



Research Article

# Efficacy of *Elaeagnus umbellata* leaves on prevention of cadmium-induced toxicity in HepG2 cells

Jae-Yeul Lee<sup>1,2†</sup>, Seun-Ah Yang<sup>3†\*</sup>, Won-Bin Bae<sup>3</sup>

<sup>1</sup>Institute of Natural Science, Keimyung University, Daegu 42601, Korea

<sup>2</sup>Department of Applied Chemistry, Kumoh National Institute of Technology, Gumi 39177, Korea

<sup>3</sup>Department of Food Science and Technology, Keimyung University, Daegu 42601, Korea

**Abstract** *Elaeagnus umbellata* leaves have been reported to suppress inflammation, allergic responses, lung cancer proliferation and oral bacterial growth. Cadmium (Cd) is a heavy metal that has been found to cause many toxicities, including liver toxicity. The aim of this study was to evaluate the capacity of 70% ethanol extract of *E. umbellata* leaves (EUL) to protect human hepatocytes from Cd toxicity. After exposure of HepG2 cells to Cd at 10  $\mu$ M for 24 h, cell viability, expression levels of apoptosis- and antioxidant-related proteins, reactive oxygen species (ROS) accumulation and Cd uptake were assessed. EUL protected HepG2 cells from Cd-induced apoptosis as determined by MTT assay. A decrease in caspase-3 and p-p53 protein levels was observed in cells pretreated with EUL prior to Cd exposure. Furthermore, the Cd-induced increase in intracellular DCF fluorescence was attenuated by EUL, indicating that the Cd-induced apoptosis preventing effect was associated with the suppression of ROS accumulation. Moreover, EUL's effects on the inhibition of p38, JNK, and AKT phosphorylation also appear to be associated with protection against Cd toxicity. Moreover, EUL upregulated Cd-depressed expression of Nrf2, HO-1, catalase, and MT-1,2 proteins, suggesting that Cd uptake-induced apoptosis in HepG2 cells may be inhibited by EUL's antioxidative potential.



OPEN ACCESS

**Citation:** Lee JY, Yang SA, Bae WB. Efficacy of *Elaeagnus umbellata* leaves on prevention of cadmium-induced toxicity in HepG2 cells. Korean J Food Preserv, 30(5), 797-810 (2023)

**Received:** August 16, 2023

**Revised:** September 24, 2023

**Accepted:** September 29, 2023

**Keywords** *Elaeagnus umbellata* leaves, cadmium, HepG2 cell, apoptosis, oxidative stress

<sup>†</sup>These authors contributed equally to this study.

**\*Corresponding author**

Seun-Ah Yang

Tel: +82-53-580-5117

E-mail: seunahy@kmu.ac.kr

Copyright © 2023 The Korean Society of Food Preservation. This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/4.0>) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

## 1. Introduction

Exposure to cadmium (Cd), a toxic heavy metal, occurs through inhalation, ingestion, and dermal contact, with occupational exposure being a common concern. Cd exhibits toxicity across multiple targets, such as the liver and kidney (Genchi et al., 2020; Matović et al., 2015). Earlier *in vivo* explorations have substantiated the intricate correlation between Cd-triggered cytotoxicity and apoptosis in the hepatic tissues of both murine and rat models (Habeebu et al., 1998; Tzirogiannis et al., 2003). Moreover, parallel *in vitro* examinations have further affirmed that Cd elicits the fragmentation of DNA—an unmistakable

hallmark of apoptosis—within HepG2 cells, a lineage of human hepatocellular carcinoma cells (Aydin et al., 2003; Fatur et al., 2002; Shimoda et al., 2001).

The involvement of key molecular players, such as p53, Bcl-2 family proteins, caspases, and MAPK signaling pathways, has been identified in Cd-induced apoptosis. Cd activates p53, a tumor suppressor protein, which promotes apoptosis through the regulation of pro-apoptotic genes. The balance between anti-apoptotic (e.g., Bcl-2) and pro-apoptotic (e.g., Bax) members of the Bcl-2 family influences apoptotic cell fate. Caspases, as central executioners of apoptosis, cleave downstream targets to execute cell death. Furthermore, MAPK signaling pathways, including JNK, p38, and ERK, have been implicated in Cd-induced apoptosis.

In recent years, *in vitro* cell-based models have played a vital role in toxicological research, enabling the assessment of Cd toxicity and elucidating underlying mechanisms. Numerous studies utilizing HepG2 cells have shed light on the mechanisms underlying Cd-induced hepatotoxicity, including oxidative stress, mitochondrial dysfunction, apoptosis, and inflammation. These cellular responses contribute to liver damage and may lead to the development of liver diseases.

Antioxidants and antioxidant enzymes such as N-acetylcysteine, glutathione, catalase, vitamin E, and superoxide dismutase are effectively applied to reduce Cd-induced hepatotoxicity (Aronis et al., 2003; Mattie and Freedman, 2001; Poliandri et al., 2003; Pourahmad and O'Brien, 2000). *Crocus sativus* and green tea extract had also been reported to inhibit Cd-induced hepatocyte toxicity in adult rats (Abdelrazek et al., 2016; Yari et al., 2016). Additionally, research has explored the detoxifying effects of *Rhizoma coptidis* (RC) aqueous extract and the intracellular distribution and metabolism of

ginsenosides in animal livers (Park et al., 2013).

*Elaeagnus umbellata*, also known as autumn olive, is a deciduous shrub cultivated in South Korea. The fruit and leaves of this plant are edible. The fruit, known as “Mokbanha” in traditional Korean medicine, has been reported to have effects on conditions such as diarrhea (Rafique et al., 2016), bleeding, and indigestion (Lee et al., 2011). It is also known to contain approximately 7–8 times more lycopene than tomatoes (Wang et al., 2020). Studies have reported various pharmacological activities of *E. umbellata* leaves, including anti-bacterial, anti-inflammation (Kang et al., 2020), skin whitening and wrinkle-improving effects (Park et al., 2019), and anticancer activity (Kim et al., 2016). However, no studies have reported the efficacy of the extract from *E. umbellata* leaves against hepatotoxicity caused by Cd.

Although research on the major active components of *E. umbellata* leaves is still limited, it is known to contain gallic acid, kaempferol, catechin, and other compounds (Abri and Maleki, 2016; Yoon et al., 2007). Gallic acid is widely recognized for its antioxidant properties (Yen et al., 2002) and has been reported to exhibit anti-inflammatory activity (Kroes et al., 1992), anti-allergic effects (Kim et al., 2006), inhibit lung cancer proliferation (Ohno et al., 1999), and inhibit the growth of oral bacteria (Kang et al., 2008). The exceptional antioxidant properties of green tea, which is rich in gallic acid, are well known. Similarly, kaempferol, a phenolic component abundant in plants, has been investigated for its anti-inflammatory activity (Devi et al., 2015) and its ability to alleviate oxidative stress in nerve cells (Sharma et al., 2007).

In this study, we aimed to investigate the cellular protective effects of ethanol extract from *E. umbellata* leaves (EUL) against Cd-induced cytotoxicity in

HepG2 cells. Moreover, the potential of the extract to prevent reactive oxygen species (ROS) levels, apoptosis, and Cd accumulation induced by Cd treatment was also examined.

## 2. Materials and methods

### 2.1. Materials

Cadmium chloride [CdCl<sub>2</sub>], 4',6-Diamidino-2'-phenylindole dihydrochloride (DAPI), 2',7'-dichlorofluorescein diacetate (DCF-DA), 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from the Sigma-Aldrich Co. (St. Louis, MO, USA). Antibodies against phospho-extracellular signal regulated kinase 1/2 (ERK; #9101), phospho-protein kinase B (Akt; #9271), phospho-c-Jun N terminal kinase 1/2 (JNK; #9251), phospho-p38 (#9211),  $\beta$ -Actin (#3700), Caspase-3 (#14220), Phospho-p53 (#9284), Nuclear factor erythroid-derived 2-related factor 2 (NRF2; #12721), Heme oxygenase-1 (HO-1; #26416), superoxide dismutase type 1 (SOD-1; #2770), Catalase (#12980), Metallothionein (MT)-1,2, anti-mouse secondary antibody (#7076), and horseradish peroxidase linked anti-rabbit secondary antibody (#7074) were purchased from Cell Signaling Technology (Beverly, MA, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were from the Welgene (Gyeongsan-si, Korea). All other chemicals and reagents used were of analytical grade.

### 2.2. Preparation of *E. umbellata* leaves (EUL)

The preparation of EUL was adapted from the method previously described by Kang et al. (2020). *E. umbellata* plants cultivated in Hapcheon-gun, Gyeongsangnam-do, Korea, were acquired for the study. The dried leaves of *E. umbellata* were

subjected to extraction using 70% ethanol (Duksan Science, Seoul, Korea) at a ratio of 10 times its weight. The extraction process was carried out at a temperature of 80°C for a duration of 6 h. Subsequently, the obtained extract was concentrated and then subjected to lyophilization using a freeze-dryer (FDU-2100, EYELA, Tokyo, Japan), resulting in the production of EUL. The lyophilized EUL was confirmed to yield approximately 16.8%.

### 2.3. Cell culture

Human hepatoma HepG2 cells were cultured in DMEM, which was supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin. The cells were maintained in a controlled environment, specifically at a temperature of 37°C in a 5% CO<sub>2</sub>-95% air incubator with regulated humidity levels. In all experimental setups, a control group was incorporated, functioning as the vehicle control group. This control group was treated with 0.1% DMSO.

### 2.4. Assessment of cell viability

The assessment of cell viability was carried out using the MTT assay method. HepG2 cells were seeded in a 96-well plate using DMEM and were cultured at 37°C for a duration of 24 h. Following the incubation period, the cells were subjected to treatment with varying concentrations of EUL ranging from 0 to 500  $\mu$ g/mL. Additionally, cells were exposed to 10  $\mu$ M CdCl<sub>2</sub> concurrently with escalating concentrations of EUL (0-500  $\mu$ g/mL). Upon the completion of the specified incubation period, a solution of 20  $\mu$ L of MTT (1 mg/mL) was introduced to the cells. This solution was allowed to incubate with the cells for a duration of 4 h. Subsequent to the incubation, the culture media were aspirated and replaced with 100  $\mu$ L of DMSO.

The mixture was gently agitated for 10 min to facilitate complete dissolution. The absorbance of the resultant solution was measured at a wavelength of 540 nm utilizing a microplate reader (Model: CA 94089, Molecular Devices, San Jose, CA, USA).

### **2.5. Western blot analysis**

HepG2 cells were seeded in a 6-well plate at a density of  $1 \times 10^6$  cells per well, utilizing DMEM as the culture medium. The cells were then cultured at a temperature of  $37^\circ\text{C}$  for a duration of 24 h. Following the incubation period, HepG2 cells were subjected to a 24 h treatment with  $\text{CdCl}_2$  and EUL. Post-treatment, HepG2 cells were lysed utilizing lysis buffer (Pro-Preptm, iNtRON, Seongnam, Korea) supplemented with a protease inhibitor (P3100\_001, GenDEPOT, Barker, TX, USA) and a phosphatase inhibitor (P3200\_001, GenDEPOT). The lysed cells were subsequently incubated on ice for 30 min. Protein concentrations within the cell lysates were determined employing a BCA protein assay (Thermo Fisher Scientific, Waltham, MA, USA). Following protein quantification, 20  $\mu\text{g}$  of proteins were loaded and subjected to separation through 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were subsequently transferred onto PVDF membranes (Whatman GmbH, Dassel, Germany). The membranes were blocked with a solution of 5% skim milk in Tris-buffered saline containing 0.1% Tween-20 for a period of 1 h. Subsequent to blocking, the membranes were incubated with primary antibodies for 16 h at  $4^\circ\text{C}$ . Following primary antibody incubation, the membranes underwent three successive washes using Tris-buffered saline containing 0.1% Tween-20. Subsequently, horseradish peroxidase-linked secondary antibodies (Cell Signaling Technology) were incubated with the membranes for 1 h.

Protein visualization was achieved via enhanced chemiluminescence, and the images were captured and analyzed using UVP Vision Works<sup>®</sup> LS Image Acquisition & Analysis Software (Upland, CA, USA).

### **2.6. Detection of intracellular ROS level**

For the visualization of nuclear morphological alterations and reactive oxygen species (ROS) in Cd-treated cells, a double staining procedure utilizing DAPI and DCFDA dye was employed. The treated cells were fixed using a solution of 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) for a duration of 10 min. Subsequently, the cells were subjected to three consecutive PBS washes and were then incubated with a combination of 0.1 mg/mL DAPI and 25  $\mu\text{M}$  DCF-DA for 10 min at room temperature. Following the staining process, the cells were washed thrice with PBS. The levels of ROS within the cells were visualized and analyzed using a fluorescence microscope. The quantification of intra-cellular  $\text{CdCl}_2$  levels was directly performed via ICP-MS and is expressed as parts per billion (ppb) per  $1 \times 10^6$  cells.

### **2.7. Detection of intracellular Cd level**

HepG2 cells cultivated in a complete culture medium were employed to quantify the levels of Cd, and these cells were designated as the Cd-control group. Post the cultivation period, cells were subjected to two washes with phosphate-buffered saline (PBS) to eliminate any potential metal contamination originating from the culture medium. The washed cells were subsequently centrifuged (95  $\times g$ , 10 min), resuspended in PBS, and a specified volume of each sample was enumerated using a hemocytometer to achieve a consistent count of  $1 \times 10^6$  cells. The collected cells were then suspended in 180  $\mu\text{L}$  of royal water (composed of

nitric acid at a ratio of 1:3 with hydrochloric acid) and subsequently lysed for a duration of 30 min. The washing solutions along with the royal water utilized for cell dilution underwent analysis through Inductively Coupled Plasma Mass Spectrometry (ICP-MS) using an Agilent 7500cx instrument (Agilent, CA, USA). Calibration procedures were executed utilizing Cd standard solutions (Sigma-Aldrich Co.).

### 2.8. Statistical analysis

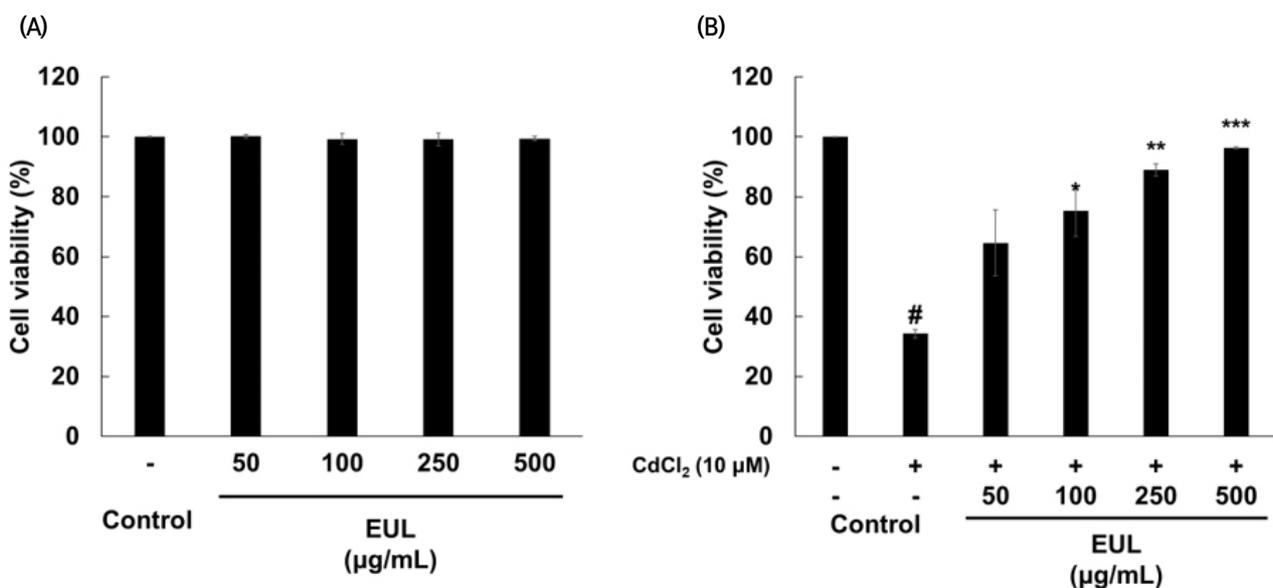
The results are expressed as means±standard deviation ellipse (SDE) values, derived from three independent experimental replicates. To determine the significance of differences between each experimental test condition and its respective control, Student's t-test was employed for each paired experiment. Significance levels were designated at \* $p<0.05$ , \*\* $p<0.01$ , and \*\*\* $p<0.001$ . Statistical analyses were performed by using Microsoft Excel software (Microsoft Co., Redmond, WA, USA).

## 3. Results and discussion

### 3.1. Protection on Cd-induced cell death

We first examined the cell viability of HepG2 cells incubated with EUL (50–500  $\mu\text{g}/\text{mL}$ ) in both the presence or absence of Cd to determine the protective effect of the extract against Cd toxicity (Fig. 1(A) and (B)). No cytotoxicity was observed by EUL treatment, but the cell viability was decreased to 34.3% after 24 h Cd (10  $\mu\text{M}$ ) exposure. Pretreatment with EUL show an increase of cell viability dose-dependently, indicating hepatoprotective effect of the extract.

In this study, the concentration of Cd applied to HepG2 cells was deliberately chosen to achieve a cytotoxic effect exceeding 50%, thereby enabling the evaluation of the preventive efficacy of EUL at different concentrations. Consequently, a concentration of 10  $\mu\text{M}$  was employed, resulting in approximately 63% cytotoxicity. Furthermore, pretreatment with EUL before the introduction of Cd exhibited a

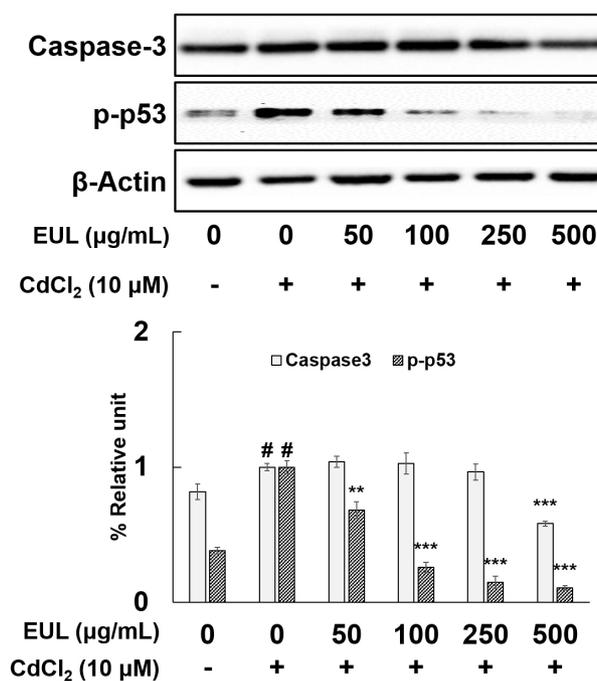


**Fig. 1.** The effects of *Elaeagnus umbellata* leaves extract (EUL) on cell viability in the absence (A) or in the presence of cadmium (B) in HepG2 cells. The cells were incubated with EUL for 24 h, then treated with CdCl<sub>2</sub> (10  $\mu\text{M}$ ) for 24 h. Results are represented as the means±SDE of percentages calculated with respect to control levels of three independent experiments. # $p<0.05$  vs. untreated cell and \* $p<0.05$ , \*\* $p<0.01$ , \*\*\* $p<0.001$  vs. CdCl<sub>2</sub> alone (n=3).

significant inhibitory effect on Cd-induced cytotoxicity in HepG2 cells. This protective impact against Cd-induced cytotoxicity in our study seems to be attributed to the prominent presence of antioxidant compounds in EUL, notably gallic acid, as evidenced in various investigations on the hepatoprotective properties of polyphenolic compounds in plant extracts against oxidative stress on HepG2 cells (Martin et al., 2008; Zhang et al., 2020). Previously, our research had identified that gallic acid and kaempferol as the primary constituents in the leaf extract of *E. umbellata*, containing 98.95 mg/100 g and 81.85 mg/100 g, respectively (Kim et al., 2016).

### 3.2. Changes in apoptotic proteins in Cd-treated HepG2 cells

In light of the pivotal role played by caspase activation and phosphorylated p53 (p-p53) in the initiation of apoptosis in response to diverse stimuli, our investigation aimed to elucidate the alterations in the levels of caspase-3 and p-p53 within HepG2 cells upon exposure to Cd. Additionally, we sought to examine the potential protective effects of EUL against Cd-induced apoptotic cell death. To achieve this, the cells underwent pre-treatment with various concentrations of EUL (50, 100, 250, 500  $\mu\text{g}/\text{mL}$ ) for 24 h, followed by subsequent exposure to Cd for an additional 24 h. Our assessment of protein expression unveiled a significant increment of approximately 1.2-fold in caspase-3 levels and a more pronounced elevation of approximately 2.6-fold in p-p53 upon Cd exposure (as depicted in Fig. 2). Remarkably, pre-treatment with EUL at the concentration of 500  $\mu\text{g}/\text{mL}$  exhibited a substantial mitigating effect. This resulted in a reduction of 42% in the expression of caspase-3 and a noteworthy decrease of 74% in



**Fig. 2.** The effects of *Elaeagnus umbellata* leaves extract (EUL) on the protein expression of caspase-3 and p-p53 in cadmium-treated HepG2 cells. The cells were incubated with EUL for 24 h, then treated with CdCl<sub>2</sub> (10  $\mu\text{M}$ ) for 24 h.  $\beta$ -Actin was used as the internal control for Western blot. Results are represented as the means  $\pm$  SDE of percentages calculated with respect to control levels of three independent experiments. # $p < 0.05$  vs. untreated cell and \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  vs. CdCl<sub>2</sub> alone (n=3).

p-p53 levels compared to Cd-treated control cells. These outcomes collectively validate the engagement of apoptosis-associated proteins in the course of Cd-induced cell demise within HepG2 cells. Furthermore, the observed protective influence of EUL, particularly in attenuating the Cd-induced alterations in these proteins—especially p-p53—affirms its role in safeguarding against the protein changes triggered by Cd. Our study corroborates earlier findings that have implicated Cd in the initiation of apoptotic cell death within HepG2 cells (Athmouni et al., 2018; Lawal et al., 2015; Oh and Lim, 2006). Notably, the use of Z-DEVD-FMK, a potent caspase 3 inhibitor, effectively thwarted Cd-induced cell death, thereby highlighting the role

of caspase 3 in cell demise at these levels. Caspases are categorized into two distinct groups: 'apoptotic initiators', represented by caspase 8, and 'apoptotic effectors', exemplified by caspase 3 (Oh and Lim, 2006). Caspase-3 activation can be initiated through the death receptor pathway, culminating in apoptotic cell death (Alarifi et al., 2013). Furthermore, exposure of HepG2 cells to Cd has been reported to trigger the phosphorylation and upregulation of the p53 protein (Seo et al., 2013). This, in turn, orchestrates the transcription of downstream proteins, including p21 and Bax, pivotal players in cellular processes linked to apoptosis (Oh et al., 2006).

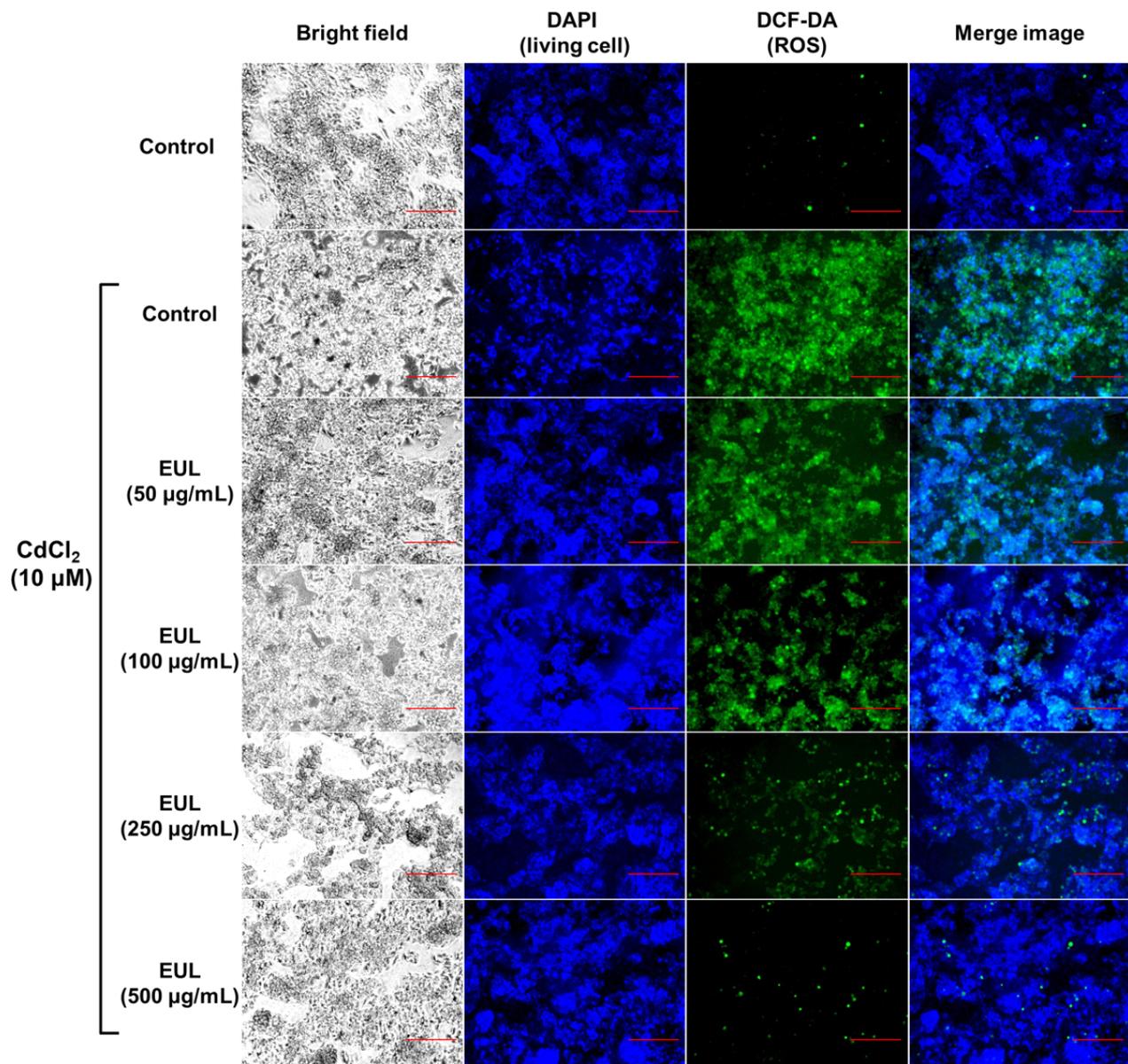
### *3.3. Effects on the Cd-induced ROS generation*

Given the well-established literature on Cd-triggered ROS production in hepatocytes (Fotakis et al., 2005), our study sought to qualitatively explore the regulatory impact of EUL on ROS levels within Cd-treated HepG2 cells. Utilizing microscopy and H<sub>2</sub>DCF-DA staining, we aimed to ascertain whether fluctuations in intracellular ROS levels correlated with the apoptosis instigated by Cd exposure. Specifically, the effect of Cd treatments (10  $\mu$ M for 24 h) was assessed, revealing a notable surge in DCF fluorescence an indicator of increased ROS accumulation within HepG2 cells in comparison to untreated control cells (Fig. 3). However, cells that were pre-treated with EUL exhibited a relatively diminished DCF fluorescence signal when contrasted with Cd-treated cells. Notably, the pre-treatment with EUL, specifically at concentrations of 250 and 500  $\mu$ g/mL, displayed significant effectiveness in curbing ROS levels relative to cells solely subjected to Cd exposure. Notably, the control cells displayed no discernible DCF fluorescence. These findings underscore the potential hepatoprotective attributes of EUL, positing its role in mitigating ROS

generation incited by Cd. Although ROS were traditionally associated with detrimental biological effects due to their propensity to induce oxidative harm to cellular constituents, it has been subsequently recognized that ROS assume a pivotal role in normal biological processes. This encompasses participation in signaling pathways governing diverse transcription factors and subsequent gene expression (Hsiao and Stapleton, 2004). It is pertinent to note that several reports have substantiated the effectiveness of free radical scavengers in impeding apoptosis initiated by Cd, acting through their antioxidative attributes. In essence, Cd triggers an initial ROS surge, consequently instigating the activation of the redox-sensitive MAPK pathway (Papaiahgari et al., 2004).

### *3.4. Effects on the protein expression of MAPKs*

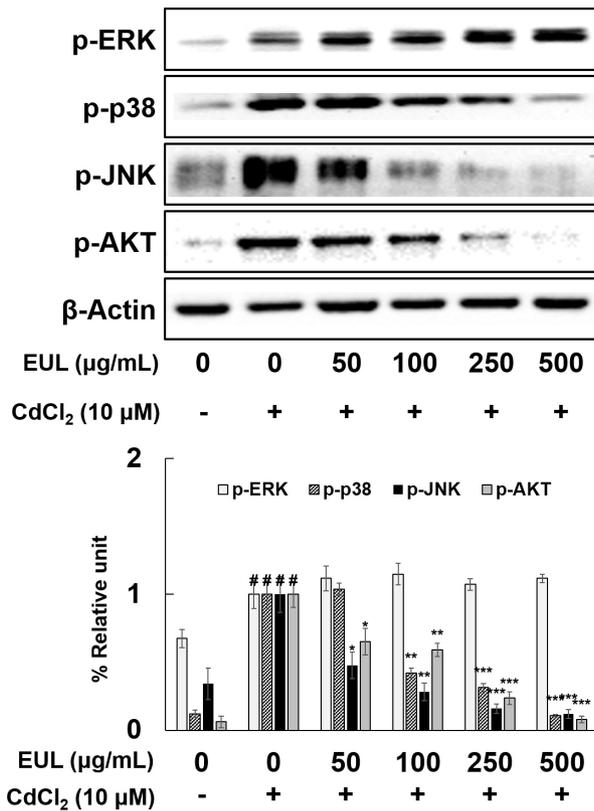
Cd activation of various signal transduction factors, including Akt, ERK, p38, and JNK in diverse cell types, including HepG2 cells, has been well-documented (Escobar et al., 2009; Tsai et al., 2016). Furthermore, Shen et al. (2004) highlighted the role of MAPK pathways in triggering the expression of antioxidant genes through a Nrf2-dependent mechanism. In order to ascertain the contribution of MAPKs to the protective effect exerted by EUL against Cd-induced cytotoxicity in HepG2 cells, we examined the activation states of AKT, JNK, ERK, and p38 within the cells. Upon treatment of HepG2 cells with Cd (10  $\mu$ M) for a duration of 24 h, phosphorylation levels of ERK, p38, JNK, and AKT exhibited significant increments of approximately 1.5-fold, 8.3-fold, 2.9-fold, and 16.7-fold, respectively (as depicted in Fig. 4). Notably, pretreatment with EUL (50-500  $\mu$ g/mL) for a period of 24 h markedly suppressed the Cd-induced escalation in phosphorylation of p38, JNK, and AKT in a dose-dependent manner.



**Fig. 3.** The effects of *Elaeagnus umbellata* leaves extract (EUL) on ROS generation in cadmium-treated HepG2 cells. The HepG2 cells ( $3 \times 10^4$  cells/well) were pretreated with EUL for 24 h with or without  $\text{CdCl}_2$  ( $10 \mu\text{M}$ ) and stained using DAPI (nuclear staining, blue signal) and DCF-DA ROS indicators (intracellular oxygen free radical, green signal). Fixation was performed by treating 25% formaldehyde for 20 min, and reacted with DCFDA ( $5 \mu\text{M}$ ) and DAPI ( $10 \mu\text{M}$ ) for 30 min. Scale bar,  $200 \mu\text{m}$ .

Intriguingly, the phosphorylation status of ERK remained unaffected following EUL pretreatment. These outcomes collectively reaffirmed that the heightened phosphorylation of MAPKs is a hallmark of Cd-induced apoptosis, and further substantiated that EUL selectively counteracted the phosphorylation of JNK, p38, and AKT. Interestingly, the phosphorylation

of ERK remained unaltered. This study thus establishes the pivotal role of Cd-induced MAPK phosphorylation in mediating apoptosis, with phosphorylated MAPKs transmitting apoptotic signals to the nucleus through the activation of sub-signaling molecules, as previously noted (Tsai et al., 2016). The current investigation reinforces



**Fig. 4.** The effects of *Elaeagnus umbellata* leaves extract (EUL) on the protein expression of MAPKs in cadmium-treated HepG2 cells. The cells were incubated with EUL for 24 h, then treated with CdCl<sub>2</sub> (10 μM) for 24 h. β-Actin was used as the internal control for Western blot. Results are represented as the means±SDE of percentages calculated with respect to control levels of three independent experiments. #p<0.05 vs. untreated cell and \*p<0.05, \*\*p<0.01, \*\*\*p<0.001 vs. CdCl<sub>2</sub> alone (n=3).

the finding that Cd-triggered phosphorylation of MAPKs is modulated by EUL, specifically inhibiting the phosphorylation of JNK, p38, and AKT. Consequently, EUL emerges as a regulator that suppresses Cd-induced apoptosis within HepG2 cells. Future research endeavors involving MAPK inhibitors are warranted for a comprehensive understanding of this intricate mechanism.

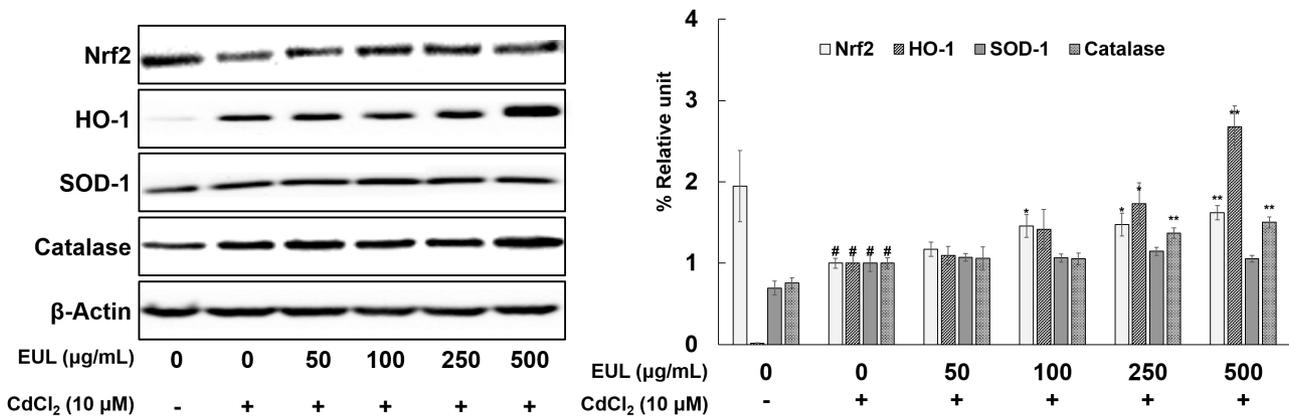
**3.5. Effects on the expression of antioxidant enzymes**

To comprehensively explore the effect of EUL on the levels of Nrf2 and its downstream target

molecules, namely HO-1, SOD-1, and catalase, in the context of Cd-induced toxicity within HepG2 cells, we conducted a rigorous analysis of protein expressions through Western blotting. As demonstrated in Fig. 5, relative to the untreated- control cells, exposure to Cd resulted in an approximate 50% reduction in Nrf2 protein expression, concomitantly with a substantial elevation in HO-1 expression. Furthermore, Cd exposure induced notable increases in SOD-1 (1.4-fold) and catalase (1.3-fold) expression levels (Fig. 5). Pre-treatment with EUL (500 μg/mL) led to a significant elevation in the protein levels of Nrf2 (1.6-fold), HO-1 (2.7-fold), and catalase (1.5-fold) in comparison to the Cd-exposed control cells. SOD-1 expression, however, remained unchanged across all tested concentrations. Our findings underscore the dose-dependent augmentation of Nrf2 expression mediated by EUL, thereby mitigating the suppression induced by Cd exposure. Additionally, the protein expression of both HO-1 and catalase demonstrated an up-regulated pattern following treatment with 10 μM Cd for 24 h. Remarkably, these elevations were further enhanced by EUL pre-treatment in HepG2 cells, underscoring the potential of EUL to mitigate Cd-induced hepatotoxicity by up-regulating key antioxidant proteins. Given the established role of Nrf2 as a pivotal cytoprotective transcription factor orchestrating the transcription of target genes encoding an array of antioxidant enzymes, our results align with the existing understanding that various antioxidant compounds exert their hepatoprotective effects by activating the Nrf2 signaling pathway (He et al., 2008; Jadeja et al., 2016; Lee et al., 2012).

**3.6. Effects on the Cd uptake and the protein expression of MT-1,2**

Intracellular Cd levels were investigated via



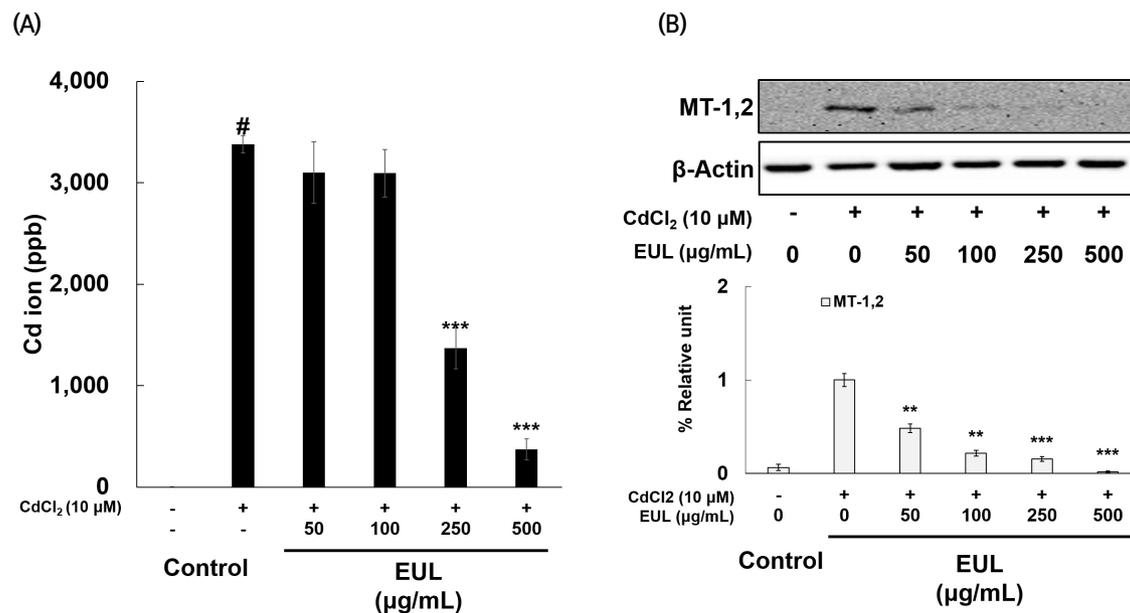
**Fig. 5.** The effects of *Elaeagnus umbellata* leaves extract (EUL) on the expression in antioxidant signaling proteins in cadmium-treated HepG2 cells. The cells were incubated with EUL for 24 h, then treated with CdCl<sub>2</sub> (10 μM) for 24 h. β-Actin was used as the internal control for Western blot. Results are represented as the means±SDE of percentages calculated with respect to control levels of three independent experiments. #p<0.05 vs. untreated cell and \*p<0.05, \*\*p<0.01 vs. CdCl<sub>2</sub> alone (n=3).

rigorous ICP-MS analysis in cells exposed to Cd. The quantification of Cd levels within these cells serves as a proxy for Cd absorption or uptake by the cultured cells. Consequently, we assessed intracellular Cd levels following exposure to 10 μM Cd in HepG2 cells, which revealed an observable elevation in intracellular Cd levels. After 24 h of Cd exposure, 3,379 ppb of Cd were detected in one million HepG2 cells (as shown in Fig. 6(A)). This finding unequivocally underscores the ability of HepG2 cells to effectively uptake Cd, affirming its capacity to traverse the cellular membrane. Intriguingly, pre-treatment with EUL at concentrations of 250 and 500 μg/mL significantly attenuated the heightened Cd concentration within the cells, reducing it by approximately 60% and 11%, respectively, compared to Cd-exposed control cells. Furthermore, the expression of metallothionein (MT)-1,2 exhibited a marked upregulation upon exposure to 10 μM Cd, relative to untreated control cells. Remarkably, EUL pre-treatment induced a progressive decline in MT expression (as depicted in Fig. 6(B)). This observation corroborates existing knowledge that Cd stimulation

leads to the induction of genes encoding MTs. Notably, MTs play a pivotal role in orchestrating protective mechanisms during Cd exposure of target organs, as previously documented (Urani et al., 2007). Collectively, our data suggest that EUL effectively mitigates Cd-induced oxidative stress by curbing the cellular uptake of Cd. This critical action ultimately contributes to hepatoprotection against Cd-induced cellular damage. Although HepG2 cells are widely used as a hepatocyte model, they may not be completely identical to normal hepatocytes. Therefore, comparative experiments with normal hepatocytes or other model systems may be necessary to ensure the accuracy of the results.

## 4. Conclusions

In the present study, we explored the potential protective effects of EUL against toxicity induced by Cd in HepG2 cells. Our observations revealed an augmentation in cell death, an upregulation of apoptotic protein expression, and an accumulation of ROS in response to Cd treatment, indicative of



**Fig. 6.** The effects of *Elaeagnus umbellata* leaves extract (EUL) on the CdCl<sub>2</sub> accumulation and the protein expression of metallothionein (MT) in cadmium-treated HepG2 cell. The cells were incubated with EUL for 24 h, then treated with CdCl<sub>2</sub> (10 μM) for 24 h. Intracellular level of Cd was determined by Inductively Coupled Plasma Mass Spectrometry (ICP-MS). Concentrations are normalized as ppb/10<sup>6</sup> cells and represent the mean±SD of at least three independent experiments. β-Actin was used as the internal control for Western blot. Results are represented as the means±SDE of percentages calculated with respect to control levels of three independent experiments. <sup>#</sup>p<0.05 vs. untreated cell and <sup>\*</sup>p<0.05, <sup>\*\*</sup>p<0.01, <sup>\*\*\*</sup>p<0.001 vs. CdCl<sub>2</sub> alone (n=3).

heightened oxidative stress. Notably, pretreatment of the cells with EUL resulted in an enhancement of antioxidant protein expression, in line with the notably robust antioxidant activity previously reported for this extract. In summary, our current data strongly imply that EUL effectively counteracts Cd-induced hepatotoxicity by impeding the activation of MAPK pathways (p38, JNK, AKT), promoting Nrf2 activation and the consequent upregulation of antioxidant proteins such as HO-1 and catalase. Additionally, EUL appears to downregulate MT expression by mitigating ROS- induced oxidative stress. Therefore, these findings underscore the potential utility of EUL as a food material, offering protective attributes against hepatic damage attributed to Cd exposure in humans.

#### Acknowledgements

This research was supported by Basic Science

Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2021R111A3055952).

#### Conflict of interests

The authors declare no potential conflicts of interest.

#### Author contributions

Conceptualization: Yang SA. Methodology: Lee JY, Yang SA. Formal analysis: Lee JY, Bae WB. Validation: Lee JY. Writing - original draft: Lee JY, Bae WB. Writing - review & editing: Lee JY, Yang SA.

#### Ethics approval

This article does not require IRB/IACUC approval because there are no human and animal participants.

#### ORCID

Jae-Yeul Lee (First author)

<https://orcid.org/0000-0003-4862-9169>

Seun-Ah Yang (First & Corresponding author)

<https://orcid.org/0000-0002-6692-1844>

Won-Bin Bae

<https://orcid.org/0009-0009-5733-5972>

## References

- Abdelrazek HM, Helmy SA, Elsayed DH, Ebaid HM, Mohamed RM. Ameliorating effects of green tea extract on cadmium induced reproductive injury in male wistar rats with respect to androgen receptors and caspase-3. *Reprod Biol*, 16, 300-308 (2016)
- Abri A, Maleki M. Isolation and identification of gallic acid from the *elaegnus angustifolia* leaves and determination of total phenolic, flavonoids contents and investigation of antioxidant activity. *Iran Chem Commun*, 4, 146-154 (2016)
- Alarifi S, Ali D, Alkahtani S, Siddiqui MA, Ali BA. Arsenic trioxide-mediated oxidative stress and genotoxicity in human hepatocellular carcinoma cells. *Onco Targets Ther*, 75-84 (2013)
- Aronis A, Melendez J, Golan O, Shilo S, Dicter N, Tirosh O. Potentiation of fas-mediated apoptosis by attenuated production of mitochondria-derived reactive oxygen species. *Cell Death Differ*, 10, 335-344 (2003)
- Athmouni K, Belhaj D, El Feki A, Ayadi H. Optimization, antioxidant properties and GC-MS analysis of *Periploca angustifolia* polysaccharides and chelation therapy on cadmium-induced toxicity in human HepG2 cells line and rat liver. *Int J Biol Macromol*, 108, 853-862 (2018)
- Aydin HH, Celik HA, Devenci R, Terzioglu E, Karacali S, Mete N, Akarca U, Batur Y. Characterization of the cellular response during apoptosis induction in cadmium-treated hep g2 human hepatoma cells. *Biol Trace Elem Res*, 95, 139-153 (2003)
- Devi KP, Malar DS, Nabavi SF, Surenda A, Xiao J, Nabavi SM, Daglia M. Kaempferol and inflammation: From chemistry to medicine. *Pharmacol Res*, 99, 1-10 (2015)
- Escobar MDC, Souza V, Bucio L, Hernandez E, Gomez-Quiroz LE, Ruiz MCG. Mapk activation is involved in cadmium-induced hsp70 expression in hepg2 cells. *Toxicol Mech Methods*, 19, 503-509 (2009)
- Fatur T, Tusek M, Falnoga I, Scancar J, Lah T, Filipic M. DNA damage and metallothionein synthesis in human hepatoma cells (hepg2) exposed to cadmium. *Food Chem Toxicol*, 40, 1069-1076 (2002)
- Fotakis G, Cemeli E, Anderson D, Timbrell JA. Cadmium chloride-induced DNA and lysosomal damage in a hepatoma cell line. *Toxicol in Vitro*, 19, 481-489 (2005)
- Genchi G, Sinicropi MS, Lauria G, Carocci A, Catalano A. The effects of cadmium toxicity. *Int J Environ Res Public Health*, 17, 3782 (2020)
- Habeebu SSM, Liu J, Klaassen CD. Cadmium-induced apoptosis in mouse liver. *Toxicol Appl Pharmacol*, 149, 203-209 (1998)
- He X, Chen MG, Ma Q. Activation of Nrf2 in defense against cadmium-induced oxidative stress. *Chem Res Toxicol*, 21, 1375-1383 (2008)
- Hsiao CJJ, Stapleton SR. Characterization of Cd-induced molecular events prior to cellular damage in primary rat hepatocytes in culture: Activation of the stress activated signal protein jnk and transcription factor ap-1. *J Biochem Mol Toxicol*, 18, 133-142 (2004).
- Jadeja RN, Upadhyay KK, Devkar RV, Khurana S. Naturally occurring Nrf2 activators: Potential in treatment of liver injury. *Oxid Med Cell Longev*, 2016, 3453926 (2016)
- Kang HR, Jung JK, Park SH, Lee JY, Yang SA. Anti-oxidant, anti-inflammatory, and anti-bacterial effects of extracts from *Elaeagnus umbellata* leaves obtained using different extract conditions. *Korean J Food Preserv*, 27, 374-384 (2020)

- Kang MS, Oh JS, Kang IC, Hong SJ, Choi CH. Inhibitory effect of methyl gallate and gallic acid on oral bacteria. *J Microbiol*, 46, 744-750 (2008)
- Kim MJ, Lim JS, Yang SA. Component analysis and anti-proliferative effects of ethanol extracts of fruits, leaves, and stems from *Elaeagnus umbellata* in HepG2 cells. *J Korean Soc Food Sci Nutr*, 45, 828-834 (2016)
- Kim SH, Jun CD, Suk K, Choi BJ, Lim H, Park S, Lee SH, Shin HY, Kim DK, Shin TY. Gallic acid inhibits histamine release and pro-inflammatory cytokine production in mast cells. *Toxicol Sci*, 91, 123-131 (2006)
- Kroes BV, Van Den Berg A, Van Ufford HQ, Van Dijk H, Labadie R. Anti-inflammatory activity of gallic acid. *Planta Med*, 58, 499-504 (1992)
- Lawal AO, Marnewick JL, Ellis EM. Heme oxygenase-1 attenuates cadmium-induced mitochondrial-caspase 3-dependent apoptosis in human hepatoma cell line. *BMC Pharmacol Toxicol*, 16, 1-13 (2015)
- Lee CH, Bae JY, Park JH. Pharmacognostical studies on the Korean folk medicine 'borisunamu'. *Korean J Pharmacogn*, 42, 1-5 (2011)
- Lee SE, Yang H, Jeong SI, Jin YH, Park CS, Park YS. Induction of heme oxygenase-1 inhibits cell death in crotonaldehyde-stimulated hepg2 cells via the pkc- $\delta$  -p38 -Nrf2 pathway. *PLOS ONE*, 7, e41676 (2012)
- Martin MA, Ramos S, Mateos R, Granado Serrano ABN, Izquierdo-Pulido MA, Bravo L, Goya L. Protection of human hepg2 cells against oxidative stress by cocoa phenolic extract. *J Agric Food Chem*, 56, 7765-7772 (2008)
- Matovic V, Buha A, Dukic-Cosic D, Bulat Z. Insight into the oxidative stress induced by lead and/or cadmium in blood, liver and kidneys. *Food Chem Toxicol*, 78, 130-140 (2015)
- Mattie MD, Freedman JH. Protective effects of aspirin and vitamin E ( $\alpha$ -tocopherol) against copper- and cadmium-induced toxicity. *Biochem Biophys Res Commun*, 285, 921-925 (2001)
- Oh SH, Choi JE, Lim SC. Protection of betulin against cadmium-induced apoptosis in hepatoma cells. *Toxicol*, 220, 1-12 (2006)
- Oh SH, Lim SC. A rapid and transient ros generation by cadmium triggers apoptosis via caspase-dependent pathway in hepg2 cells and this is inhibited through n-acetylcysteine-mediated catalase upregulation. *Toxicol Appl Pharmacol*, 212, 212-223 (2006)
- Ohno Y, Fukuda K, Takemura G, Toyota M, Watanabe M, Yasuda N, Xinbin Q, Maruyama R, Akao S, Gotou K, Fujiwara T, Fujiwara H. Induction of apoptosis by gallic acid in lung cancer cells. *Anti-Cancer Drugs*, 10, 845-852 (1999)
- Papaiahgari S, Kleeberger SR, Cho HY, Kalvakolanu DV, Reddy SP. NADPH oxidase and ERK signaling regulates hyperoxia-induced Nrf2-ARE transcriptional response in pulmonary epithelial cells. *J Biol Chem*, 279, 42302-42312 (2004)
- Park SH, Jhee KH, Yang SA. Protective effects of an ethanol extract of *Elaeagnus umbellata* leaves on  $\alpha$ -MSH-induced melanin production in b16-f0 cells and UVB-induced damage in CCD-986sk cells. *J Life Sci*, 29, 555-563 (2019)
- Park SJ, Lee JR, Jo MJ, Park SM, Ku SK, Kim SC. Protective effects of Korean red ginseng extract on cadmium-induced hepatic toxicity in rats. *J Ginseng Res*, 37, 37-44 (2013)
- Poliandri AH, Cabilla JP, Velardez MO, Bodo CC, Duvilanski BH. Cadmium induces apoptosis in anterior pituitary cells that can be reversed by treatment with antioxidants. *Toxicol Appl Pharmacol*, 190, 17-24 (2003)
- Pourahmad J, O'Brien PJ. A comparison of hepatocyte cytotoxic mechanisms for Cu<sup>2+</sup> and Cd<sup>2+</sup>. *Toxicol*, 143, 263-273 (2000)
- Rafique N, Khan T, Shah AJ. Calcium entry blocking activity of the *Elaeagnus umbellata* fruit extract explains its use in diarrhea and gut spasm. *Bangladesh J Pharmacol*, 11, 585-592 (2016)
- Seo JY, Lee YJ, Oh MH, Lee SH. *In vitro* nasal

- epithelial toxicity by cadmium accompanies up-regulation of runx3 protein with activation of pi3-kinase/akt. *Mol Cell Toxicol*, 9, 159-167 (2013)
- Sharma V, Joseph C, Ghosh S, Agarwal A, Mishra MK, Sen E. Kaempferol induces apoptosis in glioblastoma cells through oxidative stress. *Mol Cancer Ther*, 6, 2544-2553 (2007)
- Shen G, Hebbar V, Nair S, Xu C, Li W, Lin W, Keum YS, Han J, Gallo MA, Kong A-NT. Regulation of Nrf2 transactivation domain activity: The differential effects of mitogen-activated protein kinase cascades and synergistic stimulatory effect of raf and creb-binding protein. *J Biol Chem*, 279, 23052-23060 (2004)
- Shimoda R, Nagamine T, Takagi H, Mori M, Waalkes MP. Induction of apoptosis in cells by cadmium: Quantitative negative correlation between basal or induced metallothionein concentration and apoptotic rate. *Toxicol Sci*, 64, 208-215 (2001)
- Tsai JS, Chao CH, Lin LY. Cadmium activates multiple signaling pathways that coordinately stimulate akt activity to enhance c-myc mRNA stability. *PLoS One*, 11, e0147011 (2016)
- Tzirogiannis KN, Panoutsopoulos GI, Demonakou MD, Hereti RI, Alexandropoulou KN, Basayannis AC, Mykoniatis MG. Time-course of cadmium-induced acute hepatotoxicity in the rat liver: The role of apoptosis. *Arch Toxicol*, 77, 694-701 (2003)
- Urani C, Melchiorretto P, Canevali C, Morazzoni F, Gribaldo L. Metallothionein and hsp70 expression in hepg2 cells after prolonged cadmium exposure. *Toxicol in Vitro*, 21, 314-319 (2007)
- Wang T, Hou Y, Hu H, Wang C, Zhang W, Li H, Cheng Z, Yang L. Functional validation of phytoene synthase and lycopene  $\epsilon$ -cyclase genes for high lycopene content in autumn olive fruit (*Elaeagnus umbellata*). *J Agric Food Chem*, 68, 11503-11511 (2020)
- Yari A, Sarveazad A, Asadi E, Raouf Sarshoori J, Babahajian A, Amini N, Amidi F, Bahadoran H, Joghataei M, Asadi M. Efficacy of crocus sativus l. On reduction of cadmium-induced toxicity on spermatogenesis in adult rats. *Andrologia*, 48, 1244-1252 (2016)
- Yen GC, Duh PD, Tsai HL. Antioxidant and prooxidant properties of ascorbic acid and gallic acid. *Food Chem*, 79, 307-313 (2002)
- Yoon KY, Hong JY, Shin SR. Analysis on the components of the *Elaeagnus multiflora* thunb. Leaves. *Korean J Food Preserv*, 14, 639-644 (2007)
- Zhang B, Yu D, Luo N, Yang C, Zhu Y. Four active monomers from Moutan Cortex exert inhibitory effects against oxidative stress by activating Nrf2/Keap1 signaling pathway. *Korean J Physiol Pharmacol*, 24, 373-384 (2020)