential for gallotannin to exert positive effects *in vivo* to protect against stone formation.

There is previous evidence to suggest that increased expression of MCP-1 and OPN play an important role in kidney stone formation[2,22–24]). In this study, RT-PCR analysis confirmed that gallotannin attenuated MCP-1 and OPN mRNA expression in oxalate-treated HRCs. In general renal cell injury, perhaps secondary to oxalate-induced free radical generation, appears to favor CaOx crystal retention, [25–28]. In our study, gallotannin significantly attenuated the mRNA expression of NADPH oxidase subunit $p22^{phox}$ and $p47^{phox}$ in oxalate-treated HRCs. Gallotannin consistently reduced the production of ROS and MDA and also well as enhanced activity of the antioxidant enzyme SOD in oxalate-treated HRCs. These antioxidant effects of gallotannin should block pathways that could lead to crystal retention in the kidney.

In summary, gallotannin inhibited COM crystal growth and adhesion to renal epithelial cells, proliferation and migration of renal epithelial cells in a wound healing assay, and attenuated mRNA expression of MCP-1, OPN, NADPH oxidase subunit $p22^{phox}$ and $p47^{phox}$ after oxalate treatment in HRCs. Furthermore, gallotannin reduced oxalate-induced ROS and MDA generation while enhancing antioxidant enzyme activity. Overall, our findings suggest that gallotannin is a candidate for further study as a natural product that could prevent kidney stones by suppressing adhesion and retention of COM crystals within nephrons. However, additional *in vivo* studies of gallotannin are required to confirm these *in vitro* results.

Acknowledgments

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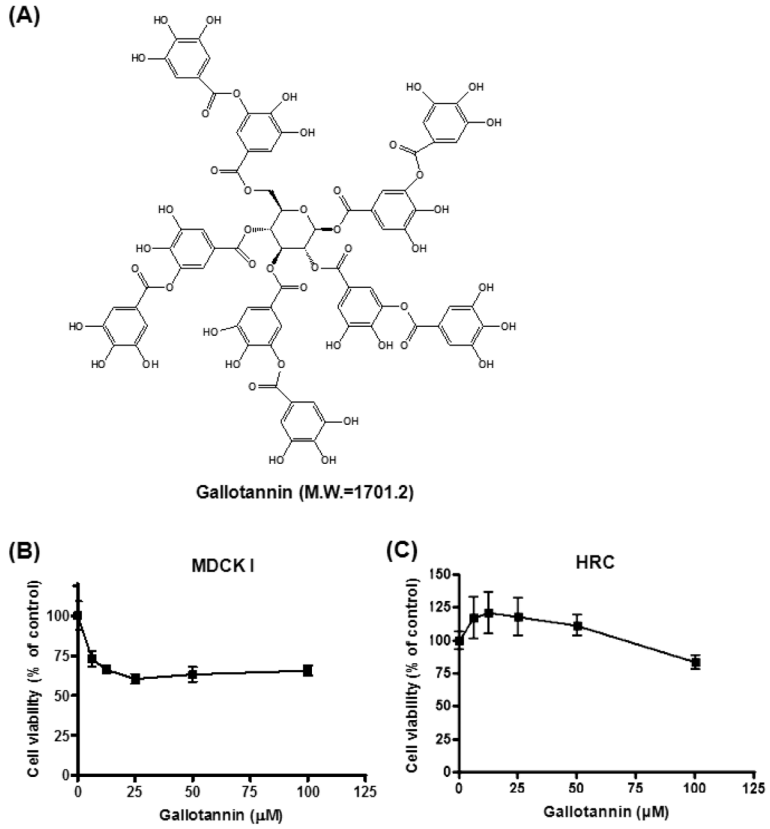

Fig. 1. Cytotoxic effect of gallotannin in MDCK I cells

(A) Chemical structure of gallotannin. Molecular weight = 1701.2. (B and C) Cytotoxicity of gallotannin was evaluated in renal epithelial cells MDCK I (B) and HRCs (C) by MTT assay. Cells were plated onto 96-well microplates (1×10^4 cells/well) and treated with various concentrations of gallotannin (0, 6.25, 12.5, 25, 50 or 100 μM) for 24 h. Data were expressed as means ± SD of three independent experiments.

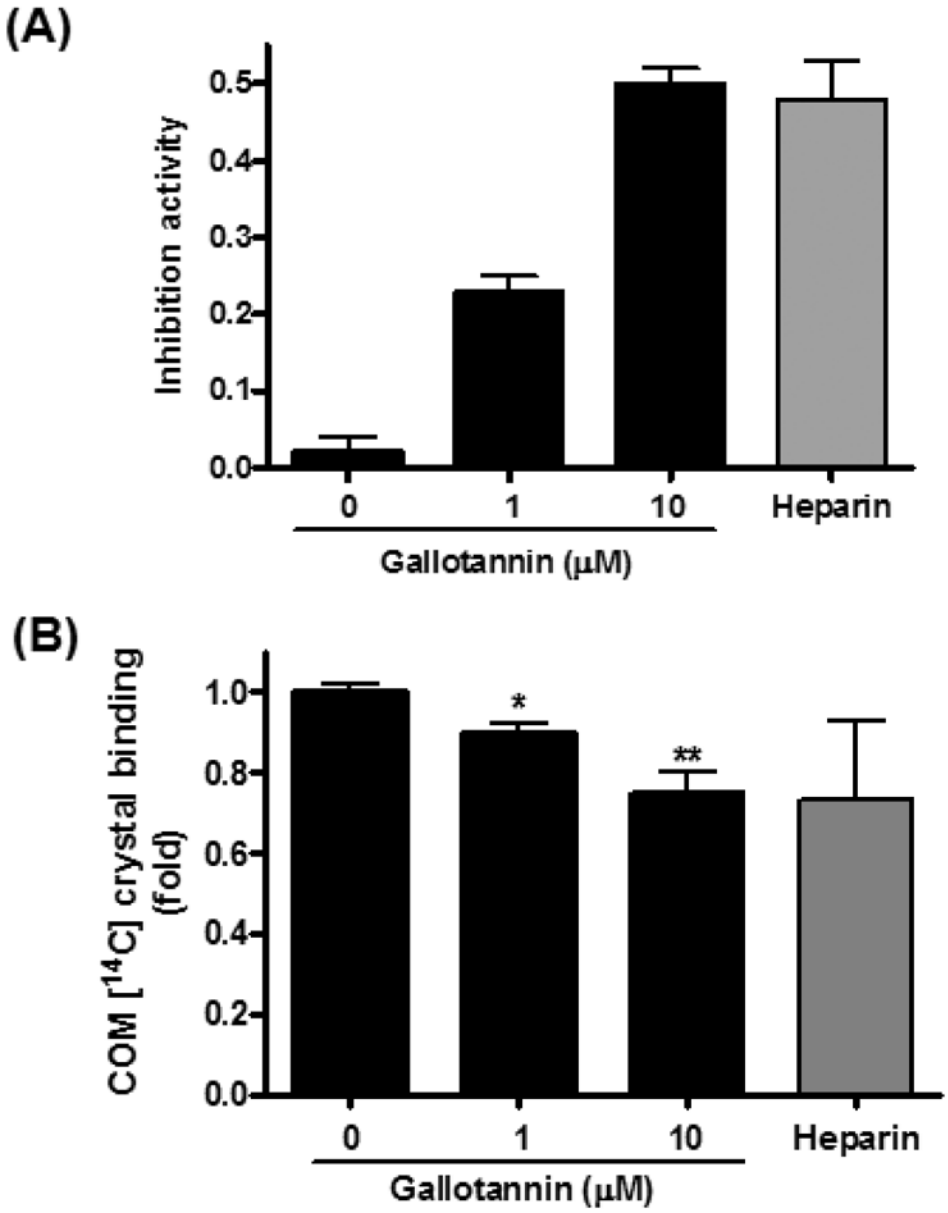

Fig. 2. Effects of gallotannin on COM crystal binding in MDCK I cells

(A) COM seed crystal slurries were incubated with gallotannin and added to calcium oxalate solution consisting of 0.5 mM calcium, 0.5 mM oxalate, 90 mM NaCl and 10 mM Tris (pH 7.2) in a quartz cuvette at 37°C and magnetically stirred at 1100 rpm. Oxalate consumption was initiated by the addition of seed crystals and monitored for 420 s with a Varian UV-Visible, continuously recording spectrophotometer (Varian Cary 3E UC-Visible spectrophotometer, Melbourne, Australia) at 214 nm. (B) [14C] COM crystals were incubated with gallotannin to pre-coat crystals and distributed homogeneously across the monolayer of MDCK I for 2 min and rinsed three times with PBS. Radioactivity of adherent crystals was counted using a beta scintillation counter (Beckman Coulter, Fullerton, CA, U.S.A.).10) Data were expressed as mean±S.D. of three independent experiments. *$p<0.05$ and **$p<0.01$ vs. untreated control, one-way analysis of variance followed by a post hoc analysis.

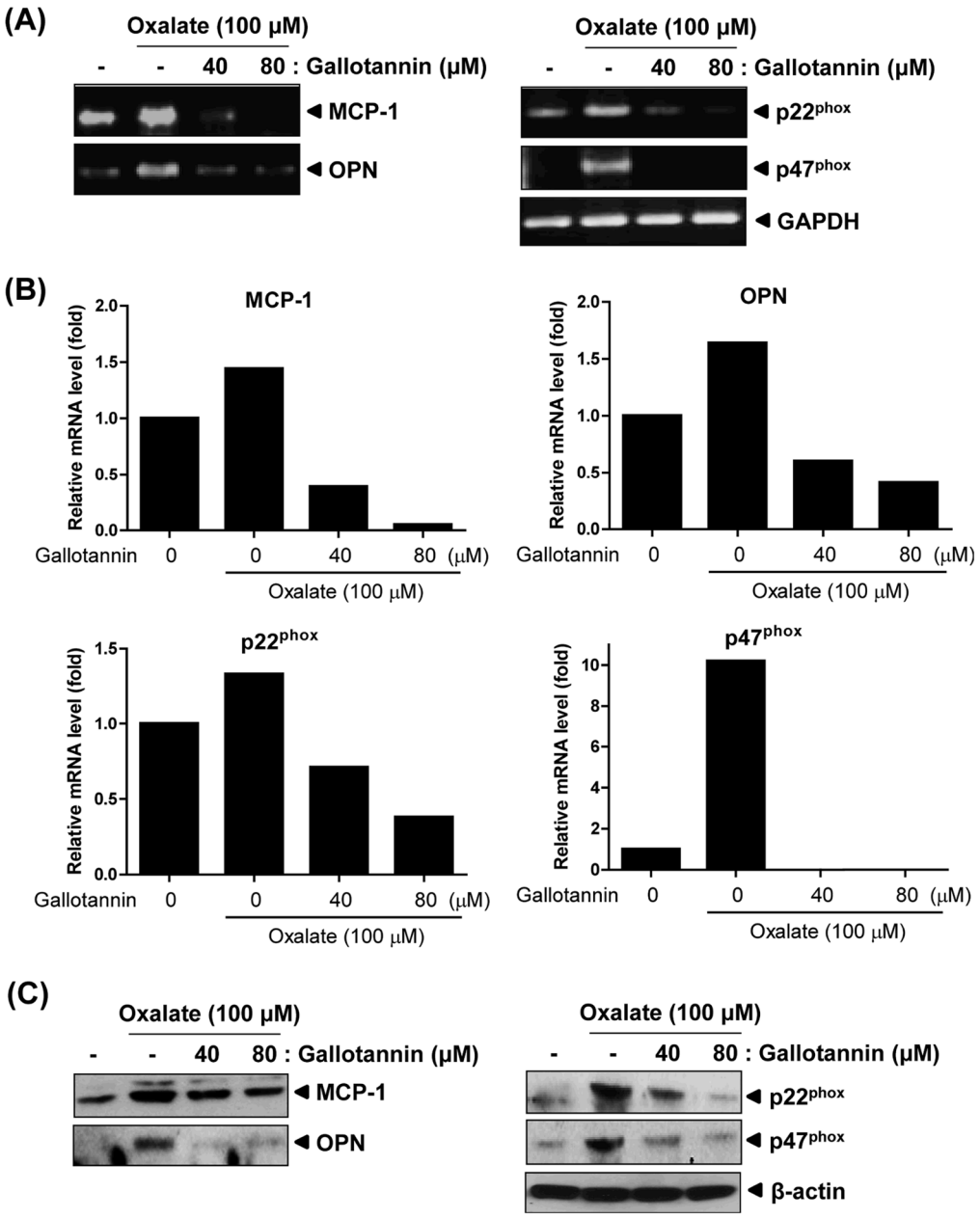

Fig. 3. Effect of gallotannin on mRNA expressions of MCP-1, OPN, p22^{phox} and p47^{phox} in oxalate-treated HRCs

Cells were exposed to oxalate (100 μM) with or without gallotannin (40 or 80 μM) for 24 h. (A) Gene expressions of MCP-1, OPN, p22^{phox}and p47^{phox} were analyzed by semi-quantitative RT-PCR. (B) Graphs represent fold changes of mRNA expression of indicated genes adjusted by *GAPDH*. (C) Cell lysates were prepared and subjected to Western blotting for MCP-1, OPN, p22phox and p47phox

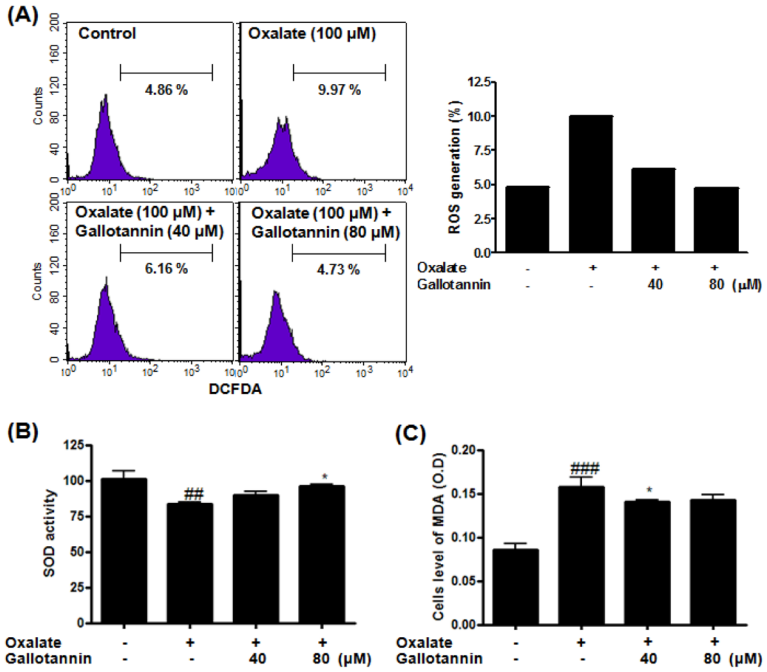

Fig. 4. Anti-oxidative effect of gallotannin in oxalate-treated HRCs

Cells were exposed to oxalate (100 μM) with or without gallotannin (40 or 80 μM) for 24 h. (A) ROS generation (%) was measured using ROS-sensitive fluorometric probe 2,7-dichlorofluorescein diacetate (DCFDA) by flow cytometric analysis. (B) SOD activity was measured by using Bioxytech SOD-525 Assay kit. (C) MDA content was measured by using Bioxytech MDA-586 Assay kit. Data were expressed as mean±S.D. of three independent experiments. ##$p<0.01$ and ###$p<0.001$ vs. untreated control *$p<0.05$ vs. oxalate control, two-way analysis of variance followed by a post hoc analysis.