Antioxidant Action of Ellagic Acid Ameliorates Paraquat-Induced A549 Cytotoxicity

Yong-Sik Kim,* Tamanna Zerin, # and Ho-Yeon Song*

Department of Microbiology, College of Medicine, Soonchunhyang University; Cheonan, Chungnam 330–090, South Korea. Received November 12, 2012; accepted January 11, 2013

Ellagic acid (EA) is a natural dietary polyphenol whose benefits in a variety of diseases shown in epidemiological and experimental studies involve anti-inflammation, anti-proliferation, anti-angiogenesis, anti-carcinogenesis and anti-oxidation properties. This study aimed to evaluate the effect of EA against paraquat (PQ)-induced oxidative stress. PQ decreased the viability of A549 cells in dose- and time-dependent manners, which was associated with the massive generation of reactive oxygen species (ROS). However, cell viability was significantly recovered by the treatment of EA, from 47.01±1.59% to 66.04±2.84%. The release of lactate dehydrogenase (LDH) was also decreased with the treatment of EA in PQ-treated A549 cells. EA induced the level of expression and activation of nuclear factor-erythroid 2-related factor (Nrf2) and its target cytoprotective and antioxidant genes, heme oxygenase-1 (HO-1) and quinone oxidoreductase 1 (NQO1). The antioxidant potential of EA might be directly correlated with the increased expression of HO-1 and NQO1, whose expression may have surmounted the oxidative stress generated by PQ. Notably, EA treatment significantly reduced the levels of biochemical markers as lipid peroxidation, reduced the intracellular ROS level, and surmounted total glutathione level in A549 cells. Data indicate that the antioxidant and cytoprotective properties of EA reduce PQ-induced cytotoxicity in human alveolar A549 cells.

Key words ellagic acid; paraquat; reactive oxygen species; nuclear factor-erythroid 2-related factor; lipid peroxidation; glutathione level

Over the last 50 years, 1,1′-dimethyl-4,4′-bipyridinium dichloride (paraquat, PQ) has become the most extensively studied, and controversial herbicide. PQ has become increasingly notorious due to its severe acute toxicity and the lack of any effective treatment. It is well established that PQ induces toxicity mainly through its metabolism and subsequent generation of reactive oxygen species (ROS) by redox cycling. Toxicity is severe in the lung due to accumulation of PQ against a concentration gradient. Ellagic acid (EA) is a natural polyphenol that is abundant as ellagitannins in raspberries, strawberries, grapes, and nuts. EA is released from ellagitannins by the gut microflora. EA possesses a wide array of biological functions including anti-inflammatory, anti-proliferation, anti-angiogenesis, anti-carcinogenesis, anti-oxidation, inhibition of lipid peroxidation, and anti-apoptosis in a number of in vitro and in vivo models. However, the beneficial effects of EA on PQ-induced oxidative damage are not elucidated yet.

Upon oxidative stress, the anti-oxidant related genes are regulated through the anti-oxidant response element (ARE), which is a cis-acting enhancer sequence found in the promoter region. Nevertheless, previous research has ascertained that nuclear factor-erythroid 2 (NF-E2)-related factor (Nrf2) is the pivotal transcription factor mediating ARE-driven induction of anti-oxidant proteins and phase-II enzymes. Many phase-II enzymes as well as other cytoprotective enzymes that are involved in detoxification and cytoprotection, such as heme oxygenase-1 (HO-1), and reduced nicotinamide adenine dinucleotide phosphate (NAD(P)H): Quinone Oxidoreductase 1 (NQO1) are regulated by the Nrf2/Kelch-like ECH-associated protein 1 (Keap1) transcription system. Keap1 can bind to Nrf2 and functions as a negative regulator of Nrf2. Upon oxidative stress, Nrf2 dissociates from Keap1, which may be achieved either by oxidation/modification of essential cysteines or by removing Zn from the cysteine residue.

In this study, we used human alveolar epithelial A549 cells to investigate the protective effect of EA on PQ-induced oxidative damage. EA-mediated protection involved reduction of lipid peroxidation, and ROS generation through modulating the expression of Nrf2 and its target genes HO-1 and NQO1. The data allow us to propose that EA can protect human alveolar epithelial A549 cells from PQ-induced oxidative damage.

MATERIALS AND METHODS

Chemicals and Antibodies Paraquat dichloride (PQ; 1,1′-dimethyl-4,4′-bipyridinium dichloride), ellagic acid (EA; 4,4′,5,5′,6,6′-hexahydroxyphenic acid 2,6,2′,6′-dilactone), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), Triton-X100, and Trypan blue stain were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.). Total glutathione detection kit, ROS detection kit, lactate dehydrogenase (LDH) assay kit, and nuclear/cytosol fraction kit were purchased from Dojindo laboratories (Kumamoto, Japan), Enzo Life Sciences (Farmingdale, NY, U.S.A.), Roche (Pleasanton, CA, U.S.A.), and Biovision (Mountain View, CA, U.S.A.), respectively. Antibodies to Nrf2 (sc-13032), HO-1 (sc-10789), and NQO1 (sc-16464) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.) and β-actin antibody (ab6276) was purchased from Abcam (Cambridge, MA, U.S.A.). Horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (IgG) (sc-2004) and anti-mouse IgG (sc-2005) were also obtained from Santa Cruz Biotechnology.

Cell Culture and Treatment Human lung carcinoma A549 cells were purchased from American Type Culture Collection (Manassas, VA, U.S.A.) and were maintained in standard Dulbecco’s Modified Eagle’s Medium (DME/M) Ham’s 1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C, 5% CO₂.
F-12 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 1% (v/v) antibiotic/antimycotic cocktail (100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B; Invitrogen, Carlsbad, CA, U.S.A.) at 37°C under saturating humidity in 5% CO₂/95% air.

**MTT Assay** MTT is the basis of a commonly used cell viability assay that is based on the conversion of MTT to formazan crystals by mitochondrial dehydrogenase. A549 cells (5×10⁵ cells/100 μL) were seeded in 96 well plates overnight and treated with specified concentrations of drugs for specified periods. The assay was performed as previously described. The visual inspection of cell viability was assessed by phase-contrast microscopy using Axiocamt-25 Microscope (Carl Zeiss, Jena, Germany).

**LDH Release Assay** A549 cells (5×10⁴ cells/100 μL) were seeded in 96 well plates overnight and then treated with specified concentrations of PQ, EA, and/or both for 72 h. The supernatant (100 μL) was transferred to a fresh 96 well plate and an equal volume of freshly prepared reaction mixture was added according to the company’s instruction (Roche, Pleasanton, CA, U.S.A.). Real-time PCR was performed in triplicate using the following protocol: 95°C for 10 s, 42°C for 5 min followed by 40 cycles of 95°C for 10 s, 42°C for 5 s, and 72°C for 20 s. The values for target gene expression were normalized to endogenous control, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and quantified relative to the expression of GAPDH in control samples. Relative quantification was performed using 2−ΔΔCT formula, where −ΔΔCT=(C_target−C_GAPDH) experimental sample−(C_target−C_GAPDH) control sample. The primer pair sequences are listed in Table 1.

**Cytoplasmic and Nuclear Protein Extraction** Cells were harvested by trypsin treatment, total RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA, U.S.A.), quantified using a ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, U.S.A.) at 260 nm. The purified total RNA was quantified using a NanoDrop spectrophotometer (NanoDrop, Wilmington, DE, U.S.A.) at 260 nm. The purified total RNA was quantified using a ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, U.S.A.) at 260 nm. The purified total RNA was used as a control. Each supernatant (100 μL) was transferred to a fresh 96 well plate and an equal volume of freshly prepared reaction mixture was added according to the company’s instruction (Roche, Pleasanton, CA, U.S.A.). The absorbance was measured at 490 nm using a Victor™ X3 multilabel reader (Perkin Elmer, Waltham, MA, U.S.A.) following 30 min incubation at room temperature in dark.

**RNA Extraction and Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)** Cells were treated with the desired concentrations of drugs for desired periods and were lysed with malondialdehyde (MDA) lysis buffer. The supernatant (200 μL) was collected by centrifugation, 600 μL of the TBA solution was added and incubated at 95°C for 1 h. The mixture was cooled to room temperature in an ice bath for 10 min. Finally, 200 μL of the mixture was placed in a 96-well microplate for colorimetric analysis. The absorbance was read at 532 nm using a Victor™ X3 multilabel reader (Perkin Elmer, Waltham, MA, U.S.A.). The concentration of MDA level was calculated using MDA as a reference standard.

**Detection of Intracellular ROS** A549 cells with a density of 1×10⁵/well were seeded in black 96-well plates overnight. Following incubation, cells were washed with 1×HBSS and then incubated with 10 μM 2,7-dichlorofluorescein diacetate (H₂DCFDA) (Invitrogen) in 1×HBSS containing 0.1 mg/mL glucose for 30 min at 37°C. Cells were washed with 1×HBSS and then treated with 200 μM H₂O₂ as a ‘+’ control, N-acetyl-L-cysteine (NAC) as ‘−’ control, 10 μM of PQ, 10 μM of EA and both for indicated time periods. Intracellular proteins were extracted using a nuclear/cytosol fractionation kit (Biovision). In brief, cytosol extraction buffer-A (CEB-A: 0.2 mL) containing dithiothreitol (DTT) and protease inhibitor was added, vortexed at full speed and incubated on ice for 10 min. Ice-cold cytosol extraction buffer-B (CEB-B: 11 μL) was added, vortexed and incubated for 1 min. The supernatant containing cytoplasmic proteins was immediately collected following centrifugation at full speed. The remaining pellet was resuspended in ice-cold nuclear extraction buffer mix, strongly vortexed, centrifuged at full speed and the nuclear protein containing supernatant was collected.

**Western Blot** All the steps were performed as previously described (Zerin et al.). In brief, proteins were separated using a 4–20% sodium dodecyl sulfate polyacrylamide gradient gel (Mini-PROTEAN® TGX™ Precast Gel; Bio-Rad) at 100 V for 1.3 h and then transferred onto a polyvinylidene fluoride membrane (Trans-Blot SD Semi-Dry Cell; Bio-Rad) at 15 V for 1 h. The membranes were blocked by incubation with 5% dried skim milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 h at room temperature. Membranes were incubated overnight at 4°C with the primary antibodies (Nrf2/HO-1/NQO1/β-actin/Lamin B) and a second incubation was carried out with horseradish peroxidase-conjugated respective anti-rabbit or anti-mouse antibody for 1.3 h at room temperature. The bound antibodies were visualized using enhanced chemiluminescence Western blotting detection reagents (Bio-Rad) and images were acquired using a ChemiDoc™ XRS+ System with Image Lab™ software (Bio-Rad).

**Lipid Peroxidation Assay** Cells were treated with the desired concentrations of drugs for desired periods and were lysed with malondialdehyde (MDA) lysis buffer. The supernatant (200 μL) was collected by centrifugation, 600 μL of the TBA solution was added and incubated at 95°C for 1 h. The mixture was cooled to room temperature in an ice bath for 10 min. Finally, 200 μL of the mixture was placed in a 96-well microplate for colorimetric analysis. The absorbance was read at 532 nm using a Victor™ X3 multilabel reader (Perkin Elmer, Waltham, MA, U.S.A.). The concentration of MDA level was calculated using MDA as a reference standard.

**Table 1. Primer Pair Sequences Used in This Study**

| Gene   | Primer sequences                          | Amplicon sizes |
|--------|------------------------------------------|----------------|
| Nrf2 (F) | 5′-GGCGACGGAAGGATGATGAC-3′               | 99 bp          |
| Nrf2 (R) | 5′-GTTGGCGATCCACTGTGTTT-3′               |                |
| HO-1 (F) | 5′-GCAACGCGACACTGC-3′                    | 245 bp         |
| HO-1 (R) | 5′-TTGGGCTCGACGCTCTTG-3′                 |                |
| NQO1 (F) | 5′-GGCAGACCTTTGTATCCAG-3′                |                |
| NQO1 (R) | 5′-CGTCTCCTTCATTCCATCCAG-3′              |                |
| GAPDH (F) | 5′-TCCATCACCATCTCCCA-3′                  | 380 bp         |
| GAPDH (R) | 5′-CATCACGCCACAGTTC-3′                    |                |
fluorescence was detected on Victor™ X3 multilabel reader (Perkin Elmer, Waltham, MA, U.S.A.) with an excitation of 485 nm and emission of 530 nm. Fluorescence values of wells without cells were subtracted from the respected values of tested samples. Fluorescent images were taken by Axiovert-25 Microscope (Carl Zeiss, Jena, Germany).

Measurement of Total Glutathione The total amount of glutathione (GSH) was evaluated following the instructions provided by the company (Dojindo Laboratories, Kumamoto, Japan). Briefly, 5×10^5 cells were collected and lysed by 10 mM HCl with repeated freezing and thawing, and then 5% 5-sulfosalicylic acid was added. The samples were centrifuged and the supernatant was collected for the quantification of total GSH. The required volume of co-enzyme, enzyme, and buffer solutions were added to the 96-well plate according to the manufacturer’s instructions. Following 5 min incubation at 37°C, GSH standard and sample solutions were added to the micro-well plate and incubated at 37°C for 10 min. The substrate solution was then added to each well and incubated for 10 min at room temperature. The absorbance was measured at a wavelength of 405 nm using a Victor™ X3 multilabel reader (Perkin Elmer).

Data Analyses At least three individual experiments were conducted for each experiment and satisfactory correlation was achieved between the results of each individual experiments. Differences between groups were analyzed using one-way analysis of variance followed by the Student’s t-test with a p-value <0.05 considered as statistically significant. Data are expressed as the mean±standard deviation.

RESULTS

Cytoprotective Effect of EA on PQ-Induced Cytotoxicity in A549 Cells Prior to investigating the cytoprotective effect of EA on PQ-induced conditions, we evaluated the cytotoxic effect of PQ and EA in A549 cells by MTT analysis. PQ showed a dose-dependent (50–200 µM) and time-dependent (12–96 h) cytotoxicity in A549 cells. We chose 100 µM concentration for further experiments, since it produced approximately 50% cell viability at 96 h (Fig. 1A). However, EA was not cytotoxic to A549 cells at concentrations up to 80 µM for 72 h (Fig. 1B). Phase contrast images showed the representative fields when cells were treated with 100 µM PQ, 10 µM EA or both for 72 h. Treatment with PQ showed a significant decrease of cells comparative to cells treated with EA or PQ/EA (Fig. 1C). In order to test the cytoprotective effect of EA against PQ-induced cytotoxic condition, A549 cells were co-treated with 100 µM PQ and 10 µM EA. Interestingly, at 96h, co-treatment improved the viability of A549 cells from 47.01±1.59 to 66.04±2.84% relative to PQ treatment only (Fig. 1D). PQ exposure (100 µM) induced the release of LDH through damaged cell membranes, but co-treatment with EA (10–80 µM) significantly reduced LDH release at 72 h (Fig. 1E).

Induction of Nrf2 and Its Target, HO-1, and NQO1 Expression by EA The expression of Nrf2 was greatly induced as early as at 3 h when A549 cells were treated with 10 µM EA only and continues until 48 h. Nevertheless, induction of the expressions of HO-1 and NQO1 began from 6 h, but HO-1 expression continued until 24 h. NQO1 expression was reduced at 24 h but was again induced at 48 h (Fig. 2A). Next,
we examined the nuclear translocalization of Nrf2 in A549 cells treated with 100 µM PQ, 10 µM EA, or both for 6 h. EA increased nuclear translocalization alone or in association with PQ compared to C or PQ only (Fig. 2B). Only marginal induction of Nrf2 gene expression was observed in cells treated with PQ only at both 6 h and 12 h, but protein induction was found at only 12 h. Only marginal induction of HO-1 gene was observed at 6 h when cells were treated with EA and/or PQ, but induction was highly increased at 12 h that was supported by the protein expression data. HO-1 mRNA expression was higher at 6 h but decreased at 12 h when treated with PQ only, but the protein level did not show any significant changes at any time point. The level of gene expression of NQO1 was markedly induced at both 6 h and 12 h for cells treated with EA and/or PQ (Figs. 3A, B). The expressions of Nrf2, HO-1 and NQO1 in A549 cells treated with 100 µM PQ only, 10 µM EA only, or both for 6 h and 12 h were examined. At 6 h, the expression of Nrf2 and NQO1 was greatly induced by PQ/EA co-treatment, but not HO1. However, at 12 h, the expression of Nrf2, HO-1, and NQO1 was induced compared to control and PQ only treatment (Figs. 3C, D).

**EA Reduces ROS Generation, Lipid Peroxidation, and Total Glutathione Level Induced by PQ Treatment in A549 Cells** We investigated whether EA itself or in combination with PQ had any effect on intracellular ROS level. In Figs. 4A and B, a significantly increased fluorescence was detected when cells were treated with H2O2 (a ROS inducer as positive control) and 100 µM PQ. NAC (ROS inhibitor as negative control) showed a decreased fluorescence level compared to control (cells treated with medium only). EA treatment significantly reduced ROS level in PQ-induced A549 cells following 6 h treatment. To assess the role of EA in lipid peroxidation induced upon PQ exposure, cellular MDA level was measured. At first, lipid peroxidation in 100 µM PQ treated A549 cells was detected in a time-dependent manner and a gradually increased MDA level was observed with time from 0–96 h (Fig. 5A). However, 10 µM EA did not produce any significant changes in MDA level compared with control up to 96 h (figure not given). Co-treatment of 100 µM PQ and 10 µM EA produced a significant decline of MDA level compared with 100 µM PQ at both 72 h and 96 h (Fig. 5B). The altitude of total GSH level was detected for cells treated with 100 µM PQ and/or 10 µM EA for 24, 48 and 72 h. Total GSH was markedly reduced when cells were treated with PQ only and the reduction was higher at 72 h compared with 48 h. EA itself did not show any effect on GSH level, but co-treatment with PQ increased the level of GSH level significantly when compared with only PQ treatment at 72 h. A very similar trend of GSH curve was found at any time points (Fig. 5C).

**DISCUSSION**

The hospital fatality rate of patients who have ingested PQ is around 55% with no significant changes between the survivors and non-survivors in respect to their characteristics. The high fatality rate is attributed to the high inherent toxicity of PQ and the lack of any effective treatment. Increased generation of ROS by PQ is the leading cause of cell death and the plant polyphenolic anti-oxidant might have the potential to reduce the level of ROS. The present study...
investigated the protective role of EA against PQ-induced cytotoxicity in human alveolar A549 cells. It was observed that 10 \( \mu \)M EA improved cell viability and decreased LDH release against 100 \( \mu \)M PQ-derived oxidative stress in A549 cells.

The concentration of EA used here was similar to that used in another study to protect hepatocytes from mitochondria generated ROS.\(^{17}\) EA induced and activated the cytoprotective transcription factor Nrf2, a well-known transcription factor that activates the transcription of several phase II detoxifying enzymes, even at very early time (3 h) and, consequently,
HO-1 and NQO1 expressions were induced as early as 6 h. The marked augmentation of the cytoprotective proteins HO-1 and NQO1 may play an indispensable role in down-regulating PQ-induced cytotoxicity in A549 cells. HO-1 has potent regulatory activities during physiological stress and considered as a ‘therapeutic funnel.’ There is evidence that NQO1 is responsible for cellular defense mechanism against redox cycling and oxidative stress, as well as against carcinogenesis. Therefore, the induction of HO-1 and NQO1 could be closely associated with the protection of A549 cells against PQ-induced oxidative stress. One recent study revealed that Resveratrol, another polyphenol, also strongly activated Nrf2 for the protection of PQ induced ROS production, inflammation, and fibrotic reactions.

Treatment with EA inhibited lipid peroxidation and generation of free radical derivatives that is apparent from the decreased level of lipid peroxidation biomarkers in MOLT-4 human leukemia cells. A very recent article showed that EA pretreatment markedly reduces UVA-induced accumulation of MDA in keratinocytes. These findings further support our data where 100 µM PQ gradually increased MDA accumulation with time. When PQ-exposed cells were co-treated with EA, a marked decrease of cellular MDA level was observed. GSH, a non-protein thiol, is present extensively in all cell types that participate in the detoxification and protection of cells against oxidative stresses. PQ showed a large reduction of cellular GSH that might be the reason of weakening cellular defense against the devastating oxidative stress generated by PQ. An elevation of GSH level is therefore important to recover PQ-induced stress. EA improved the level of total GSH level in PQ-exposed conditions that might be responsible to suppress the PQ generated stress. PQ is conducted as a common

Fig. 5. (A) A549 Cells Were Treated with 100 µM PQ for 0–96 h, (B) 100 µM PQ and/or 10 µM EA for 72 h and 96 h to Detect the MDA By-Product of Lipid Peroxidation and (C) Cells Were Treated with 100 µM PQ and/or 10 µM EA for 24, 48, and 72 h for the Detection of Total GSH Level. Asterisk (*) denotes significant differences relative to control and the pound sign (#) denotes a significant difference relative to PQ (p<0.05).

Fig. 6. Schematic Diagram Showing the Protective Function of EA against PQ Induced ROS Stress. Here, we postulated that EA reduces ROS level and lipid peroxidation and thus maintain total intracellular glutathione level. EA plays the role by activating Nrf2 and its target gene expression (HO-1, and NQO1) that are responsible for the detoxification and elimination of potentially harmful PQ. ARE indicates antioxidant response element.
inducer of ROS that was supported by our study where 100 µM PQ sufficiently induced intracellular ROS level in A549 cells. Treatment of 10 µM EA in PQ-treated A549 cells sufficiently reduced the generation of intracellular ROS.

In this study, the significant protection against PQ-induced cytotoxicity following EA treatment is mainly attributed through recovery on ROS generation, lipid peroxidation, LDH release and total GSH level. The beneficial effect of EA may manifest through the enhancing effect of the endogenous release and total GSH level. The beneficial effect of EA may be a desirable food supplement for a variety of oxidative stress conditions. Further research based on detailed molecular mechanisms and clinical trials might be required in PQ-induced intoxication.

Acknowledgement
This research was supported by the Soonchunhyang University Research Grant for Yong-Sik Kim (20120684).

REFERENCES

1) Dinis-Oliveira RJ, Duarte JA, Sánchez-Navarro A, Remião F, Bastos ML, Carvalho F. Paraquat poisonings: mechanisms of lung toxicity, clinical features, and treatment. Crit. Rev. Toxicol., 38, 13–71 (2008).

2) Adam A, Smith LL, Cohen GM. An assessment of the role of redox cycling in mediating the toxicity of paraquat and nitrofurantoin. Environ. Health Perspect., 85, 113–117 (1990).

3) Rannels DE, Kameji R, Pegg AE, Rannels SR. Spermidine uptake by type II pneumocytes: interactions of amine uptake pathways. Am. J. Physiol., 257, L346–L353 (1989).

4) Larrosa M, Tomás-Barberán FA, Espin JC. The dietary hydrolysable tannin punicalagin releases ellagic acid that induces apoptosis in human colon adenocarcinoma Caco-2 cells by using the mitochondrial pathway. J. Nutr. Biochem., 17, 611–625 (2006).

5) Falsaperla M, Morgia G, Tartarone A, Ardito R, Romano G. Support ellagic acid therapy in patients with hormone refractory prostate cancer (HRPC) on standard chemotherapy using vinorelbine and estramustine phosphate. Eur. Urol., 47, 449–454, discussion, 454–455 (2005).

6) Labrecque L, Lamy S, Chapus A, Mihoubi S, Durocher Y, Ma Q. Low concentrations of PDGF and VEGF receptors by ellagic acid, a dietary-derived phenolic compound. Carcinogenesis, 26, 821–826 (2005).

7) Rogerio AP, Fontanari C, Borduelli E, Keller AC, Russo M, Soares EG, Albuquerque DA, Faccoli LH. Anti-inflammatory effects of Lactoensia pacari and ellagic acid in a murine model of asthma. Eur. J. Pharmacol., 580, 262–270 (2008).

8) Singh K, Khanna AK, Visen PK, Chander R. Protective effect of ellagic acid on t-butyl hydroperoxide induced lipid peroxidation in isolated rat hepatocytes. Indian J. Exp. Biol., 37, 939–940 (1999).

9) Yu YM, Chang WC, Wu CH, Chiang SY. Reduction of oxidative stress and apoptosis in hyperlipidemic rabbits by ellagic acid. J. Natr. Biochem., 16, 675–681 (2005).

10) Friling RS, Bensimon A, Tichauer Y, Daniel V. Xenobiotic-inducible expression of murine glutathione S-transferase Ya subunit gene is controlled by an electrophile-responsive element. Proc. Natl. Acad. Sci. U.S.A., 87, 6258–6262 (1990).

11) Itoh K, Chiba T, Takahashi S, Ishii T, Igarashi K, Katoh Y, Oyake T, Hayashi N, Satoh K, Hatayama I, Yamamoto M, Nabeshima Y. An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. Biochem. Biophys. Res. Commun., 236, 313–322 (1997).

12) Brigelius-Flohé R, Banning A. Part of the series: from dietary antioxidants to regulators in cellular signaling and gene regulation. Sulforaphane and selenium, partners in adaptive response and prevention of cancer. Free Radic. Res., 40, 775–787 (2006).

13) Dinkova-Kostova AT, Holtzclaw WD, Wakabayashi N. Keap1, the sensor for electrophiles and oxidants that regulates the phase 2 response, is a zinc metalloprotein. Biochemistry, 44, 6889–6899 (2005).

14) Itoh K, Wakabayashi N, Katoh Y, Ishii T, Igarashi K, Engel JD, Yamamoto M. Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. Genes Dev., 13, 76–86 (1999).

15) Zerin T, Kim YS, Hong SY, Song HY. Protective effect of methylprednisolone on paraquat-induced A549 cell cytotoxicity via induction of efflux transporter, P-glycoprotein expression. Toxicol. Lett., 208, 101–107 (2012).

16) Sabzghabae AM, Eizadi-Mood N, Montazeri K, Yaraghi A, Golabi M. Fatality in paraquat poisoning. Singapore Med. J., 51, 496–500 (2010).

17) Hwang JM, Cho JS, Kim TH, Lee YJ. Ellagic acid protects hepatocytes from damage by inhibiting mitochondrial production of reactive oxygen species. Biomed. Pharmacother., 64, 264–270 (2010).

18) Szabo ME, Gallayas E, Bak I, Rakotovao A, Boucher F, de Leiris J, Nagy N, Varga E, Tosaki A. Heme oxygenase-1-related carbon monoxide and flavonoids in ischemic/reperfused rat retina. Invest. Ophthalmol. Vis. Sci., 45, 3727–3732 (2004).

19) Asher G, Lotem J, Cohen B, Sachs L, Shaul Y. Regulation of p53 stability and p53-dependent apoptosis by NADH quinone oxidoreductase 1. Proc. Natl. Acad. Sci. U.S.A., 98, 1188–1193 (2001).

20) Jasswal AK. Regulation of genes encoding NAD(P)H: quinone oxidoreductases. Free Radic. Biol. Med., 29, 254–262 (2000).

21) He X, Wang L, Szklarz G, Bi Y, Ma Q. Resveratrol inhibits paraquat-induced oxidative stress and fibrogenic response by activating the nuclear factor erythroid 2-related factor 2 pathway. J. Pharmacol. Exp. Ther., 342, 81–90 (2012).

22) Mertens-Talcott SU, Talcott ST, Percival SS. Low concentrations of quercetin and ellagic acid synergistically influence proliferation, cytotoxicity and apoptosis in MOLT-4 human leukemia cells. J. Nutr., 133, 2669–2674 (2003).

23) Hseu YC, Chou CW, Senthil Kumar KJ, Fu KT, Wang HM, Hsu LS, Kuo YH, Wu CR, Chen SC, Yang HL. Ellagic acid protects human keratinocyte (HaCaT) cells against UVA-induced oxidative stress and apoptosis through the upregulation of the HO-1 and Nrf-2 antioxidant genes. Food Chem. Toxicol., 50, 1245–1255 (2012).

24) Pari L, Sivasankari R. Effect of ellagic acid on cyclosporine A-induced oxidative damage in the liver of rats. Fundam. Clin. Pharmacol., 22, 395–401 (2008).