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Protective effects of *Quercus salicina* blume leaves aqueous extracts against cadmium-induced oxidative stress and cell cytotoxicity in HK-2 cells

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ABSTRACT

Background: *Quercus salicina* Blume is an evergreen plant growing southern parts of Korea and Japan, and has been used for the treatment of dysentery, dermatitis, and hemorrhage from possible effects such as anti-inflammatory, anti-endemic and litholytic activities. We investigated the underlying mechanisms of *Quercus salicina* Blume leaf extracts (QS) protection against cadmium treatment utilizing human kidney origin HK-2 cells.

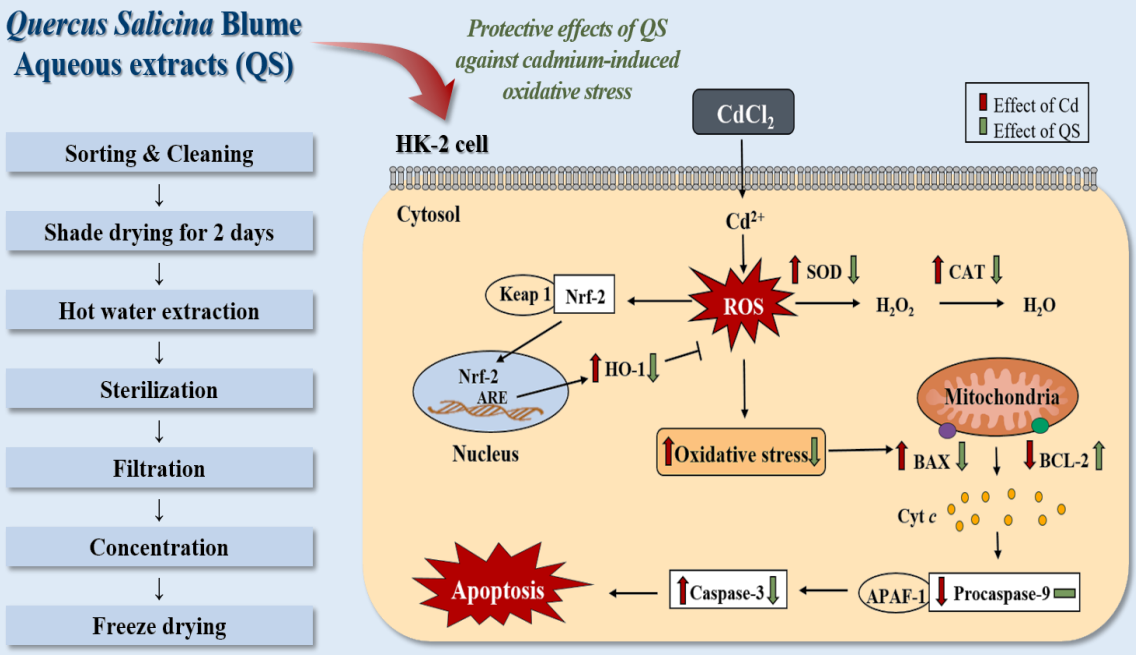
Methods: To ascertain the functional constituents of the hot water-extracted QS, we conducted analyses to determine the total phenolic and flavonoid contents. Subsequently, the antioxidant activity was assessed using DPPH (2,2-diphenyl-1-picrylhydrazyl) and FRAP (ferric ion reducing antioxidant power) methods. After treating HK-2 cells with QS and Cd, we confirmed the production of reactive oxygen species (ROS) and the expression of related proteins through western blotting. This investigation aimed to assess the antioxidant and anti-apoptotic effects of QS against cadmium-induced oxidative stress.

Results: QS exhibited strong antioxidative potential, since the samples exhibited significantly high DPPH and FRAP values. Upon exposure of HK-2 cells to cadmium, inducing oxidative stress, the application of QS effectively reinstated cellular antioxidative functions, leading to an augmentation in cell viability. Notably, the presence of QS attenuated the heightened expression of oxidative marker enzymes induced by cadmium treatment, including SOD (superoxide dismutase) 1, catalase, Nrf-2, and Heme-oxygenase. The apparent disappearance of the apoptotic

bodies indicates a potential apoptosis controlling effect of QS. Furthermore, the considerable increase in sub-G1 phase with the addition of cadmium was diminished under QS treatment indicating the ability of QS to reduce the degree of apoptosis related cell cycle phase. The apoptotic quenching effects of QS were accompanied by the downregulation of pro-apoptotic protein Bax and up-regulation of pro-apoptotic Bcl-2. The examination of cleaved caspase-9 and caspase-3 has provided evidence that the pronounced anti-apoptotic activities observed in cadmium-challenged HK-2 cells significantly diminished in the presence of QS.

Conclusions: QS is a highly effective antioxidant and reduces cell cytotoxicity caused by cadmium through antioxidant functions and anti-apoptotic capabilities.

Keywords: *Quercus salicina* Blume leave, cadmium, antioxidant, anti-apoptosis



INTRODUCTION

Cadmium (Cd) belongs to the heavy metals group and is considered an important environmental hazard for both humans and wildlife. It can be toxic even at low concentrations, and consumption of this toxic Cd for long periods of time may lead to detrimental effects in health [1]. US-EPA has declared Cd along with arsenic, chromium, and lead as the most alarming heavy metal in the environment [2]. Cd exhibits its toxic effects mainly in the kidney, liver, and neuronal cells. Its toxic targets include bone and blood cells. Cadmium in the form of cadmium ions cause acute and chronic toxicity in organisms [3]. Given its facile transfer from soil to plants, cadmium contamination is frequently identified in various human food sources, particularly in staples like rice, which holds significant importance in many Asian diets [4]. Prolonged exposure to low-dose cadmium in humans has been associated with kidney

tubular impairment, potentially leading to a decline in the reabsorptive capacity for essential nutrients, including vitamins and minerals [3, 5]. Drawing from various studies, the suggested mechanism of cadmium-induced kidney tubular injury involves an accumulation of reactive oxygen species (ROS), leading to heightened oxidative stress [3, 6]. Cadmium accumulation in the proximal tubule of the nephron has been reported to cause dysfunction of the mitochondrial electron transport chain, resulting in electron leakage and ROS production [6]. In recent years a human proximal tubular cell line, HK-2 cell line, has been used to study nephrotoxicity caused by cadmium [7]. Numerous studies have presented that cadmium is capable of inducing both apoptotic and necrotic cell death [1, 6, 8]. Furthermore, many reports claim that functional foods containing biologically active compounds, such as plant extracts and herbal medicines could ameliorate cadmium-injured nephrotoxicity through their antioxidative activities [9, 10]. In recent decades, the prevailing antioxidant hypothesis has centered on polyphenols or flavonoids and how they confer health benefits in preventing and treating non-communicable degenerative diseases. [11]. Following an exhaustive search for the precise mechanisms behind the preventive health attributes of these natural antioxidant constituents, numerous vital biological activities were discovered. In addition to their fundamental antioxidant role, these natural components are increasingly recognized for their significant anti-inflammatory capabilities, which play a pivotal role in countering the emergence of various degenerative diseases [12]. The extensive research has been focused on the control of acute and chronic inflammatory symptoms [13]. Acute inflammation is connected to a process of repairing body functions in normal state. Sustained inflammation, commonly termed chronic inflammation, may contribute to the development of conditions such as atherosclerosis, rheumatoid arthritis, inflammatory bowel disease, and even diabetes [14]. *Quercus salicina* Blume is known to possess various phytochemicals and thus reported to have anti-oxidative, anti-inflammatory, anti-hemolytic, and anti-cancer effects [15, 16]. Quercus (oak) trees have been widely utilized for medicinal purposes due to the medicinal properties of their leaves, bark, and fruit [17]. They have been traditionally used to treat burns, hemorrhage, and gastrointestinal diseases, such as dysentery [16]. This study aims to evaluate the antioxidant effects of *Quercus salicina* Blume leaves and its protective effects against cadmium-induced oxidative stress and apoptosis, which cause kidney cell damage.

MATERIALS AND METHODS

Chemicals: Cadmium chloride (CdCl2), Folin & Ciocalteu's reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and 2'7'-dichlorofluorescin diacetate (DCF-DA) were procured from Sigma-Aldrich (St. Louis, MO, USA). The Penicillin-streptomycin solution and fetal bovine serum were sourced from Corning microplates (Tewksbury, MA, USA), while the RPMI-1640 medium was acquired from Welgene (Kyungsang-do, Korea). Leaves of Quercus salicina Blume were generously provided by Bosung afforestation in Jeonnam, Korea. All remaining reagents utilized in the study were of analytical grade.

Preparation of QS: To prepare the *Quercus salicina* Blume leaf extracts (QS), the leaves of *Quercus salicina* Blume underwent a 48-h drying process, achieving a 6% final moisture content. Following drying, the samples were subjected to extraction using hot distilled water (1:100) for one hour. The obtained solution underwent sterilization at 100 ℃ for 20 minutes, followed by filtration and concentration over a 2-hour period using a Rotary Evaporator. The concentrated solution was subsequently subjected to freeze-drying. To achieve the target concentrations of

5, 10, 15, 30, 50, 75, and 100 µg/mL, the resulting powder was further diluted with 95% ethanol.

Total phenolic and flavonoid contents: The determination of Total Phenolic Content (TPC) followed the method outlined by Folin and Denis [18] with slight adjustments. Specifically, 0.2 mL of the diluted sample, prepared at a 1:9 ratio with distilled water, was mixed with 0.2 mL of 2N Folin-Ciocalteu's reagent. After 3 minutes, 0.4 mL of 10% sodium carbonate solution was added, and the solution was allowed to incubate in darkness at room temperature for 1 hour. Following incubation, the absorbance was assessed at 725 nm employing an ELISA microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). The determination of TPC in the sample was calculated by referring to a standard curve developed with gallic acid, and the outcome was expressed as milligrams of gallic acid equivalents per g (mg GAE/g).

The determination of Total Flavonoid Content (TFC) was conducted following the methodology described by Moreno et al. [19], with minor modifications. In this procedure, 0.1 mL of QS was introduced into a test tube and combined with 0.02 mL of 10% aluminum nitrate (Al (NO3)3·9H2O) and 0.02 mL of 1 M potassium acetate (CH3COOK). Following the addition of 0.86 mL of ethyl alcohol, the solution underwent a 30-minute reaction period at room temperature in the absence of light. Subsequently, the absorbance was gauged at 415 nm, and a standard curve was constructed utilizing quercetin. The Total Flavonoid Content (TFC) in the sample was then quantified using the standard curve and expressed as milligrams of quercetin equivalents per 100 g (mg QE/100 g).

DPPH free radical scavenging assay: The DPPH free radical scavenging assay was performed using the Blois [20] method with minor modifications. QS was diluted with 95% ethyl alcohol into concentrations of 0. 10, 25, 50, 100, 250, and 500 µg/mL. Then, 20 µL of sample and 180 µL of 0.2 mM DPPH reagent (ethanol) were added to each well of a 96-well plate and reacted for 30 min in the dark at 37 ℃. Absorbance measurements were conducted at 517 nm employing an ELISA microplate reader, with quercetin, α-tocopherol, and L-ascorbic acid utilized as positive controls. All analyses were performed in triplicate, and the DPPH free radical scavenging activity of the sample was calculated as follows:

$$(1)\quad DPPH\ (\%) = \frac{(Control\ O.D. - Sample\ O.D.)}{Control\ O.D.} \times 100$$

Ferric reducing antioxidant power (FRAP) assay: The FRAP assay was performed in accordance with the method outlined by Benzie and Strain [21], with slight adjustments. To prepare the FRAP reagent, a mixture of 0.3 M sodium acetate solution (pH 3.6, $C_2H_3NaO_2 \cdot 3H_2O$), 10 mM TPTZ in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H_2O$ was combined in a 10:1:1 ratio (v/v/v). Various concentrations of QS (0, 10, 25, 50, 100, and 250 µg/mL) were achieved through dilution with 95% ethyl alcohol. The diluted solution was incubated at 37 ℃ for 15 min. Subsequently, each well of a 96-well plate received 180 µl of the FRAP reagent and 20 µl of the sample and was left to react at room temperature for 30 min. Absorbance readings were taken at 595 nm using an ELISA microplate reader, and each analysis was carried out in triplicate. For calibration, Quercetin, α-tocopherol, and L-ascorbic acid served as positive controls, while iron (II) sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$) was employed as the standard to generate the calibration curve. The results are expressed in terms of $FeSO_4 \cdot 7H_2O$ equivalents (mM).

Cell lines and cell viability measurement: Cultured HK-2 cells (human renal proximal tubular epithelial cells), procured from the Korean Cell Line Bank in Seoul, Korea, were cultured in RPMI-1640 medium supplemented with 10% inactivated fetal bovine serum

and 100 U/ml penicillin. The cultivation was carried out at 37 ℃ within an environment saturated with 95% air and 5% CO_2. A total of 1×10^5 cells were distributed into individual 96-well plates. Following a 24-hour incubation period, the cells underwent treatment with different concentrations of test samples for a duration of 4 hours. Following the sample treatment, 100 µL of MTT solution (diluted in RPMI 1640 without phenol red) was added to each well and incubated for an additional 4 h. The plates were then subjected to centrifugation for 10 min at 3,000 rpm. Subsequently, the MTT solution was removed, and 100 µL of DMSO was introduced into each well. To determine the absorption values at 540 nm, a microplate reader was employed. Cell viability was determined using the following formulation:

$$(2)\quad Cell\ viability\ (\%) = \frac{A_e}{A_c} \times 100$$

A_e and A_c correspond to the absorption values of the experimental group (measured during treatment) and the control group (untreated), respectively.

Reactive oxygen species measurement: DCF-DA assay was performed to measure the level of ROS production in HK-2 cells following treatment with QS and cadmium. After dispensing 160 µL of HK-2 cells (1×10^5 cells/mL) into a 96-well plate and stabilizing for 24 h, cadmium was treated by concentration or 25 µM cadmium and various concentrations of QS were treated together. The medium was aspirated, followed by a wash with cold PBS. Subsequently, 200 µL of DCF-DA was added and incubated at 37 ℃ for 30 minutes in a humidified atmosphere containing 95% air and 5% CO2. After washing with PBS, the chemiluminescence was determined by spectrofluorometer (Agilent Tech, Santa Clara, USA) at 485 nm excitation and 528 nm emission.

DAPI (4',6-diamidino-2-phenylindole) staining: For the evaluation of apoptotic changes in the cell nucleus, 1 × 10^5 HK-2 cells were seeded in a 24-well plate and incubated for 24 hours at 37 ℃ in a 5% humidified CO2 incubator. On the subsequent day, following a PBS rinse, the cells were treated with 200 µL of QS at four different concentrations (1, 5, 10, and 25 µg/mL). After the incubation for 4 h, an additional 20-h incubation period was carried out after introducing cadmium (25 µM). Following this, the culture media were aspirated, and the cells were fixed with 3.7% formaldehyde in PBS for 10 minutes, followed by a PBS rinse. DAPI staining was carried out with 50 µL of DAPI solution for 20 minutes at room temperature. After staining, the cells were washed with PBS and examined for nuclear alterations using a Confocal laser scanning microscope (LSM 510, Carl Zeiss Co., Obekochen, Germany) at 600× magnification.

Cell-cycle analysis: *T*o confirm DNA damage in HK-2 cells [22, 23], cell cycle analysis was conducted. HK-2 cells were cultured in 100 mm tissue-culture dishes. Following treatment with 25 µM Cd and QS at concentrations of 1, 5, and 10 µg/mL, they were incubated at 37 ℃ in a 5% humidified CO2 incubator for 24 hours. After two washes with PBS, cells were fixed with a PBS/methanol solution (1:2, v/v) at 4 ℃. The cell pellet was collected by centrifugation at 700 rpm for 5 minutes and then stained with 10 µg/mL propidium iodide (PI) and 10 µg/mL RNase A in the dark. Flow cytometry was performed using BD FACS Canto II flow cytometer and BD FACS Diva software (BD Biosciences,

San Jose, CA).

Western blotting analysis: After a double wash with PBS, HK-2 cells were lysed using a lysis buffer containing RIPA buffer, supplemented with protease and phosphatase inhibitors. The PierceTM BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) was then employed to quantify protein content. Subsequent to this, 20 µL aliquots of protein samples were separated on SDS-PAGE gels and electro transferred onto PVDF membranes activated with methanol (Roche, Mannheim, Germany). The membranes were then blocked with 5% skim milk and underwent an overnight incubation with primary antibodies. The primary antibodies used included SOD1 (1:500, Santa Cruz, USA), Catalase (1:500, Santa Cruz, USA), Nrf-2 (1:250, Santa Cruz, USA), HO-1 (1:1000, Rockland, USA), BAX (1:250, Santa Cruz, USA), BCL-2 (1:500, Santa Cruz, USA), Procaspase-9 (1:250, Santa Cruz, USA), Caspase 3 (1:1000, Santa Cruz, USA), and β-actin (1:1000, Santa Cruz, USA). Following the incubation with primary antibodies, the membranes were washed and exposed to horseradish peroxidase-conjugated secondary anti-rabbit antibodies (Cell Signaling Technology, Danvers, MA, USA) or secondary anti-mouse antibodies for 1 hour. Protein bands on the membranes were visualized using ClarityTM Western ECL Substrate (Bio-Rad, Hercules, CA, USA) and imaged with ChemiDoc (Bio-Rad, Hercules, CA, USA).

Statistical analysis: The data were expressed as Mean ± Standard Deviation (SD). Group differences were assessed using one-way analysis of variance (ANOVA), followed by the Duncan's multiple range test, utilizing SPSS (version 21.0, SPSS Inc., Chicago, IL, USA).

RESULTS

Total phenolic and flavonoid content (TPC and TFC): The results of TPC and TFC measurements of *Quercus salicina* Blume leaves extracts (QS) are shown in Table 1. The TPC of QS leaves was 287.12 mg GAE/g (gallic acid equivalent), and the TFC was 23.58 mg QE/g (quercetin equivalent), respectively.

Table 1. Contents of total phenolic compounds and flavonoids of *Quercus salicina* Blume leaves extracts (QS).

Components	Contents
Total phenolic (mg GAE/g[1)])	287.12 ± 1.21
Total flavonoid (mg QE/g[2)])	23.58 ± 2.78

Values are presented as means ± SD.

[1)] mg gallic acid equivalents per gram.

[2)] mg Quercetin equivalents per gram.

Antioxidant Activities of Quercus salicina Blume leaf extracts: At the concentrations of 10, 25, 50, 100, 250, 500 µg/mL, QS exhibited DPPH free radical scavenging activities of 33.9, 53.5, 73.0, 85.9, 89.8, 91.6% respectively. FRAP values of QS were 0.12, 0.15, 0.32, 0.46, 0.98 mM $FeSO_4$ at the concentrations of 10, 25, 50, 100, 250 µg/mL, respectively. In summary, these findings collectively indicate a significant ($P < 0.05$) concentration-dependent increase in the antioxidant activity of QS.

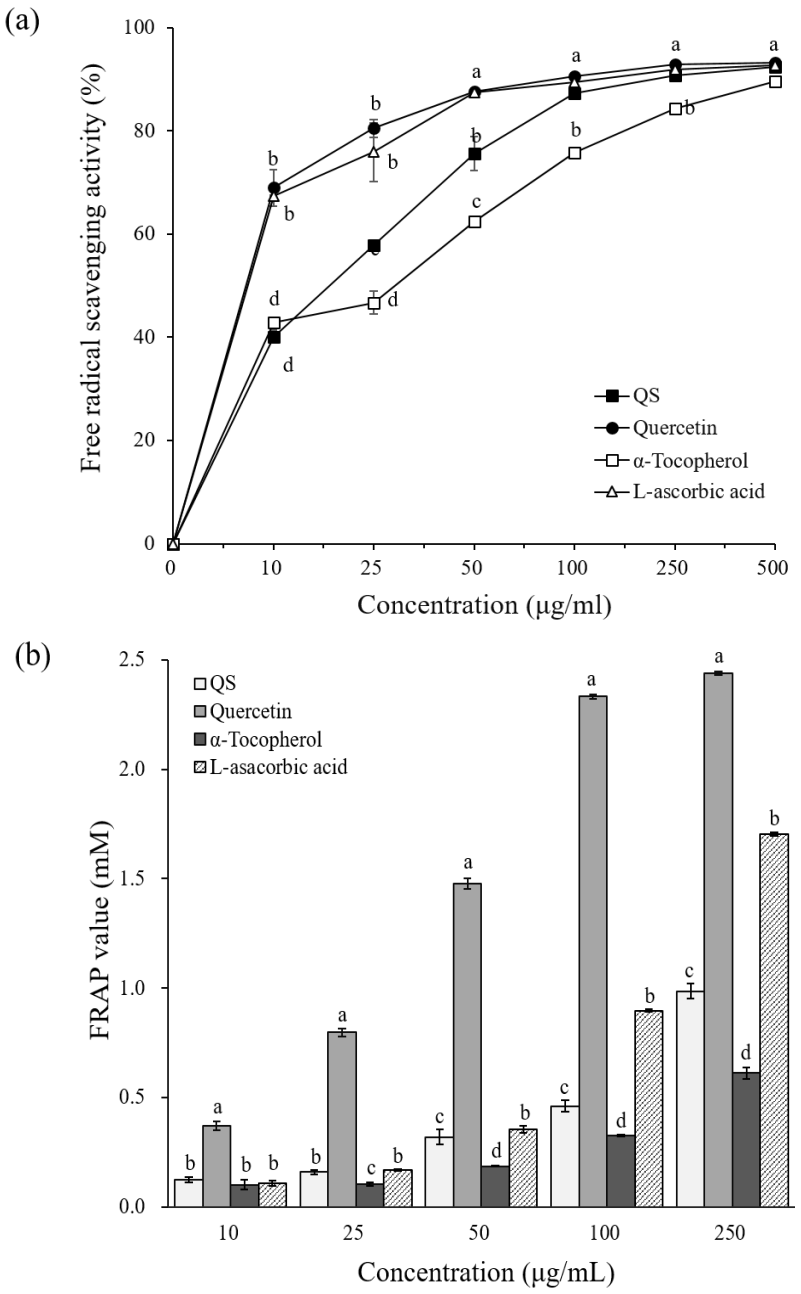


Figure 1. Antioxidant Activities of *Quercus salicina* Blume leave extracts (QS): free radical scavenging activity (a), and ferric reducing antioxidant power (b).

The protection of QS on cadmium-induced cell cytotoxicity: First, to test cell viability of HK-2 cells against the treatment of cadmium, an MTT assay was performed. The concentrations of 0, 1, 5, 10, 20, 25, 50, 75, and 100 μM cadmium were applied to the cells (Fig.2a), and the apparent decrease in viability was observed. At the same time the cytotoxicity of various concentrations of QS (1, 5, 10, 25, 50, 75, and 100 μg/mL) to HK-2 cells was tested (Fig.2b). After treating HK-2 cells with QS for 24 h at the concentrations of 1-50 μg/mL, higher than 90% survival rates. Hence, for subsequent experiments, we employed QS concentrations of up to 50 μg/mL, as they had no substantial impact on cell proliferation and viability. To test the kidney cell protective effect of QS, 25 μM cadmium were treated for 20 h after pre-incubation with QS (0~50 μg/mL) for 4 h in HK-2 cells. The study revealed that the reduced cell viability, decreased to

70.95% by Cd treatment, was restored with the addition of QS. Notably, the highest level of protection was observed, particularly at 10 µg/mL (Fig. 2c). In further experiments, 25 µM cadmium and 1, 5, 10 µg/mL QS were used.

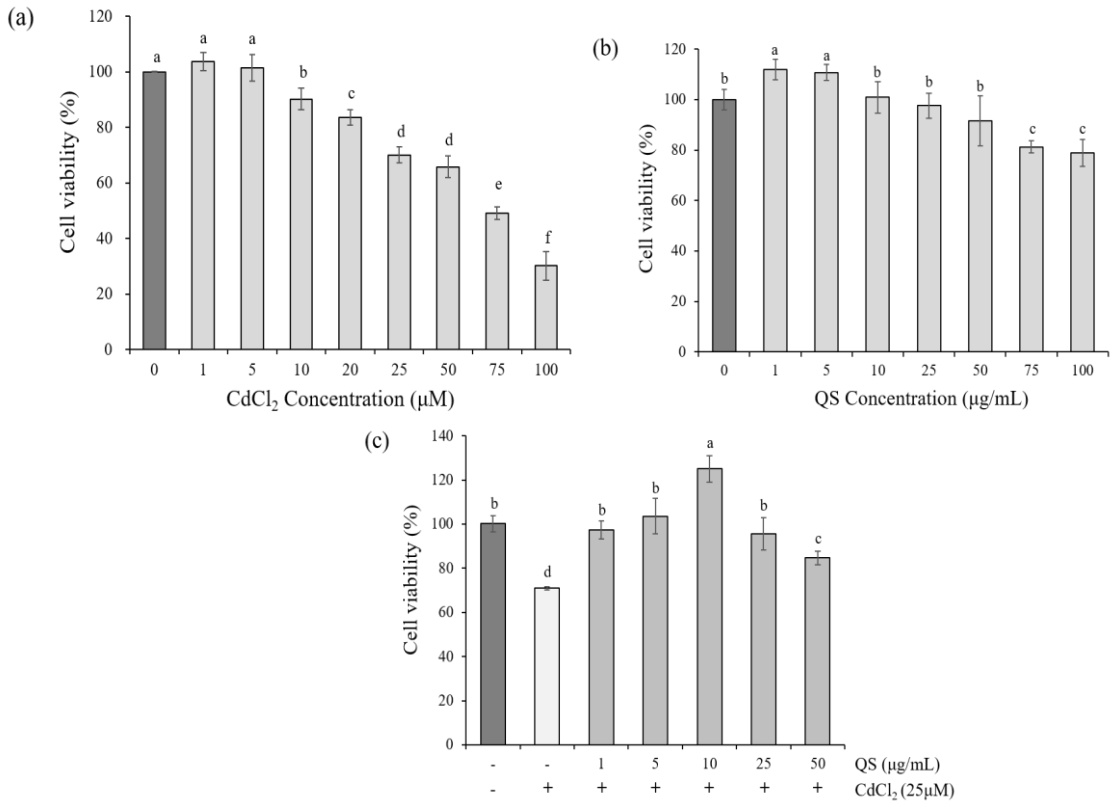


Figure 2. Effect of $CdCl_2$ and *Quercus salicina* Blume leave extract (QS) on cell viability in HK-2 cells (a, b), and protective effect of Q**S** on cadmium-induced cell cytotoxicity (c).

Quenching of Reactive Oxygen Species of QS in cadmium-treated HK-2 cells: The exposure of HK-2 cells to cadmium led to an elevation in ROS production in a concentration-dependent manner, as depicted in Figure 3a. When HK-2 cells were treated with QS and then cadmium challenge was performed, there were considerable ROS scavenging effects by QS pretreatment were shown with DCF-DA assays (Fig. 3b).

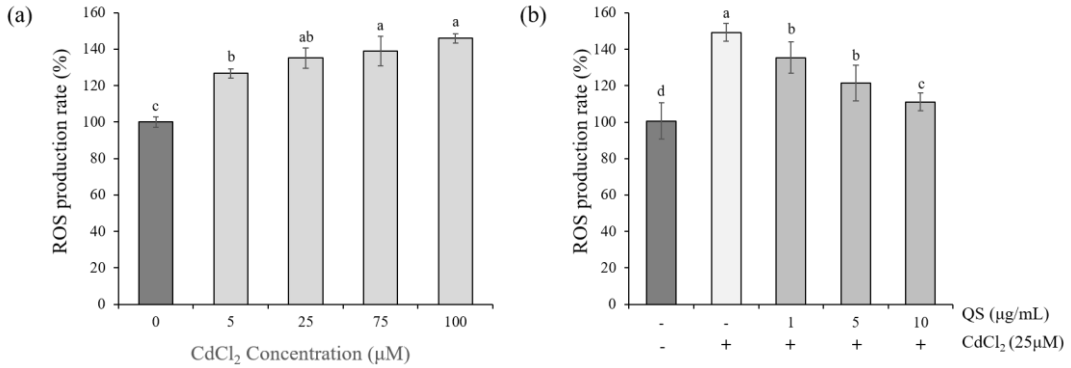


Figure 3. The impact of CdCl2 on reactive oxygen species (ROS) production (a) and the influence of Quercus salicina Blume leaf extract (QS) on cadmium-induced reactive oxygen species (ROS) production (b) in HK-2 cells were investigated.

Effect of QS on the oxidative stress induced enzyme levels in the presence of cadmium: To elucidate whether ROS quenching was accompanied with ROS related enzyme control, western blotting was performed. The protein levels of all four enzymes, superoxide mutase (SOD) 1, catalase (CAT), nrf-1, and heme oxygenase-1 (HO-1), were elevated when cadmium was introduced. Nevertheless, the inclusion of QS reduced the expression of these enzymes (Fig. 4). These findings suggested that the increased oxidative defense enzyme load induced by cadmium was mitigated by pre-treatment with QS in HK-2 cells.

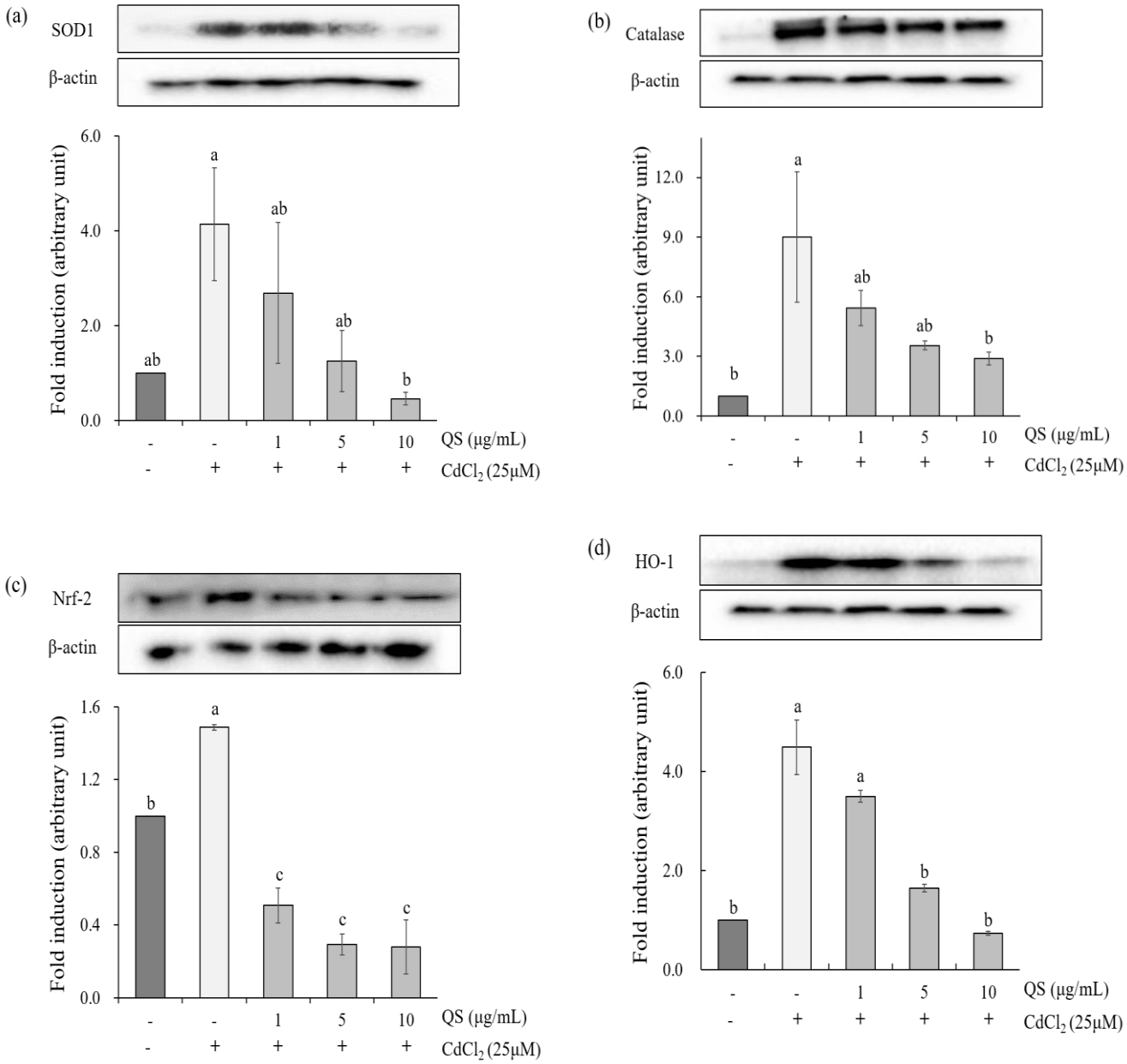


Figure 4. Effect of *Quercus salicina* Blume leave extract (QS) on the oxidative stress induced enzyme levels in the presence of cadmium: SOD 1 (a), catalase (b), Nrf-2 (c), and HO-1 (d).

Effect of QS on apoptosis control in the presence of cadmium: To elucidate whether ROS quenching was accompanied with apoptosis control, the alteration in apoptotic body formation caused by cadmium would

be prevented by QS, DAPI staining assays were performed. As shown in Fig. 5 the induction of anti-apoptosis was observed with QS at the concentrations of 5, 10 µM of QS, and at the concentrations of 5, 10 µM of QS the apparent decrease in the apoptotic bodies were found indicating QS protected HK-2 cells from apoptotic damage of cadmium.

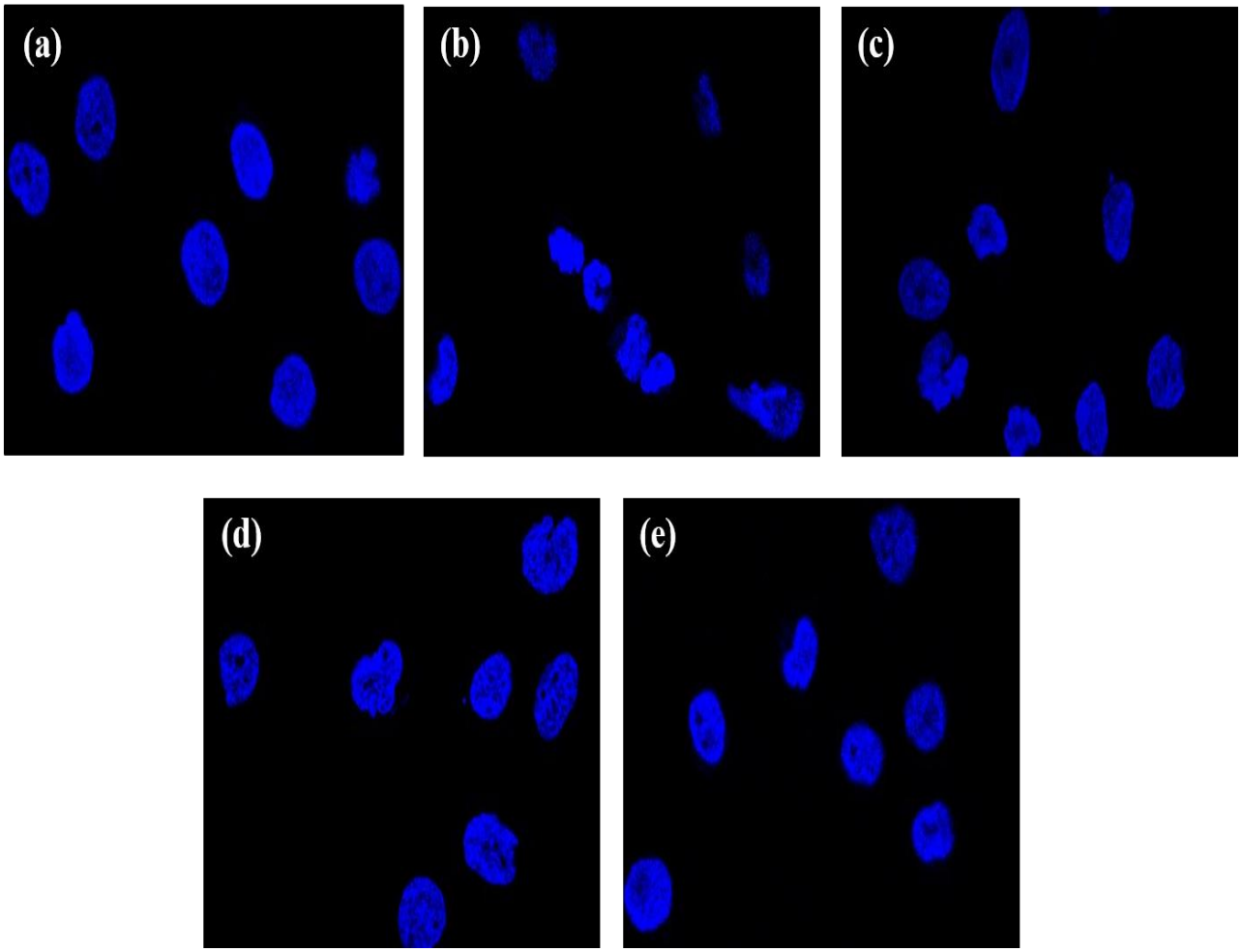


Figure 5. Effect of *Quercus salicina* Blume leave extract (QS) on nuclear morphological changes in $CdCl_2$ treated HK-2 cells: Cells (1×10^5/well) were treated with PBS alone (a), QS 0 µg/mL (b), 1 µg/mL (c), 5 µg/mL (d), and 10 µg/mL (e) in the presence of 25 µM $CdCl_2$. The apoptotic body was observed by DAPI staining by confocal laser scanning microscope (×600).

Cells in sub-G1 phase represent cells that will have fractional DNA content that has undergone apoptosis [24, 25]. To further validate the regulatory impact of QS on apoptosis induced by cadmium oxidative stress, the percentage of cells in the sub-G1 phase was calculated following cell cycle analysis using flow cytometry (Fig. 6). The Cd-only treatment group exhibited a significantly higher percentage of cells in the sub-G1 phase compared to the PBS-only control group. This suggested that apoptosis occurred in HK-2 cells by Cd toxicity. However, the cell ratio in the sub-G1 phase tended to decrease by QS treatment compared to the cadmium-treated group, confirming that cadmium-induced apoptosis was regulated by QS, like the results of nuclear morphological changes through DAPI staining.

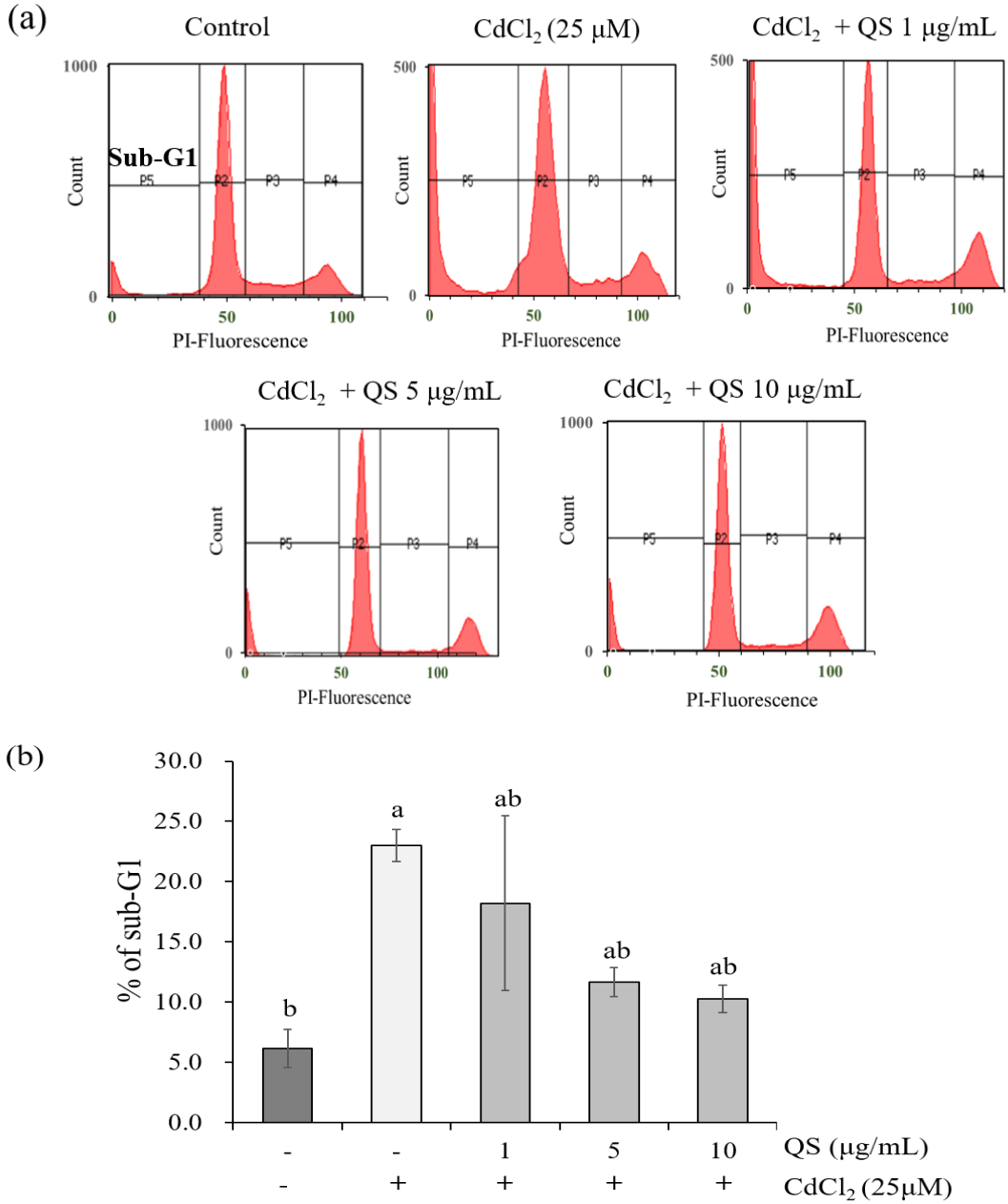


Figure 6. Effect of *Quercus salicina* Blume leave extract (QS) on changes in the percentage of the sub-G1 portion of the cell cycle: HK-2 cells were treated with PBS alone (control), QS 0 μg/mL, 1 μg/mL, 5 μg/mL, and 10 μg/mL in the presence of 25 μM $CdCl_2$, and examined by flow cytometry. Histogram represents PI fluorescence intensity (a), and the sub-G1 portion of the samples is expressed as a percentage (b).

Effect of QS on apoptosis related protein expressions in the presence of cadmium: To confirm whether QS affects apoptosis related protein expressions, western blotting was performed (Fig. 7). The presence of cadmium led to an increase in the protein expression of the pro-apoptotic marker Bax (Fig. 7a). However, the addition of QS resulted in a concentration-dependent reduction of Bax expression. In contrast, anti-apoptotic Bcl-2 expressions were increased by the addition of cadmium (Fig. 7b), and this was reversed by the treatment of QS (1-10 μg/mL). Also, procaspase-9 expression was elevated by the cadmium treatment, and this was reversed by pre-treatment with QS in a concentration-dependent manner. The increased caspase-3 expression was reversed by the QS.

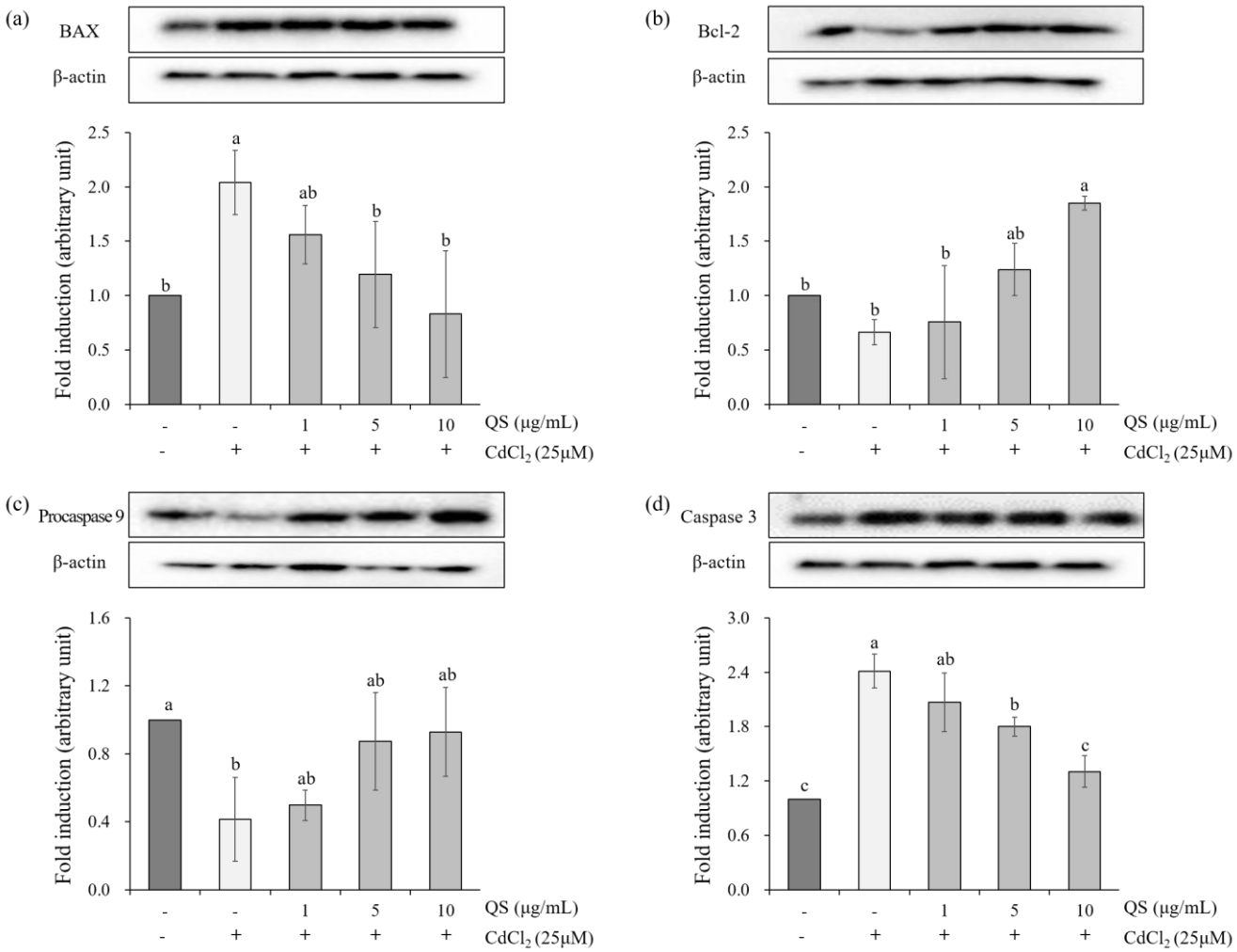


Figure 7. Effect of *Quercus salicina* Blume leave extract (QS) on the protein expression related to apoptosis in $CdCl_2$ treated HK-2 cells: BAX (a), Bcl-2 (b), procaspase 9 (c), and caspase 3 (d).

DISCUSSION

Cadmium is regarded as one of the most serious toxic pollutants possibly from high mobility and lower threshold to bring toxicity [1]. Cadmium in the form of cadmium ion is known to cause both acute and chronic toxicity in organisms [6]. These toxicities are often linked to oxidative stress which in turn is linked to cellular damage and abnormalities [3]. Some reports claim that cadmium at low concentration is capable of inducing DNA oxidative damage and lessening genetic stability of cells [26]. HK-2 cells have been developed for investigating nephrotoxicity *in vitro*, and the HK-2 cells have been used for studying nephrotoxicity of heavy metals such as cadmium [1, 6, 8]. Cadmium is a food contaminant that can be highly transferred from soil to plants, making the diet the primary source of exposure. In response, the World Health Organization (WHO) has set a safe intake limit of 7 μg cadmium/week/kg body weight, grounded on renal cadmium concentrations ranging from 100-200 μg/g wet weight. This is equivalent to the urinary threshold limit of 5-10 μg/g creatinine [27]. Nonetheless, various studies have claimed that adverse kidney effects can occur as little as less than 0.5 μg/g creatinine [3, 27]. Acute exposure of HK-2 cells to cadmium has been shown to cause apoptosis by stimulating pro-apoptotic proteins including caspases [28]. Apoptosis is linked to increased reactive oxygen species production triggered by cadmium. Since cadmium causes oxidative stress, an adequate antioxidative system would be a cell defense mechanism. Key protective antioxidative enzymes encompass superoxide dismutase (SOD), catalase

(CAT), Nrf-2, and Heme oxygenase-1 (HO-1) [29, 30]. *Quercus salicina Blume,* native to Korea and Japan, is known to have health-promoting attributes including antioxidant, anti-inflammatory, antibacterial, anti-edema, stone dissolving, and diuretic properties [15, 16]. This study had two main objectives. The first goal was to evaluate the free radical scavenging effect after quantifying the total phenol and flavonoid content. The second objective was to evaluate the protective effect against oxidative stress induced by cadmium in HK-2 kidney-origin cells in vitro. The analysis revealed total polyphenol contents of 287.12 mg GAE/g and total flavonoid contents of 23.58 mg QE/g. The DPPH radical scavenging assay and FRAP value assay also indicated that QS possess high antioxidative potential.

In general, research on plant leaves has primarily focused on their antioxidant attributes, which have demonstrated efficacy in *in vitro* systems [9, 15]. This study broadened its scope to evaluate the extract's capability to scavenge reactive oxygen species (ROS) in the presence of cadmium-induced stress in HK-2 cells. The introduction of QS to Cd-treated HK-2 cells revealed a noticeable reduction in cytotoxicity. The expression of antioxidant-related proteins, including SOD 1, catalase, Nrf-2, and heme oxygenase-1, in HK-2 cells exhibited an increasing trend upon treatment with cadmium alone, but their expression was downregulated upon co-treatment with QS. Antioxidant defense systems in the body against oxidative stress are largely divided into enzymatic and non-enzymatic defense mechanisms [31]. Enzymatic mechanisms include SOD, catalase, and glutathione peroxidase (Gpx). SOD converts O_2^- into H_2O_2, catalase breaks down H_2O_2 into water and oxygen, and Gpx reacts glutathione with H_2O_2 to produce water [32]. Non-enzymatic mechanisms, on the other hand, react with ROS such as $^1O_2$ and OH^- that are not targeted by antioxidant enzymes. Antioxidants such as vitamin A, vitamin E, bilirubin, and uric acid are among the non-enzymatic defenses. Unlike antioxidant enzymes in the body, non-enzymatic defenses can be supplied from external sources. Plant polyphenol compounds have high antioxidant activity, and numerous studies have investigated the cellular-level antioxidant activity of polyphenols derived from plants. Nrf-2 is a type of non-enzymatic defense mechanism that acts as a nuclear transfer factor, binding to Kelch-like ECH-associated protein 1 (Keap 1) in a stable state [33]. However, in oxidative stress environments such as exposure to ultraviolet rays, H_2O_2, cytokines, and heavy metals, Keap 1 may become deformed or phosphorylated, causing separation of Nrf-2 from Keap 1. In response to oxidative stress, free Nrf-2 moves into the nucleus and binds to ARE, which leads to the expression of antioxidant-related proteins such as HO-1. Alam et al. [34] reported that treatment with 10 μM cadmium in MCF-7 cells sequentially activated p38 and Nrf-2, resulting in an increased expression of HO-1. Similarly, Stewart et al. [35] found that exposure of HepG2 cells to 50 μM cadmium showed a significant increase in Nrf-2 and HO-1 expression compared to untreated cells. In this study, the treatment of HK-2 cells with cadmium-induced oxidative stress revealed that QS functions as a non-enzymatic defense mechanism. It effectively reduced intracellular oxidative stress and downregulated the expression of both enzymatic and non-enzymatic defense mechanisms. Furthermore, apoptotic stress caused by cadmium in HK-2 cells seemed to be restored since it was shown that pro-apoptotic protein, Bax expression was decreased by the addition of QS, whereas anti-apoptotic protein, Bcl_2 expression was elevated by QS. Moreover, the examination of procaspase-9 and caspase-3 has shown that the significant anti-apoptotic activities by QS were

evident when HK-2 cells in the presence of cadmium compared to the cadmium challenge alone.

CONCLUSIONS

In this study, *Quercus salicina* Blume leave extract (QS) was prepared using a hot water extraction method. The prepared QS contained phytochemicals such as polyphenols and flavonoids, and the antioxidant activity of QS increased in a concentration-dependent manner when evaluated by DPPH and FRAP. QS exhibited considerable protective properties by modulating the antioxidant activity and regulating certain antioxidant enzymes under conditions of cadmium-induced toxicity. The anti-apoptotic effect of QS was identified to be mediated, in part, by reducing Bax activities and increasing Bcl2 activities. Additionally, there was an elevation of procaspase-9 along with an attenuation of caspase-3 activities. These findings offer evidence for the health-promoting potential of QS in the presence of cadmium exposure. The results suggest that QS possesses antioxidant activities and provides protection against heavy metals, making it valuable as a potential anti-oxidative functional food aimed at addressing heavy metal pollution.

Abbreviations: QS: *Quercus salicina* Blume leave extracts, Cd: cadmium, $CdCl_2$: Cadmium chloride, ROS: reactive oxygen species, DPPH: 2,2-diphyenyl-1-picrylhydrazyl, FRAP: ferric ion reducing antioxidant power, MTT: 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, DCF-DA: 2'7'-dichrorofluorescin diacetate, TPC: total phenolic content, TFC: total flavonoid content, GAE: Gallic acid equivalent, QE: quercetin equivalent, DAPI: 4',6-diamidino-2-phenylindole, Na_2CO_3: sodium carbonate solution, CH_3COOK: potassium acetate, PI: propidium iodide, SOD: superoxide mutase, CAT: catalase, HO-1: heme oxygenase-1

Competing Interests: The authors declare that they have no conflicts of interest.

Author Contributions: YJK participated in experimentation, data collection, statistical analysis, and drafting the manuscript. JL provided advice on the development of protocol of the study. AO designed and supervised the study and completed the manuscript.

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