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Daphne jejudoenosis and its bioactive compounds protect kidney proximal tubular cells against hydrogen peroxide-induced oxidative stress

Daeun Moon[1], Ji-Yeon Lee[2,3], Yoon-A. Kang[3], Ji-Yeong Bae[2,3*] and Jinu Kim[1,2*]

Abstract
Reactive oxygen species (ROS), particularly hydrogen peroxide (H_2O_2), are implicated in oxidative stress and kidney tubular injury. *Daphne jejudoensis* (DJ), a plant native to Jeju Island, has demonstrated anti-inflammatory properties, but its effects on kidney diseases remain unexplored. This study investigated the impact of DJ extract and its compounds, daphnin and daphnetin, on H_2O_2-induced cell death and ROS production in kidney proximal tubular HK-2 cells. Extracts from the DJ plant parts significantly enhanced cell viability in H_2O_2-exposed cells. The DJ leaf extract significantly reduced levels of superoxide anion and hydroxyl radical and prevented the decrease in superoxide dismutase (SOD) activity. Fractions from 70% ethanol DJ leaf extract exhibited protective effects against H_2O_2-induced injury, with the ethyl acetate fraction standing out for its ability to enhance antioxidant enzyme activities. Daphnin and daphnetin, major bioactive compounds of DJ extract, significantly increased cell viability and reduced ROS production in H_2O_2-exposed cells, restoring SOD and catalase activities. Conclusively, DJ extract and the main compounds, daphnin and daphnetin, protect kidney proximal tubular cells against H_2O_2-induced oxidative stress by reducing ROS production and enhancing antioxidant defenses. These findings suggest the potential therapeutic use of DJ in kidney diseases associated with oxidative stress.

Keywords *Daphne jejudoensis*, Hydrogen peroxide, Oxidative stress, Kidney proximal tubular cell, Daphnin, Daphnetin

Introduction

Reactive oxygen species (ROS) are oxygen-containing chemically reactive molecules that are natural byproducts of normal cellular metabolism, particularly generated in the mitochondria [1]. While ROS play a crucial role in cell signaling and maintaining homeostasis, excessive ROS can induce oxidative stress, damaging cellular protein, lipids, and DNA, which is associated with various diseases [2]. Among ROS, hydrogen peroxide (H_2O_2) is relatively stable and membrane-permeable, serving as a crucial signaling molecule that regulates processes such as cell growth and immune responses [3]. Elevated levels of H_2O_2 can cause oxidative stress, leading to kidney

*Correspondence:
Ji-Yeong Bae
jybae@jejunu.ac.kr
Jinu Kim
jinu.kim@jejunu.ac.kr
[1]Department of Anatomy, Jeju National University College of Medicine, 102 Jejudaehak-ro, Jeju 63243, Republic of Korea
[2]Interdisciplinary Graduate Program in Advanced Convergence Technology & Science, Jeju National University, Jeju 63243, Republic of Korea
[3]College of Pharmacy, Jeju National University, 102 Jejudaehak-ro, Jeju 63243, Republic of Korea

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tubular injury in various conditions, such as kidney ischemia and reperfusion injury [4], cisplatin nephrotoxicity [5], glomerulonephritis [6], and ureteral obstruction [7]. The proximal tubules in the kidney are more susceptible to cell injury and death than the distal tubules in these situations [8]. Therefore, understanding the mechanisms of hydrogen peroxide-induced injury in proximal tubular cells is crucial for studying and treating kidney diseases.

Daphne jejudoensis (DJ), a plant belonging to the Thymelaeaceae family, is native to Jeju Island in the Republic of Korea and is commonly found in the Gotjawal forest areas [9]. Initially classified as conspecific with *Daphne kiusiana*, DJ was identified as a distinct species in 2013 due to several morphological differences, including smooth calyx surfaces, hairless calyx tubes and lobes, and elliptic leaves [10]. Several species of the genus *Daphne* are used in traditional medicine in Korea, Tibet, China, and the Middle East for treating various conditions, including cancer [11], allergic asthma [9], rheumatism [12], and viral infections [13]. Specifically, *Daphne kiusiana* has been shown to effectively inhibit inflammation [14, 15]. Recently, DJ extract has also demonstrated significant decreases in the expression of tumor necrosis factor, interleukin 1 beta, interleukin 6, nitric oxide synthase 2, and prostaglandin-endoperoxide synthase 2, in lipopolysaccharide-exposed periodontal ligament cells [16]. Despite these findings, the effects of DJ extract on kidney diseases remain largely unexplored. In this study, we investigated the impact of DJ extract on H_2O_2-induced cell death and ROS in kidney proximal tubular cells. Additionally, we fractionated the extract and isolated its main compounds to test their effects on H_2O_2-induced cell death and ROS production

Materials and methods

Extraction and fractionation of DJ

The dried and powdered leaves of DJ (995.8 g) were soaked in 70% ethanol and sonicated (Powersonic 520, hwashintech, Republic of Korea) for 30 min, three times. The extracted supernatant was collected by centrifugation at 4,500 rpm for 5 min (Avanti J-15R, Beckman Coulter, Brea, CA, USA) and filtered with Whatman NO. 1 filter paper (Maidstone, United Kingdom). The filtrate was evaporated under reduced pressure using a rotary evaporator (Heidolph, Schwabach, Germany). The total extract of DJ (160 g) was further fractionated using sequential solvent partitioning by different solvents of increasing polarity, including hexane, methylene chloride (MC), ethyl acetate (EA), butanol, and water, to obtain different solvent fractions. The percentage yield of the dried fractions was calculated, and the fractions obtained were stored in a refrigerator at−4 °C prior to analysis.

Cell culture and exposure to hydrogen peroxide

The HK-2 human kidney proximal tubular cell line (KCLB, Seoul, Korea; product no. 22190) was cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum at 37 °C with 5% CO_2, as described previously [17]. After reaching 80% confluence on culture dishes, the culture medium was replaced with a serum-free medium. Subsequently, the cells were treated with DJ extract and its compounds, including daphnin (Sigma-Aldrich, St. Louis, MO, USA; product no. PHL85730) and daphnetin (Sigma-Aldrich; product no. PHL89621), for 60 min. They were then exposed to 600 μM hydrogen peroxide (H_2O_2; Sigma-Aldrich; product no. H1009) or distilled water (control) for 6 h. All types of DJ extracts were dissolved in the culture medium and administered after filtration using a 0.20 μm pore size filter (Advantec, Dublin, CA, USA; product no. 25AS020AS).

Cell viability

A yellow water-soluble tetrazolium dye, thiazolyl blue tetrazolium bromide (MTT; Biosesang, Seongnam, Korea; product no. M1029), was used to measure cell viability, as described previously [18]. After removing the culture medium, HK-2 cells on 24-well plates were incubated at 37 °C with 300 μl of 5 mg/ml MTT in phosphate-buffered saline (PBS) for 30 min. After removing the MTT solution, 300 μl of dimethyl sulfoxide (DMSO) was added to each well and incubated for 5 min. To quantify the purple-colored formazan product, the absorbance of 100 μl of the solution from wells incubated with DMSO was measured on a 96-well plate at a wavelength of 595 nm with a reference wavelength of 620 nm using the SpectraMax i3x multi-mode microplate reader (Molecular Devices, San Jose, CA, USA) in the Bio-Health Materials Core-Facility at Jeju National University.

Superoxide anion and hydroxyl radical levels

The fluorescent dyes, dihydroethidium (DHE; Sigma-Aldrich; product no. 37291) and 2',7'-dichlorofluorescin diacetate (DCFDA; Sigma-Aldrich; product no. D6883), were employed to assess the production of intracellular superoxide anion and hydroxyl radical, respectively. HK-2 cells attached to 60 mm dishes were detached using 0.05% trypsin-ethylenediaminetetraacetic acid and then incubated with 1 μM DHE and 10 μM DCFDA for 30 min. After isolating into a single-cell population, the DHE and DCFDA fluorescence were detected through the phycoerythrin-area (PE-A) and fluorescein isothiocyanate-area (FITC-A) channels, respectively, using the CytoFLEX flow cytometer and CytExpert software purchased from Beckman Coulter (Indianapolis, IN, USA). The single-cell population was defined by side scatter-height versus side scatter-width, followed by forward

scatter-height versus forward scatter-width in the flow cytometer.

Superoxide and hydroxyl radical scavenging capacities

Scavenging capacities for superoxide and hydroxyl radical were assessed using commercial kits purchased from MyBioSource (San Diego, CA, USA; product no. MBS9718966 and MBS9719201, respectively) following the manufacturer's instructions. These assays, based on the methods of Ukeda [19] and Floyd [20], respectively, were modified to improve robustness and convenience. Absorbance measurements were taken using a spectrophotometer (Cole-Parmer, Staffordshire, UK) at 450 nm for superoxide scavenging capacity and 520 nm for hydroxyl radical scavenging capacity.

Enzyme activity

HK-2 cells were maintained in ice during sample preparation and assay. The protein concentration in the homogenates was determined using the Bradford reagent (Sigma-Aldrich; product no. B6916) and then normalized to units per milligram of protein for enzyme activity. Catalase and superoxide dismutase (SOD) activities were determined using commercial kits (Northwest Life Science Specialties, Vancouver, WA, USA; product no. NWK-CAT01 and Cayman Chemical, Ann Arbor, MI, USA; product no. 706002, respectively) according to the manufacturer's instructions. These assays are based on the respective methods of Beers [21] and Marklund [22], with the following modifications made to enhance robustness and convenience. One unit (U) of catalase is defined as the amount of enzyme that decomposes 1.0 mmol of hydrogen peroxide per minute at 25 °C. One unit (U) of SOD was defined as the amount of enzyme required to exhibit 50% dismutation of the superoxide radical, measured by the change in absorbance per minute at 25 °C. The absorbances in the assays were measured using the Cole-Parmer spectrophotometer at 240 nm and 450 nm for catalase and SOD activities, respectively.

Liquid chromatography conditions for the identification of main compounds in DJ samples

The determination of daphnetin and daphnin content was carried out by high performance liquid chromatography (HPLC) analysis. The fraction samples were prepared in HPLC grade methanol (Thermo Fisher Scientific, USA) at a concentration of 10 mg/ml and filtered using PTFE 0.45 μm syringe filters (LKLAB, Republic of Korea). HPLC analysis was performed on an Agilent infinity 1260 series HPLC equipped with a J'sphere ODS H80 column (4.5 × 250 mm, 4 μm, S-8 nm; YMC, Tokyo, Japan). The injection volume was 5 μl with a flow rate of 1.2 ml per minute, and the column temperature was maintained at 25 °C. The mobile phase was composed of water with 0.1% formic acid (Solvent A) and acetonitrile with 0.1% formic acid (Solvent B). A gradient program was adapted as follows: 0–5 min, B 0–10%; 5–15 min, B 10–20%; 15–25 min, B 20–30%; 25–45 min, B 30–40%; 45–55 min, B 40–60%; 55–60 min, B 60–80%; 60–65 min, B 80–100%; 65–75 min, B 100–0%. The UV wavelength for detection was sel at 280 nm.

Statistical analysis

Numeric data were subjected to statistical analysis using SigmaPlot 14.0 (Systat Software, San Jose, CA, USA), as previously outlined [23]. The normal distribution was evaluated via the Shapiro–Wilk normality test. If the data did not meet the criteria for normality, a logarithmic transformation was applied. Parametric data were analyzed using one-way analyses of variance (ANOVA) for a single independent variable and two-way and three-way ANOVA for two or more independent variables. A Holm-Sidak's post hoc test was then performed. In graphical representations, data were depicted as mean ± standard error of the mean (SEM) with individual data points. A significant level of 0.05 was deemed statistically significant. The supplementary material contains all raw numeric data and corresponding statistical outcomes.

Results

Protective effect of DJ plant leaf extract against H_2O_2-induced injury in kidney proximal tubular cells

To assess the impact of DJ on H_2O_2 exposure in kidney proximal tubular cells, the plant was divided into four parts: flower, branch, root, and leaf. Subsequently, cell viability in HK-2 cells treated with these extracts and exposed to H_2O_2 was measured using the MTT assay. After 6 h of H_2O_2 exposure, cell viability decreased to 66.5% (Fig. 1A). Treatment with extracts from all plant parts at 100 μg/ml significantly enhanced cell viability (Fig. 1A). Notably, leaf extract at 1 and 10 μg/ml also significantly increased cell viability, while extracts from other parts did not. A two-way ANOVA of cell viability revealed that both the plant part and the dose had significant effects (F = 2.821, p = 0.044 and F = 29.653, $p < 0.001$, respectively); however, the interaction between the plant part and the dose was not significant (F = 0.651, p = 0.750). These findings suggest that the DJ plant, especially leaf, extract has a protective effect against H_2O_2-induced injury in kidney proximal tubular cells.

DJ leaf extract mitigates H_2O_2-induced ROS in kidney proximal tubular cells

To investigate whether DJ extract affects ROS production induced by H_2O_2, we evaluated to evaluate superoxide anion and hydroxyl radical levels using DHE and DCFDA dyes in flow cytometry, respectively. Among the plant

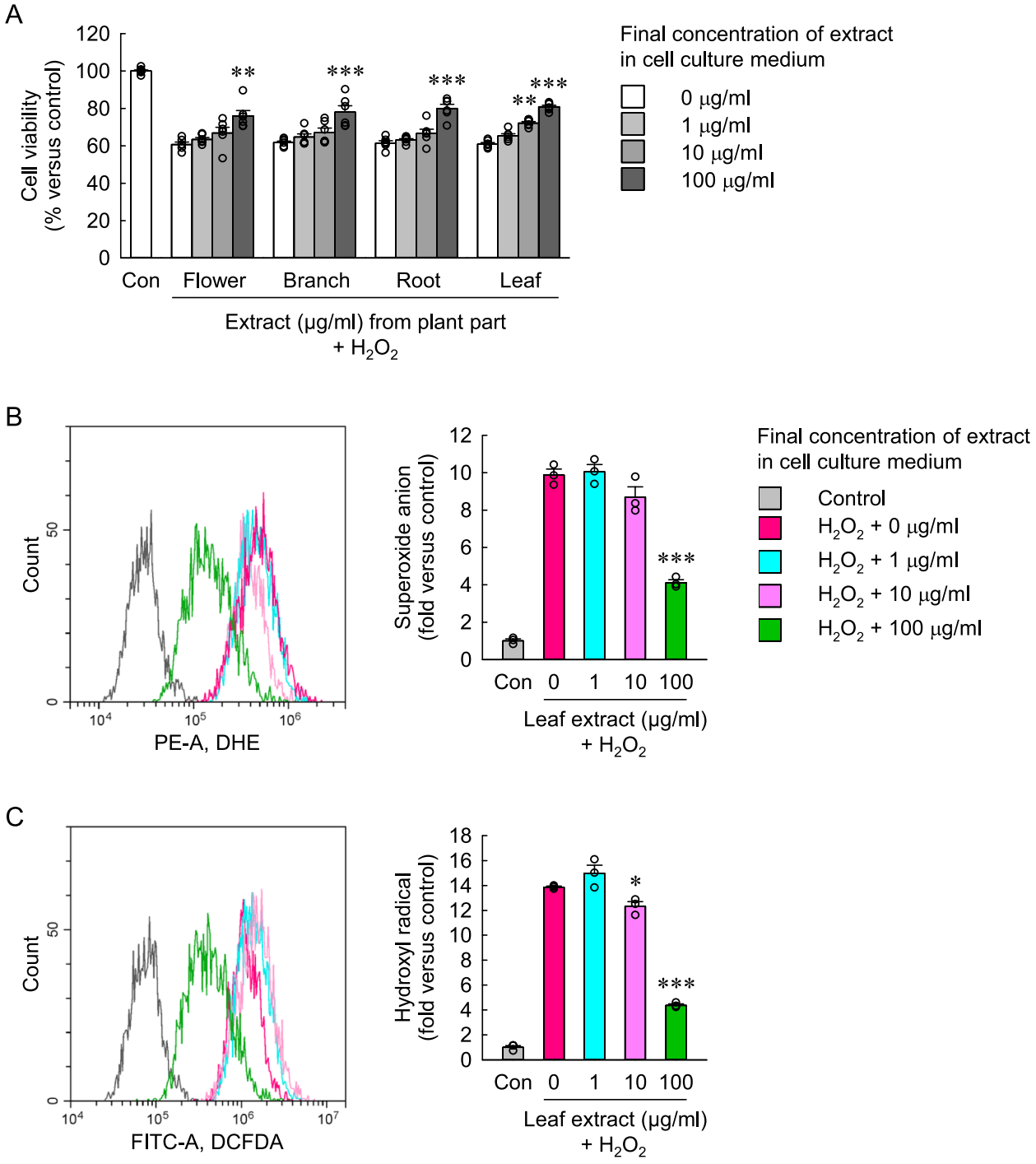


Fig. 1 DJ extract attenuates ROS production induced by H_2O_2 exposure in human kidney proximal tubular cells. HK-2 cells were treated with 70% ethanol extracts of DJ flower, branch, root, and leaf for 60 min, followed by exposure to 600 μM H_2O_2 for 6 h. (**A**) Cell viability was assessed using the MTT assay. (**B**) Representative histogram of DHE fluorescence intensity using flow cytometry in the left histogram graphs. Quantification of superoxide anion levels using mean values of DHE in the right bar graphs. (**C**) Representative histogram of DCFDA fluorescence intensity using flow cytometry in the left histogram graphs. Quantification of hydroxyl radical levels using mean values of DCFDA in the right bar graphs. Statistical significance was determined by two-way ANOVA (**A**) and one-way ANOVA (**B**, **C**) followed by Holm-Sidak's post hoc test. Con denotes control. *p<0.05, **p<0.01, ***p<0.001 versus 0 μg/ml

parts, the leaf extract had the most significant effect on cell viability and was selected for further study (Fig. 1A, $p < 0.01$ at 10 μg/ml and $p < 0.001$ at 100 μg/ml versus 0 μg/ml, respectively). Exposure to H_2O_2 dramatically increased the superoxide anion level by 9.9-fold compared to the control (Fig. 1B). However, treatment with the extract at 100 μg/ml significantly reduced the elevated level (Fig. 1B). Similarly, the hydroxyl radical level increased by 13.9-fold after H_2O_2 exposure compared to the control, but treatment with the extracts at 10 and 100 μg/ml significantly reduced this increase (Fig. 1C). Statistical analyses using one-way ANOVA on superoxide anion and hydroxyl radical showed a significant effect among groups ($F = 54.267$, $p < 0.001$ and $F = 373.027$, $p < 0.001$, respectively). These findings suggest that DJ leaf extract attenuates ROS production induced by H_2O_2 exposure in kidney proximal tubular cells.

DJ leaf extract enhances intracellular antioxidant enzymes in H_2O_2-exposed kidney proximal tubular cells

Since many plant-derived extracts have been reported to possess antioxidant capacity [24], we tested whether DJ leaf extract had the ability to scavenge superoxide anion and hydroxyl radical. The results showed that the DJ leaf extract did not significantly increase the level of scavenging capacities for superoxide and hydroxyl radical (Fig. 2A,B). This suggests that the DJ leaf extract does not directly reduce superoxide anion and hydroxyl radical ($F = 0.351$, $p = 0.789$ and $F = 0.905$, $p = 0.456$, respectively, as determined by one-way ANOVA). Given this, we next tested whether the DJ leaf extract stimulated the activity of intracellular antioxidant enzymes, including SOD and catalase, in HK-2 cells. Exposure to H_2O_2 significantly reduced SOD activity, but treatment with the DJ leaf extract at 10 and 100 μg/ml significantly restored the enzyme activity (Fig. 2C). Consistently, exposure to H_2O_2 significantly reduced catalase activity, but treatment with the DJ leaf extract at 100 μg/ml significantly attenuated this reduction in enzyme activity (Fig. 2D). Statistical analysis using one-way ANOVA on SOD activity and catalase activity revealed significant effects among the groups ($F = 23.173$, $p < 0.001$ for SOD activity and $F = 5.380$, $p = 0.007$ for catalase activity). Taken together, these findings suggest that DJ leaf extract attenuates ROS production by enhancing the activation of intracellular antioxidant enzymes in H_2O_2-exposed kidney proximal tubular cells.

Protective effects of fractions of DJ leaf extract against H_2O_2-induced injury in kidney proximal tubular cells

After fractionating the 70% ethanol extract of DJ leaves into hexane, MC, EA, butanol, and water in order, we investigated whether these fractions exhibited a protective effect against H_2O_2-induced injury in kidney proximal tubular cells. Treatment with the hexane fraction at 100 μg/ml, the MC fractions at 10 and 100 μg/ml, and the EA fractions at 10 and 100 μg/ml significantly alleviated the decrease in cell viability after 6 h of H_2O_2 exposure (Fig. 3A). However, the butanol and water fractions had no impact on cell viability (Fig. 3A). Statistical analysis using two-way ANOVA on cell viability showed significant effects of fraction ($F = 10.516$, $p < 0.001$), dose ($F = 18.647$, $p < 0.001$), and the interaction between fraction and dose ($F = 5.975$, $p < 0.001$). These findings suggest that fractions of DJ leaves protect kidney proximal tubular cells against H_2O_2-induced injury.

Fractions of DJ leaf extract enhance antioxidant defense system in H_2O_2-exposed kidney proximal tubular cells

We investigated the effects of fractions of DJ leaf extract in 70% ethanol on ROS production and antioxidant enzyme activity in H_2O_2-exposed kidney proximal tubular cells. Treatment with hexane, MC, and EA fractions at 100 μg/ml significantly reduced the superoxide anion level in H_2O_2-exposed HK-2 cells (Fig. 3B). Especially, the concentration of 10 μg/ml in MC and EA fractions also showed a significant reduction of superoxide anion level in H_2O_2-exposed HK-2 cells (Fig. 3B). Although the superoxide anion level is slightly higher at 100 μg/ml compared to 10 μg/ml, this difference is not statistically significant ($p = 0.224$) and is therefore not considered a meaningful effect. Statistical analysis using two-way ANOVA on superoxide anion showed a significant effect of dose ($F = 22.247$, $p < 0.001$), but no significant effects of fraction ($F = 2.657$, $p = 0.091$) and the interaction between fraction and dose ($F = 1.412$, $p = 0.251$). Consistently, treatment with hexane, MC, and EA fractions at 100 μg/ml significantly reduced the hydroxyl radical level in H_2O_2-exposed HK-2 cells (Fig. 3C). Especially, the concentration of 10 μg/ml in the EA fraction also showed a significant reduction of superoxide anion level in H_2O_2-exposed HK-2 cells (Fig. 3C). Statistical analysis using two-way ANOVA on superoxide anion showed significant effects of fraction ($F = 4.283$, $p = 0.026$) and dose ($F = 49.678$, $p < 0.001$), but no significant effect of the interaction between fraction and dose ($F = 1.064$, $p = 0.411$). Among the fractions, the EA fraction at 10 μg/ml had the most significant effect on reducing ROS production and was selected for evaluating antioxidant enzyme activities (Fig. 3B,C). Treatment with the EA fraction at 10 and 100 μg/ml restored the SOD activity reduced by H_2O_2 exposure in HK-2 cells (Fig. 3D). Additionally, concentrations of 10 and 100 μg/ml in the EA fraction prevented the decrease in catalase activity in H_2O_2-exposed HK-2 cells (Fig. 4E). Statistical analysis using one-way ANOVA on SOD and catalase activities showed significant effects among groups ($F = 9.740$, $p < 0.001$ and $F = 17.835$, $p < 0.001$, respectively). These

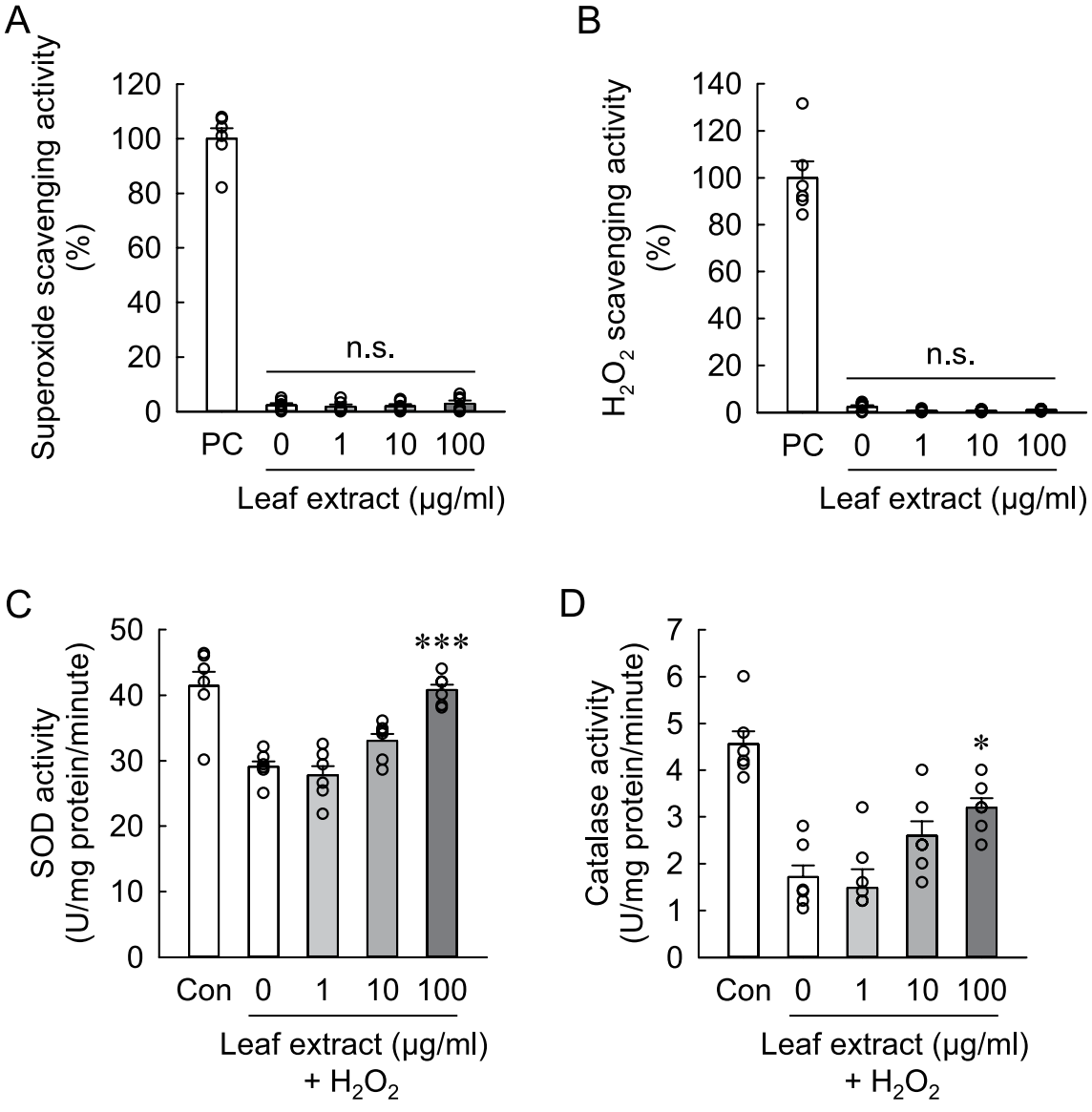


Fig. 2 DJ leaf extract inhibits reductions in SOD and catalase activities in human kidney proximal tubular cells after H_2O_2 exposure. HK-2 cells were treated with DJ leaf extracts in 70% ethanol for 60 min, followed by exposure to 600 μM H_2O_2 for 6 h. (**A**, **B**) Superoxide and hydroxyl radical scavenging capacities. (**C**, **D**) Activities of SOD and catalase. All statistical significance was determined using one-way ANOVA followed by Holm-Sidak's post hoc test. Con, PC, and n.s. denote control, positive control, and no significant difference, respectively. *$p<0.05$, ***$p<0.001$ versus 0 μg/ml

findings suggest that fractions of DJ leaf extract stimulate the antioxidant defense system following H_2O_2 exposure in kidney proximal tubular cells.

Daphnin and daphnetin enhance cell viability in H_2O_2-exposed kidney proximal tubular cells

The main compounds, daphnin and daphnetin, in extracts from various parts of the DJ plant in 70% ethanol (Supplementary Fig. 1) and fractions of the 70% ethanol leaf extract (Supplementary Fig. 2) were assessed through HPLC analysis. Next, we investigated the impact of these main compounds on H_2O_2-induced cell death and ROS production. At doses of daphnin and daphnetin that were not toxic in the control group, treatment with daphnin at 30 μM and daphnetin at 3, 10, and 30 μM significantly increased cell viability in H_2O_2-exposed HK-2 cells (Fig. 4A, B). The results of two-way ANOVA on cell viability are as follows: For daphnin, there was a significant effect of the H_2O_2 factor ($F=218.194$, $p<0.001$), while the dose factor ($F=1.626$, $p=0.199$) and the interaction between the two factors ($F=1.732$, $p=0.176$) were not significant. Similarly, for daphnetin, there was a significant effect of the H_2O_2 factor ($F=79.922$, $p<0.001$), while the dose factor ($F=1.231$, $p=0.311$) and the interaction between the two factors ($F=2.023$, $p=0.126$) were not significant.

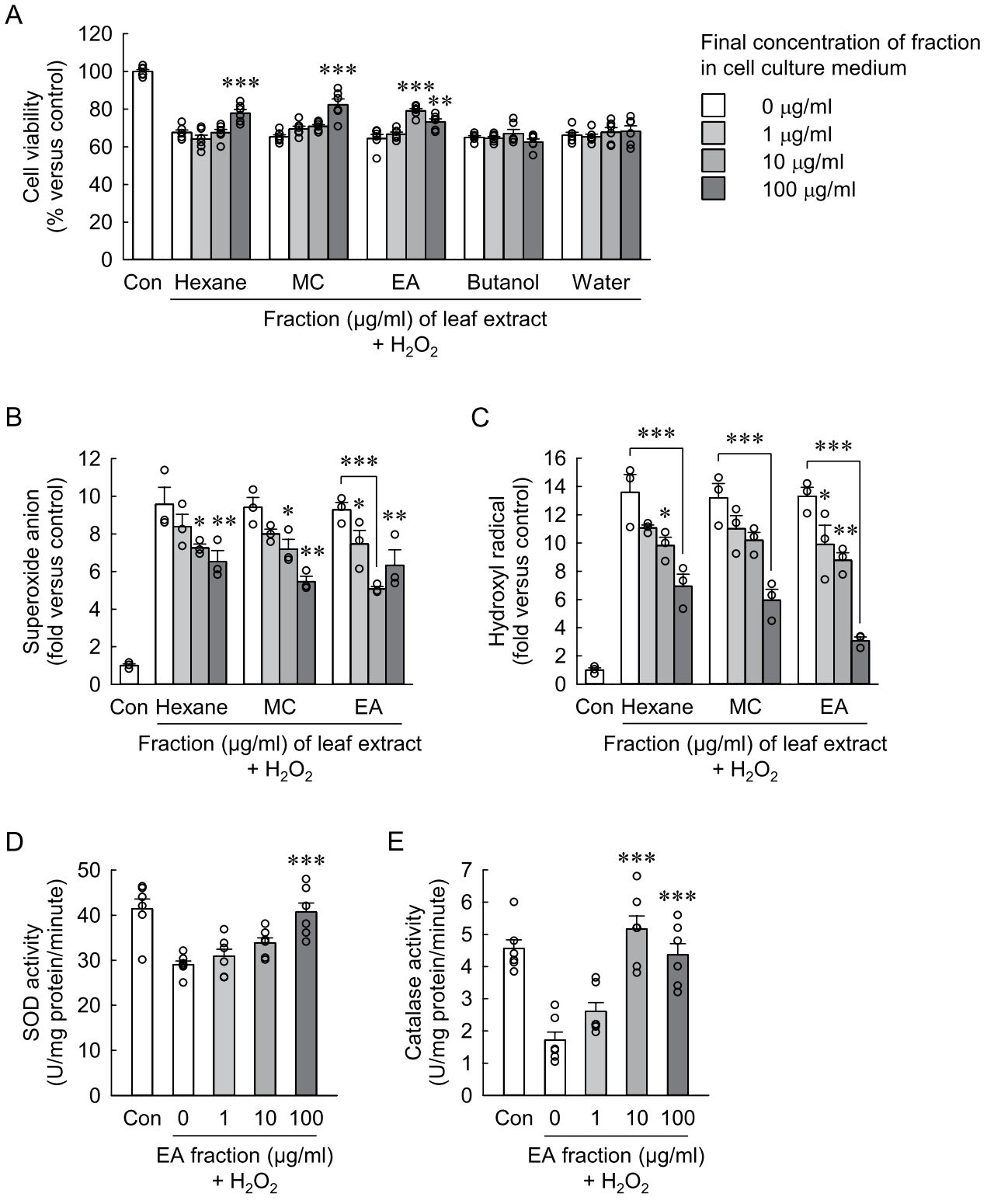


Fig. 3 Fractions of DJ leaf extract protect human kidney proximal tubular cells against ROS induced by H_2O_2. HK-2 cells were treated with hexane, MC, EA, butanol, and water fractions of DJ leaf extract for 60 min, followed by exposure to 600 μM H_2O_2 for 6 h. (**A**) Cell viability. (**B**, **C**) Levels of superoxide anion and hydroxyl radical were measured by mean values of DHE and DCFDA, respectively, using flow cytometry. (**D**, **E**) Activities of SOD and catalase. Statistical significance was determined by two-way ANOVA (**A**, **B**, **C**) and one-way ANOVA (**D**, **E**) followed by Holm-Sidak's post hoc test. *$p<0.05$, **$p<0.01$, ***$p<0.001$ versus 0 μg/ml

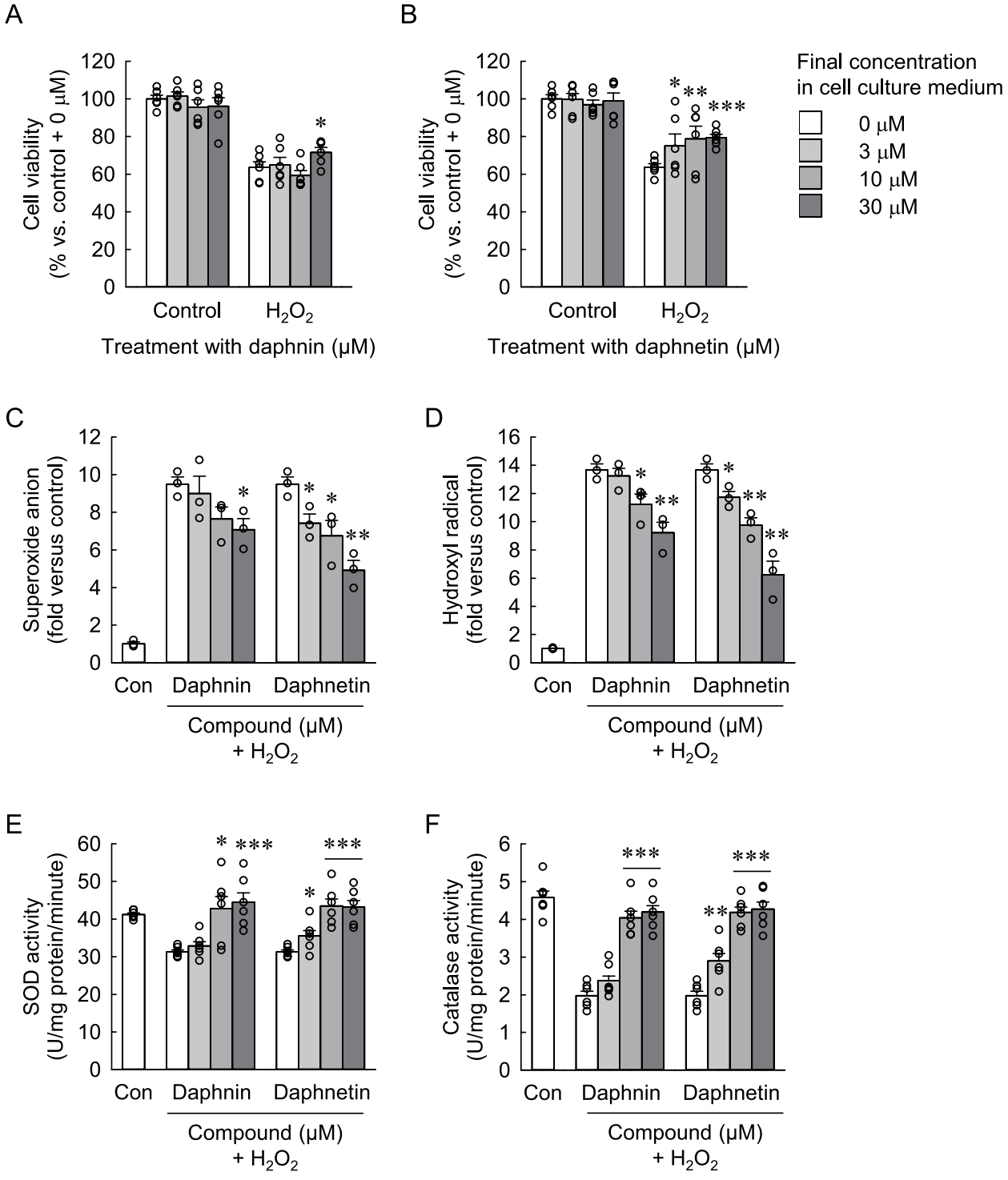


Fig. 4 Treatments with daphnin and daphnetin protect human kidney proximal tubular cells against ROS induced by H_2O_2. HK-2 cells were treated with daphnin and daphnetin for 60 min, followed by exposure to 600 μM H_2O_2 for 6 h. (**A**, **B**) Cell viability. (**C**) Superoxide anion levels. (**D**) Hydroxyl radical levels. (**E**) SOD activities. (**F**) Catalase activities. All statistical significance was determined by two-way ANOVA by Holm-Sidak's post hoc test. *$p<0.05$, **$p<0.01$, ***$p<0.001$ versus 0 μg/ml

Daphnin and daphnetin prevent oxidative stress in H_2O_2-exposed kidney proximal tubular cells

We then examined the effects of both daphnin and daphnetin on H_2O_2-induced oxidative stress in kidney proximal tubular cells by evaluating ROS production and antioxidant enzyme activity. Treatment with daphnin at 30 μM and daphnetin at 10 and 30 μM significantly reduced superoxide anion production induced by H_2O_2 exposure in HK-2 cells (Fig. 4C). The results of the statistical analysis using two-way ANOVA on superoxide anion production had significant effects of the compound type factor ($F = 7.008$, $p = 0.018$) and the dose factor ($F = 11.578$, $p < 0.001$), but no significant interaction between the two factors ($F = 1.129$, $p = 0.367$). Intracellular levels of hydroxyl radical were also significantly reduced, reaching levels comparable to the control, by treating H_2O_2-exposed HK-2 cells with daphnin and daphnetin at both doses of 10 and 30 μM (Fig. 4D). Especially, the dose of 3 μM in daphnetin more decreased hydroxyl radical level in H_2O_2-exposed HK-2 cells (Fig. 4D). The results of two-way ANOVA on hydroxyl radical production revealed significant effects of the compound type factor ($F = 4.833$, $p = 0.043$), the dose factor ($F = 33.882$, $p < 0.001$), and the interaction between the two factors ($F = 3.413$, $p = 0.043$). Furthermore, decreased activities of SOD and catalase after H_2O_2 exposure were completely restored by treatment with daphnin and daphnetin at 10 and 30 μM in HK-2 cells (Fig. 4E,F). Daphnin and daphnetin at 3 μM also significantly increased SOD and catalase activities in H_2O_2-exposed HK-2 cells (Fig. 4E,F). Statistical analysis using two-way ANOVA on SOD activity for daphnin and daphnetin showed a significant effect of the dose factor ($F = 21.402$, $p < 0.001$), but no significant effect of the compound type factor ($F = 0.317$, $p = 0.576$) and interaction between the two factors ($F = 0.370$, $p = 0.775$). Similarly, two-way ANOVA on catalase activity for daphnin and daphnetin also showed a significant effect of the dose factor ($F = 14.788$, $p < 0.001$), but no significant effect of the compound type factor ($F = 2.009$, $p = 0.164$) and interaction between the two factors ($F = 0.764$, $p = 0.521$). Taken together, these findings suggest that both daphnin and daphnetin prevent an imbalance between ROS production and the antioxidant system in H_2O_2-exposed kidney proximal tubular cells, thereby providing protection against oxidative stress.

Discussion

Acute kidney injury is commonly due to acute tubular necrosis (ATN), particularly in proximal tubules [25]. It usually represents a reversible loss of kidney function resulting from nephrotoxic and/or ischemic insults [25, 26]. The initiation and maintenance of ATN are caused by the excessive or aberrant generation of ROS [26]. Among ROS, H_2O_2 is relatively stable and can diffuse through cell membranes, making it a significant player in cellular signaling pathways and in the oxidative stress response to cellular components such as proteins, lipids, and DNA [27]. In the current study, we have shown for the first time to our knowledge that DJ extract, especially its main compounds daphnin and daphnetin, provides significant protection against H_2O_2-induced oxidative stress and cell death in kidney proximal tubular cells. This protective effect is primarily achieved through the reduction of ROS production and the stimulation of antioxidant enzyme activities, including SOD and catalase.

The 70% ethanol extract of DJ leaves exhibits antioxidant ability by quenching stable free radicals, including 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), through donating electrons or hydrogen atoms, and by reducing ferric ions to ferrous ions as measured by the ferric reducing antioxidant power (FRAP) assay [9]. In the current study, the DJ leaf extract did not have a direct antioxidant ability to remove superoxide and hydroxyl radical. Although the DPPH, ABTS, and FRAP assays primarily measure the overall antioxidant capacity of plant extracts, they do not specifically measure superoxide or hydroxyl radical scavenging [28]. Intriguingly, treatment with the DJ leaf extract significantly reduced superoxide and hydroxyl radical production and enhanced SOD and catalase activities in proximal tubular cells. These findings suggest that DJ leaf extract attenuates superoxide and hydroxyl radical through an intracellular antioxidant system rather than directly.

Daphnetin, a natural coumarin derivative, effectively mitigates oxidative stress in various injured cells, including *tert*-butyl hydroperoxide-exposed macrophages [29], oleic acid-exposed hepatocellular carcinoma [30], and arsenic-exposed lung epithelial cells [31]. Daphnin, a precursor of daphnetin, converts to daphnetin through hydrolysis, involving the cleavage of a glycosidic bond [32]. This process results in the release of glucose and the formation of daphnetin, suggesting a similar effect of daphnetin [32]. The current study identified daphnin and daphnetin as key compounds within DJ extracts through HPLC analysis, providing a clear direction for future research. Both compounds significantly increased cell viability and reduced ROS production in H_2O_2-exposed kidney proximal tubular cells at non-toxic doses. Additionally, they restored the activities of SOD and catalase, which were diminished by H_2O_2 exposure. They also showed a consistent effect in reducing superoxide anion and hydroxyl radical levels. Although the butanol and water fractions contained high levels of daphnin, their daphnetin content was low (Supplementary Fig. 2). Consistently, daphnetin significantly improved cell viability compared to daphnin ($F = 7.250$, $p = 0.009$; Fig. 4A,B) and more effectively reduced superoxide anion production

($F = 7.008$, $p = 0.018$; Fig. 4C) and hydroxyl radical production ($F = 7.250$, $p = 0.009$; Fig. 4D). These results indicate a lack of protective activity in the butanol and water fractions despite their high daphnin content and support daphnetin as the principal active component. Notably, daphnetin's impact on superoxide anion was strongly significant, with significant reductions observed at a dose of 3 μM. Overall, these findings suggest daphnetin may have a more robust antioxidant effect compared to daphnin, highlighting their critical roles in the extract's protective effects against oxidative stress.

Daphnetin has been shown to protect kidneys against nephrotoxicity induced by cisplatin [33, 34], as well as gentamicin [35] and diabetic nephropathy [36]. This reno-protective action is associated with a reduction in ROS [33, 36], a decrease in lipid peroxidation [34], and an upregulation of antioxidant enzymes [34, 35]. These effects are primarily mediated through the targeting of nuclear factor erythroid 2-related factor 2 (NRF2) signaling pathway [33–36]. The NRF2 pathway is a master regulator of the cellular antioxidant response, and its activation leads to the transcription of genes encoding antioxidant enzymes, including SOD and catalase [37, 38]. In the current study, we evaluated the protective effects of daphnin and daphnetin on ROS production and antioxidant enzyme activities in H_2O_2-exposed kidney proximal tubular cells. Our finding that daphnin and daphnetin restored SOD and catalase activities provides indirect evidence of NRF2 pathway involvement, as the upregulation of these enzymes is a primary downstream effect of NRF2 activation. These results provide insight into their roles in safeguarding against oxidative stress-induced damage to kidney parenchyma. However, further research is necessary to determine whether this protection is dependent on the NRF2 pathway in both in vitro and in vivo models of oxidative stress.

In experimental studies with rodents, it has been observed that the maximum tolerable dose of daphnetin when administered orally exceeds 100 mg/kg body weight [39]. Furthermore, no allergenic effects are detected when conducting skin allergy tests at concentrations up to 4 mg/ml [39]. In terms of genetic safety, daphnetin is found to be non-mutagenic in the bacterial reverse mutation test [32]. Protective effects of daphnetin at a dosage of 100 μM for a Duration of 12 h have been noted in hippocampal neuronal cells, guarding them against the harmful effects of glutamate [40]. Additionally, treatment with daphnetin ranging from 0 to 160 μM does not adversely affect the survival of microglial cells [41]. Collectively, both in vitro and in vivo assessments have established that daphnetin does not exhibit significant toxicity within the doses that are pharmacologically applicable. Consistent with the in vitro studies, the current study demonstrated that treatments with 30 μM of daphnetin and daphnin did not reduce cell viability. These results suggest that daphnetin and daphnin are generally safe within a certain dosage range.

In conclusion, the current study presents compelling evidence that extracts from DJ offer significant protection against oxidative stress in kidney proximal tubular cells. The compounds daphnetin and daphnin are identified as particularly promising for further investigation. These findings open up possibilities for new therapeutic strategies to treat kidney damage caused by oxidative stress. Future research should aim to clarify the specific molecular mechanisms that confer these protective effects and to assess the effectiveness of these compounds in vivo. In essence, this study contributes important insights to the domain of nephroprotective agents and underscores the medicinal promise of natural compounds in the treatment of kidney disease.

Abbreviations

ABTS	2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
ANOVA	Analyses of variance
ATN	Acute tubular necrosis
DCFDA	2′,7′-Dichlorofluorescin diacetate
DHE	Dihydroethidium
DJ	*Daphne jejudoensis*
DPPH	2,2-Diphenyl-1-picrylhydrazyl
EA	Ethyl acetate
FITC-A	Fluorescein isothiocyanate-area
FRAP	Ferric reducing antioxidant power
H_2O_2	Hydrogen peroxide
HPLC	High performance liquid chromatography
MC	Methylene chloride
MTT	Thiazolyl blue tetrazolium bromide
NRF2	Nuclear factor erythroid 2-related factor 2
PE-A	Phycoerythrin-area
ROS	Reactive oxygen species

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13765-025-01037-2.

Additional file 1: Tables. Raw numeric data and statistical results.

Additional file 2: Supplementary Figures. UV chromatograms of DJ extracts and fractions.

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Author contributions

DM performed experiments using cells and wrote the original draft. JYL, YAK, and JYB collected plant materials, prepared plant extracts and fractions, and performed HPLC analysis. JK conceived the experiment. DM and JK analyzed and interpreted the numeric data. JK and JYB secured funding and edited the manuscript. All authors red and approved the final manuscript.

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Data availability

The datasets used and/or analyzed during the current study are available from the corresponding authors on reasonable request.

Declarations

Competing interests

The authors declare that they have no competing interests.

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